Hepatoprotective efficacy of methanolic extract of *Indigofera suffruticosa* (Mill) on paracetamol–induced liver damage in mice

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ABSTRACT – Background – *Indigofera suffruticosa* Mill (Fabaceae) is abundant in northeastern Brazil and popularly used in the treatment of infectious and inflammatory processes. Several biological properties, such as anti-inflammatory, anticerine, antitumor, hepatoprotective and low toxicity, are reported for this plant. Objective – This study investigated hepatoprotective activity and the antioxidant effect of methanolic extract of *I. suffruticosa* leaves (MEIS) on Swiss albino mice submitted to experimental models of acetaminophen-induced liver injury. Methods – MEIS (50 mg/kg; p.o.) was standardized according to the LD50 and its hepatoprotective property on Swiss albino mice evaluated during a 7-day period. On the eighth day, the acetaminophen-induced hepatic injury was performed. Histomorphometric analysis of liver tissue, antioxidant activity and serum levels of alanine aminotransferase (AST), aspartate aminotransferase (ALT) and bilirubin were measured. Results – MEIS (50 mg/kg; p.o.) restored serum enzyme levels and results were close to those of positive control (silymarin) when compared to the negative control. Histopathological and histomorphometric analyzes confirmed MEIS hepatoprotective activity, showing reorganization of structural units of cells, nuclei and sinusoidal capillaries of hepatocytes, reducing the damage on liver tissue and increasing organ regeneration rate. MEIS showed high antioxidant potential at concentrations of 1000 and 500 µg/mL. Conclusion – This study suggests that MEIS has hepatoprotective activity and high antioxidant potential.


INTRODUCTION

The liver plays an important role in maintaining metabolic homeostasis and biotransformation of chemicals in the body. Hepatic diseases are a worldwide health problem, and are triggered mainly by viruses, metabolic diseases or chemical compounds. The treatment of such diseases is carried out conventionally using synthetic pharmaceuticals, or, secondarily, by using isolated compounds or parts of medicinal plants used in popular medicine. However, there are still few drugs used to treat liver diseases with significant effects on humans. Thus, medicinal plants with hepatoprotective or curative activity used for the treatment of hepatic disorders become important subjects of studies to elucidate their mechanism of action and characterize compounds that can be used for the development of new hepatoprotective drugs.

Some experimental models are used to demonstrate the hepatoprotective action of certain plants, especially against paracetamol-induced liver damage. Paracetamol may promote hepatocellular injury through three mechanisms, independently or in association. The first and most common mechanism is ingestion of doses higher than 10 g by adults and up to 150 mg/kg by children, popularly known as “overdose”. The second mechanism is the cytochrome P450 (CYP) over activation, resulting from the use of enzyme-inducing drugs and chronic alcohol abuse. Finally, the third mechanism occurs with glucagon depletion in hepatocytes through alcohol intake or malnutrition. *Indigofera suffruticosa* Mill (Fabaceae) is abundant in northeastern Brazil, and has intense popular use for treatment of infections, inflammations and other processes, without reports of harmful side effects to humans. The phytochemical investigation of leaf extracts of *I. suffruticosa* revealed the presence of alkaloids, flavonoids, steroids, proteins, carbohydrate, triterpenes and indigo coumarins and, the chemical properties of two compounds isolated from *I. suffruticosa* leaves (indigo and indirubin) were identified, isolated and purified by Vieira et al.

Later, the bis-indolic alkaloid fraction of leaves of *I. suffruticosa* was investigated and proved to be a phytotherapeutic agent of liver. Pharmacological studies showed aqueous extracts of leaves of *I. suffruticosa* have anti-inflammatory, antimicrobial and embryotoxic properties, besides being used as alternative anticerine and antitumor therapy. The aqueous leaf extract of *I. suffruticosa* has been shown to inhibit egg hatching, larval eclyse and to have detergent effects on oviposition of *Aedes aegypti* mosquitoes. Mice carrying sarcoma 180 had positive hepatic response to subchronic treatment with methanolic extract of *I. suffruticosa* leaves. The structural organization of the renal tubules and hepatocyte nuclei of mice were preserved after treatment with *I. suffruticosa*. The aqueous extract of *I. suffruticosa* leaves has low toxicity, with 98% viability of larvae.

