Evaluation of Soybean Methanol Fraction on Acute Inflammation

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ABSTRACT Soybeans have been of interest of researchers because of the presence of isoflavones, a subclass of flavonoids, which have demonstrated anti-inflammatory activity. The aim of this study was investigate the anti-inflammatory activity of the methanol fraction from soybean, which contains mainly isoflavone glucosides and malonylglucosides. The anti-inflammatory activity of the methanol fraction from soybean was studied using croton oil–induced mouse ear edema and carrageenan-induced pleurisy models. The methanol fraction inhibited the ear edema in a dose-dependent manner: 0.625 mg/kg by 44.23% (P < .05), 1.25 mg/kg by 60.68% (P < .01), and 2.5 mg/kg by 65.68% (P < .01). Myeloperoxidase enzyme activity was reduced at the dose of 2.5 mg/kg (64.79%, P < .05). No effects were seen on carrageenan-induced pleurisy at different doses of the methanol fraction (100 or 400 mg/kg). These results demonstrated that the methanol fraction containing conjugated isoflavones showed topical anti-inflammatory activity. There was no acute toxicity in Swiss mice after oral administration of the fraction, at doses of 1,000, 2,000, 3,000, and 4,000 mg/kg.

KEY WORDS: • acute toxicity • anti-inflammatory activity • ear edema • isoflavone • soybean

INTRODUCTION

Soybeans, rich in isoflavones, are widely consumed based on their health benefits, including the prevention of chronic degenerative diseases related to climacterium: cancers of the breast and colon, diabetes, osteoporosis, atherosclerosis, and depression. There is a presumed relationship between the protective effects and isoflavones, the main compounds present in soybeans.1,2 Isoflavones have been shown to possess several biological activities: estrogenic, anti-estrogenic, anticarcinogenic, antioxidant, anti-inflammatory, and antidiabetic.3,4 It has been suggested that isoflavones prevent chronic degenerative diseases through reduction of inflammatory processes. The pathways involved include estrogenic and antioxidant mechanisms, reducing the production of interleukin-6 by inhibition of transcription factor nuclear factor-κB, activation of peroxisome proliferators (peroxisome proliferator-activated receptors α and γ), and inhibition of cyclooxygenase-2 and/or nuclear factor-κB inhibitory activity.5 It has been of interest that the conjugated isoflavone–rich methanol fraction activated peroxisome proliferator-activated receptors α, β, and γ in vitro, in a dose-dependent manner.6 In vitro tests have demonstrated that isoflavone glucosides and aglycones inhibit the production of arachidonic acid, prostaglandins, and leukotrienes through inhibition of the enzymes phospholipase A2, cyclooxygenase-2, and 1- and 5-lipoxygenase.7–9 Therefore, this study is to investigated the acute anti-inflammatory activity in vivo of the methanol fraction, which is rich in conjugated isoflavones, of soybean and also evaluated its acute toxicity after oral administration.

MATERIALS AND METHODS

Plant material

The soybean seeds were obtained from COAMO Co. (Campo Mourão, PR, Brazil) in October 2006.

Extraction and purification

Non-transgenic frozen soybeans (1 kg) were triturated, macerated with 12 L of hexane for 20 days, and then filtered. The obtained residues (E1; 702 g) were macerated with ethanol:water (3:2 vol/vol) for 7 days and filtered. The solvent from these extracts was evaporated under reduced pressure and lyophilized, yielding 160.6 g of ethanol extract. The ethanol extract was mixed in 2 L of methanol and centrifuged. The methanol fraction was evaporated to afford 48.0 g of methanol fraction.1 The composition of this fraction was analyzed by high-performance liquid chromatography–ultraviolet, the procedure for which is described in a previous study.6
**Animals**

Male Swiss mice (weighing 25–30 g) and male Wistar rats (weighing 180–220 g) were used. The animals were housed with a 12-hour light/dark cycle in a temperature-controlled room with free access to water and food. The experimental protocol followed the guidelines of the Brazilian legal guidelines for protection of animals.