Declared conflict of interest of all authors: none

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**ARTICLE**

**ABSTRACT**

**INTRODUCTION**

**RESULTS**

**CONCLUSION**
Due to the anti-inflammatory, anticancer and antitumor properties, and because it is a liver protective agent with previously reported low toxicity, we hypothesize the plant can be used as a hepatoprotective phytotherapeutic agent. This study investigated the effect of *I. suffruticosa* on the hepatoprotective activity as well as its antioxidant effect through an experimental model of paracetamol-induced hepatic lesion in Swiss albino mice.

**METHODS**

**Chemicals**

Methanol, distilled water, 10% formalin, sodium chloride (0.9% NaCl), hematoxylin, eosin, silymarin and acetaminophen were supplied by Merck (Germany).

**Plant material and extraction**

Leaves of *I. suffruticosa* were collected in the city of Recife, state of Pernambuco, Brazil, in July 2014. The sample was certified by the biologist Marlene Barbosa from the Department of Botany at the Federal University of Pernambuco (UFPE) and it is deposited under the n. 43694 in the Herbarium of the Center for Biological Sciences – (UFPE). Leaves of *I. suffruticosa* (1.3 kg) were washed in running water, pulverized and extracted with methanol (MeOH) at room temperature for 48 hours. Then, the solvent was filtered and evaporated at 35°C under reduced pressure. The methanolic extract of *I. suffruticosa* leaves (MEIS) yielded 54.5 g. Fresh dilution of dried extract in saline solution (0.9% NaCl) was prepared on the day of experiments.

**Experimental animals**

Eighteen healthy male Swiss albino mice, aged 40 days, weighing on average 35 to 45 g were acquired from the Aggeu Magalhães Research Center (Pernambuco, Brazil). Animals had free access to water and feed. They were kept under standard environmental conditions of temperature, humidity 12:12 light-dark cycle. The Committee on Animal Research and Ethics of UFPE approved the experiments under opinion n. 500/12, process n. 23076.025195/2012-64 of 2012.

**Experimental design and treatment arrangement**

Swiss albino mice were randomly divided into three groups (n=6 animals/group) and orally pre-treated during seven days before paracetamol-induced liver damage, according their respective group: G1 – treated with saline (15 mL/kg; p.o.), G2 – treated with Silymarin (25 mg/kg; v.o.) and G3 – treated with MEIS (50 mg/kg; v.o.). The *I. suffruticosa* extract dose was based on LD50. In the eighth day, after fasting for 12 h, the mice were administered with paracetamol (300 mg/kg; v.o.). Twenty four hours later, blood was gained through cardiac puncture, and serum was separated for later determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin. Following they were euthanized cervical dislocation for collection of liver samples.

**Levels of transaminases**

Serum enzyme levels (alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin) of groups G1, G2 and G3 of Swiss albino mice submitted to pretreatment with IS extract prior to paracetamol-induced hepatic injury were analyzed using commercial kits according to the manufacturer’s protocols.

**Histopathological and histomorphometric analysis of liver**

The liver of control groups (G1 and G2) and treatment group (G3) were fixed in 2.5% formaldehyde and stained with hematoxylin and eosin (HE) (20). Thirty histological sections were analyzed through four random fields. The histological sections were digitized using a digital video camera (Leica DFC 280, Wentzler, Germany) coupled to a light microscope (Leica Microscopy & Systems GmbH, Wentzler, Germany) under 40x final magnification. Morphometric evaluation, the perimeter of hepatocyte nuclei and the diameter of sinusoidal capillaries were determined using the Scion Image software (Scion Corporation, New York, USA).

**In vitro antioxidant assay – DPPH radical scavenging activity**

The antioxidant activity of MEIS was determined by using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Briefly, different concentrations of MEIS (1,000, 500, 250 and 125 µg/mL) and DPPH (0.4mM) were prepared in ethanol. For determination of MEIS antioxidant activity, sample were mixed with DPPH solution (1:1; v/v) for 40 minutes at room temperature. After incubation period, the absorbance of the solution was measured at 517 nm using a microplate reader and related to the negative control (DPPH and ethanol). All tests were carried out in triplicate, and the ability to eliminate DPPH (% of antioxidant activity) was calculated using the following equation:

\[
\text{Elimination [DPPH]} \% = \left( \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs control}} \right) \times 100
\]

**Statistical analysis**

The results for levels of transaminases, as well as histopathological and histomorphometric parameters of hepatocytes nuclei and hepatocyte nuclei were expressed as mean ± SEM (standard error of the mean) and analyzed by Kruskal-Wallis test at P<0.001. The antioxidant activity was analyzed by Tukey test (P≤0.05) using the Assistat software – version 7.6 beta.