**Croton oil–induced ear edema in mice**

Edema was induced by applying 20 μL of croton oil diluted in a vehicle of acetone/water (7:3 vol/vol) on the inner surface of the left ear of mice. The right ear received only the vehicle (20 μL). Indomethacin (1.0 mg per ear) and dexamethasone (0.1 mg per ear) were used as reference anti-inflammatory drugs (positive controls). Immediately after the application of croton oil, the treated groups received 20 μL of the methanol fraction at the doses of 0.625, 1.25, and 2.5 mg and the same volume of the reference drugs on the inner ear surface; the control group received 20 μL of the left ear vehicle. After 6 hours, the animals were killed, and each ear was perforated with a metal punch to provide a 6-mm-diameter disc. Edema was assessed by the difference in weight (mg) between the left and right ears. Inhibition was calculated as a percentage:

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\% \text{ inhibition} = \frac{\text{weight of left ear}_{\text{control}} - \text{weight of left ear}_{\text{treated}}}{\text{weight of left ear}_{\text{control}} - \text{weight of right ear}_{\text{vehicle}}} \times 100
\]

**Myeloperoxidase activity**

Myeloperoxidase (MPO) activity was assayed in the supernatant of homogenates of the ear sections (untreated controls and animals treated with 2.5 mg of the methanol fraction or with 0.1 mg of dexamethasone). The ear was placed in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma, St. Louis, MO, USA) (1 mL/50 mg of tissue) in a Potter homogenizer. The homogenate was shaken in a vortex-mixer and centrifuged for 5 minutes. Ten microliters of the supernatant was added to each well of a 96-well microplate, in triplicate. Two hundred microliters of the buffer solution containing o-dianisidine dihydrochloride (16.7 mg, Sigma), double distilled water (90 mL), potassium phosphate buffer (10 mL), and 1% H₂O₂ (50 μL) was added. The enzyme reaction was stopped using ethyl acetate. Enzyme activity was determined by the absorbance, measured at 460 nm in an enzyme-linked immunosorbent assay reader.

**Carrageenan-induced pleurisy in rats**

The methanol fraction of soybean was diluted in water, immediately prior to use in the pleurisy trials. The methanol fraction of soybean (100 or 400 mg/kg), indomethacin (5 mg/kg), the vehicle (water), and the saline (0.9% NaCl) were administered orally by gavage in different groups of rats that had been fasted for 15 hours, 30 minutes prior to the induction of pleurisy. The carrageenan was diluted in phosphate-buffered saline (pH 7.4). Pleurisy was induced by injection of 0.25 mL of a carrageenan suspension (200 μg) into the right pleural space. After 4 hours, the animals were killed, and the pleural exudates were collected by aspiration and transferred to conical centrifuge tubes. The total volume of the exudate was measured. For differential analysis, a sample was diluted with Turk’s solution to lyse the red blood cells. The resulting fluid was centrifuged for 10 minutes, and the cells were resuspended. The slides were prepared, dried, fixed, and stained. Mononuclear and polymorphonuclear leukocytes present in a 50-μL aliquot were determined in a Neubauer chamber by means of optical microscopy.

**Acute toxicity test**

The Swiss mice (weighing 25–30 g) after 15 hours of fasting received doses of 1,000, 2,000, 3,000, or 4,000 mg/kg methanol fraction through oral gavage. The animals were observed for 7 days. Food and water were provided throughout the experiment.

**Statistical analysis**

The results are presented as mean±SEM values. Data were subjected to an analysis of variance followed by Tukey’s post hoc test, using the Prism version 4.0 statistical program (Prism Software, Irvine, CA, USA). P<.05 was considered statistically significant.