**Levels of transaminases**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>ALT (IU/L)</th>
<th>AST (IU/L)</th>
<th>Bilirubin (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>15 mL/kg</td>
<td>94.48±6.89**</td>
<td>120.98±13.67**</td>
<td>7.9±3.5**</td>
</tr>
<tr>
<td>G2</td>
<td>25 mg/kg</td>
<td>31.36±4.78</td>
<td>36.65±5.35</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>G3</td>
<td>50 mg/kg</td>
<td>37.78±4.56*</td>
<td>39.24±5.78*</td>
<td>2.1±1.2*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM. **Statistically significant compared to animals treated with saline solution (P<0.05). *Statistically significant for animals treated with silymarin and *Indigofera suffruticosa* extract (P<0.05).
ALT (94.48±6.89), AST (120.98±15) and bilirubin (7.9±3.5) compared to animals treated with silymarin (G2): ALT (31.36±4.78), AST (36.65±5.35) and bilirubin (1.4±0.5), which reduced serum enzyme levels. Results of G2 were close to those treated with MEIS (G3): ALT (37.78±4.56), AST (39.24±5.78) and bilirubin (2.1±1.2). The reduction in serum levels of these important markers related to liver integrity suggests a hepatoprotective activity of MEIS against paracetamol-induced hepatic injury.

**Histopathological and histomorphometric analysis of liver**

Hepatic tissue samples collected from each group on the eighth day were evaluated at 400x magnification. A photomicrograph of each group is shown in **FIGURE 1**. Histological analysis of hepatic tissue after pretreatment with MEIS (50 mg/kg; p.o.) (G3) and the positive control (G2) receiving silymarin (25 mg/kg) prior to paracetamol-induced hepatic injury showed normal hepatic tissue. Lobular central vein (CV) radiating out of the vein, well-reorganized hepatocyte cords (HC) in the structural units of cells among which are the well-arranged sinusoidal capillaries (S), suggesting a possible angiogenic action compared to the negative saline control (G1). The G1 showed structural differences in the hepatic tissue whose hepatocyte cords (HC) showed no organized cellular structural units, congested sinusoidal capillaries (S) and areas of necrosis with discrete leukocyte infiltration (LI).

**Area and perimeter of hepatocyte nuclei**

The areas and perimeters of hepatocyte nuclei of the three groups were randomly compared and are shown in **FIGURES 2 and 3**. The areas and perimeters of G3 (0.106±0.025 and 1.145±0.140) showed no significant difference compared to those of G2 (0.094±0.027 and 1.082±0.150). However, the areas and perimeters of G1 (0.434±0.127 and 1.650±0.669) showed significant difference when compared to those of G2 (0.094±0.027 and 1.082±0.150) and G3 (0.106±0.025 and 1.145±0.140) (P<0.001).

**Diameter of hepatic sinusoidal capillaries**

Sinusoidal capillaries of G3, subjected to subchronic pretreatment MEIS (50 mg/kg; p.o.), G2 positive control/silymarin and G1 negative/saline solution, were randomly compared and are shown in **FIGURE 4**. The hepatic sinusoidal capillaries of G3 mice (28±11.86) showed no significant difference when compared to G2 (30.85±7.81). However, G1 (59±14) showed significant difference when compared to G2 (30.85±7.81) and G3 (28±11.86) (P<0.001).

**In vitro antioxidant activity of MEIS by DPPH free-radical scanning method**

The MEIS antioxidant potential is shown in **FIGURE 5**: 73.79% ±1.5915 of antioxidant activity at 1,000 μg/mL; 79.58% ±1.0231 at 500 μg/mL; 67.25% ±2.6531 at 250 μg/mL and 57.12% ±0.4641 at 125 μg/mL. Results suggest MEIS have great antioxidant potential at 1,000 and 500 μg/mL and may contain substances capable of donating electrons; thus reducing free radicals.
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The increase in serum enzyme levels(16) indicates interference with DNA synthesis. The same mechanisms of aqueous extraction of the same plant were demonstrated in the study by Lima et al. (2014) who showed restoration of normal physiology of serum enzyme levels. These data can be corroborated by histological analysis of liver. Histological analysis is an important tool for detection of morphological changes in the liver caused by bioactive compounds. The results of histological analysis showed structural organization in the hepatic tissue of mice subjected to paracetamol-induced injury after subchronic pretreatment with MEIS, similarly to the positive control/silymarin. However, hepatic tissue changes observed for the negative/saline solution control included: congested sinusoids, changes in hepatocyte cords and areas of necrosis and hemorrhage, characterized by leukocyte infiltration. Our data corroborate the results found by Silva et al.(21) who demonstrated that the aqueous extract of I. suffruticosa leaves preserved the liver architecture, suggesting its use as alternative hepatoprotective agent(21). In this study, there was reorganization of hepatocyte structural units and sinusoidal capillary cells, suggesting a possible angiogenic action in the liver of mice treated with MEIS and corroborating the pharmacological, anti-inflammatory, anticancer and antimicrobial properties of the extract(15,17,18).