**RESULTS**

**High-performance liquid chromatography analysis**

The isoflavone content in the methanol fraction of soybeans was obtained by high-performance liquid chromatography. The isoflavones acetylaidzin, acetylgenistin, and acetylglycitin were not found. The major compounds in the methanol fraction were daidzin, genistin, malonyldaidzin, and malonylgenistin. The concentrations of these isoflavones in the methanol fraction were, respectively, 145.67±3.14, 147.54±5.54, 276.54±4.21, and 466.54±7.37 mg of isoflavones/100 g of methanol fraction (Fig. 1). Total isoflavone content was 1,345.09 mg/100 g of fraction.

**Croton oil–induced ear edema in mice**

Croton oil application on the left ear of mice induced a cutaneous inflammation. After 6 hours, the weight of the ear increased approximately two times compared with the right ear (with no croton oil applied). As positive controls, indomethacin (1.0 mg per ear) and dexamethasone (0.1 mg per ear) significantly inhibited the ear edema by 81.4% (P<.001), respectively. The methanol fraction containing soy isoflavones at doses of 0.625, 1.25, and 2.5 mg/kg significantly reduced the ear edema in a dose-dependent manner, by 44.23% (P<.05), 60.68% (P<.01), and 65.68% (P<.01), respectively. There was no statistical difference in comparing the treatments between the positive
controls and the methanol fraction of soybean (P > .05) (Fig. 2). Application of the methanol fraction at a dose of 5.0 mg/kg caused a similar inhibition at the dose of 2.5 mg/kg.

**MPO activity**

Another event with the topical application of croton oil is a massive tissue cell infiltration, reflected by the increased MPO activity. Methanol soy fraction (2.5 mg) and dexamethasone (0.1 mg) produced a remarkable inhibition of MPO activity (64.79% and 64.64% [P < .05], respectively) (Fig. 3).

**Carrageenan-induced pleurisy in rats**

Intrapleural injection of carrageenan in groups of animals pretreated orally with saline or vehicle induced an acute inflammatory response, characterized by an increase in the volume of the pleural exudate and the number of leukocytes migrated into the cavity, compared with the base parameters (obtained from normal animals that received an injection of phosphate-buffered saline in the cavity). Methanol fraction at the doses of 100 and 400 mg/kg did not alter development of the inflammation response compared with the control group. Treatment of the animals with indomethacin, the reference anti-inflammatory drug, significantly reduced the intensity of the response. The results are presented in Figure 4 and Table 1.

**Acute toxicity test**

The methanol fraction of soybeans did not show acute toxicity in mice. By comparison with the control group, those treated did not show any differences in consumption of water, food, and body weight during the days of the observation. Furthermore, the animals showed no depressive, excitatory, or sleepiness symptoms. Macroscopic inspection indicated no alterations in the stomach, liver, spleen, and kidneys of the animals (data not shown).
The anti-inflammatory activity of the methanol fraction of soybean was studied using the following inflammation models: croton oil–induced ear edema in mice and carrageenan-induced pleurisy in rats. These are well-characterized models that permit the quantification and correlation of both exudates and cellular migration with changes of other inflammatory parameters. Croton oil is an irritant agent that causes cell damage and activates phospholipase A2, which releases arachidonic acid from the cell plasma membrane. From arachidonic acid there is a production of prostaglandins by cyclooxygenase-1 and cyclooxygenase-2 and of leukotrienes by 5-lipoxygenase. Eicosanoids promote the chemotaxis of neutrophils, and they induce biosynthesis of elastase, collagenase, and other compounds. These enzymes break down structural proteins into peptides. As a consequence, there is an increase in vascular permeability and hydrostatic pressure, resulting in edema and migration of neutrophils to the damaged tissue.13 The development of the inflammatory response induced by carrageenan (pleurisy) is characterized by an initial phase (1–2 hours) that is dependent on the release of histamine, serotonin, and bradykinin, followed by a later stage (3–4 hours) that is maintained principally by prostanoids, nitric oxide, and cytokines.14–21

The present study showed that the methanol fraction of soybean, administered topically, reduced the inflammation in the mouse ear. In this experimental model, the methanol fraction inhibited both fluid extravasation and cellular influx, with the latter indicated by a reduction in MPO activity. The enzyme MPO, found in azurophilic granules of neutrophils and other cells of myeloid origin, is considered a marker of polymorphonuclear leukocyte influx into inflamed tissues. Therefore, its inhibition may result in an anti-inflammatory activity.13 On the other hand, in the pleurisy induced by the carrageenan model, the methanol fraction did not inhibit both fluid extravasation and leukocyte migration. These results indicated that the inhibitory effect of methanol fraction depends on the administration route. Only topical applications reduced the inflammatory response, suggesting that after oral administration the absorption of their anti-inflammatory components may be affected by the gastrointestinal tract through metabolism during the first passage through the liver, or inactivation by pH differences or enzyme contents in the stomach or the small intestine. In addition, there may be differences in the drug concentrations in the target tissue, when the methanol fraction is administered orally or applied topically. After oral administration, the absorption of isoflavones requires the initial hydrolysis of glycosylated molecules by enzymes released from intestinal bacteria. Then, daidzein and genistein are methylated by bacterial enzymes, forming formononetin and biochanin A, respectively. Besides, after enterohepatic circulation, these molecules can undergo other structural changes due to reduction and oxidation reactions. The resulting metabolites are conjugated with glucuronic acid and to a lesser extent with sulfuric acid. Soybeans have a small amount of aglycones, which are readily absorbed in the stomach and conjugated in the liver. Only a small portion of free aglycones could be detected in the blood. Knowledge of the factors involved in absorption and metabolism of isoflavones, including the role of intestinal bacteria, may be important to determine the clinical efficacy. The biological role of metabolites and conjugates in the soybean fraction needs to be defined.22–26

Moreover, there may be a possibility that the methanol fraction possesses activity on the various inflammatory mediators involved in different experimental models. As demonstrated by other researchers, some plant extracts

![FIG. 4. Effect of MFS on pleurisy induced by intrapleural injection of carrageenan (Cg) in male Wistar rats (weighing 180–220 g). The animals (n = 5–10 for each group) were treated orally with the fraction at the concentrations indicated, 30 minutes prior to the injection of Cg (200 μg). Indo, administered orally at 5 mg/kg, was used as the reference anti-inflammatory drug (positive control). One group of animals receiving an intrapleural injection of saline (basal). Data are mean ± SEM volumes of pleural exudates, 4 hours after injection of Cg. *P < .001 compared with the control groups (Cg + saline) (analysis of variance, Tukey’s post hoc test).](image-url)
may present inhibitory activity varied on the inflammatory stages and the mediators in different experimental models.27,28

We cannot specifically identify what pro-inflammatory mediator is inhibited by the methanol fraction. In vitro studies suggest that the anti-inflammatory mechanisms of isoflavones are related to the inhibition of the enzymes phospholipase A2, cyclooxygenase-2, and 1- and 5-lipoxigenase.7,9

In the present study, there are findings suggesting some components of the methanol fraction possess activity against the arachidonic acid metabolites. This study attempted to demonstrate that the methanol fraction, which is rich in conjugated isoflavones, showed significant anti-inflammatory activity when administered topically to animals. This result is consistent with other studies carried out in vivo with isoflavone glucosides and malonylglucosides.29,30 Furthermore, aglycones and other compounds in the methanol fraction may contribute to the observed anti-inflammatory effects.

Soybean extracts rich in conjugated isoflavones did not show acute toxicity in mice and may be administered topically without hydrolyzing these compounds, in order to produce an anti-inflammatory effect. To our knowledge, the study is the first of its kind to its kind to report a topical anti-inflammatory activity with a product rich in conjugated isoflavones.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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