The histomorphometric analysis of the area and perimeter of hepatocyte nuclei and diameter of sinusoidal capillaries of mice subjected to paracetamol-induced injury after subchronic treatment with MEIS were similar when compared to silymarin. The groups pretreated with MEIS or silymarin showed no significant statistical differences. These data corroborate histological and morphometric analyses of experiments performed by Lima et al. (2014) who showed the absence of a structural change in the hepatic epithelium of mice carrying sarcoma 180 after treatment with Indican isolated of methanol extract from I. suffruticosa leaves. However, significant changes were observed in the control group with sarcoma 180(14). The nuclei and sinusoidal capillaries of hepatocytes after subchronic treatment with extract and silymarin showed restoration of normal physiology of serum enzyme levels and results were close to those of positive control (silymarin) when compared to the negative control. This effect was similar to the group treated with silymarin. Possibly, this effect is associated with the extract antioxidant activity. On the other hand, the negative control presented increasing ALT, AST and bilirubin serum levels. The serum enzyme levels using Caralluma umbellate, a perennial and succulent herb, were investigated. Pretreatment with ethanolic extract followed by paracetamol administration led to reduced levels of total bilirubin, cholesterol, ALT, AST, and alkaline phosphatase. The extract showed hepatoprotective activity for restoring serum enzyme levels and preventing oxidative stress(21). Similar data were described for Melia azedarach L. (cinnamon) used for treatment of leprosy and inflammatory processes. The methanolic extract of Melia azedarach (500 mg/kg) administered in rats for seven days, followed by the paracetamol administration on the 5th day showed a hepatoprotective activity, reducing levels of AST, ALT and alkaline phosphatase and increasing enzyme activity(22). The hepatoprotective potential in our study was due to the MEIS pretreatment at 50 mg/kg, which showed restoration of normal physiology of serum enzyme levels.

DISCUSSION

Liver diseases are a worldwide health problem mainly triggered by viruses, metabolic diseases or chemical compounds(2-4). The hepatoprotective activity of plant extracts and compounds has been verified through the model of paracetamol-induced intoxication (PCM)(5,24-27). The increase in serum enzyme levels is among the evidence of PCM-induced hepatic injury(29). Serum enzymes levels act as markers reflecting both hepatocellular necrosis and their release into the blood after damage to cell membrane(29,30).

I. suffruticosa has been used in traditional medicine for the treatment of infections and inflammation without reports of side effects to humans. These data were demonstrated by its properties: anti-inflammatory, embryotoxic, antimicrobial, anticancer, antitumor, hepatoprotective and low toxicity(12,15-18,20). This study investigated the effects of MEIS on paracetamol-induced liver injury and its in vitro antioxidative action on Swiss albino mice.

Mice pretreated with MEIS prior to paracetamol—induced hepatic injury showed restoration of serum enzyme levels of ALT, AST and bilirubin, reduced hepatic tissue damage and increased organ regeneration rate. MEIS (50 mg/kg; p.o.) restored serum enzyme levels and results were close to those of positive control (silymarin) when compared to the negative control. This effect was similar to the group treated with silymarin. Possibly, this effect is associated with the extract antioxidant activity. On the other hand, the negative control presented increasing ALT, AST and bilirubine serum levels. The serum enzyme levels using Caralluma umbellate, a perennial and succulent herb, were investigated. Pretreatment with ethanolic extract followed by paracetamol administration led to reduced levels of total bilirubin, cholesterol, ALT, AST, and alkaline phosphatase. The extract showed hepatoprotective activity for restoring serum enzyme levels and preventing oxidative stress(21). Similar data were described for Melia azedarach L. (cinnamon) used for treatment of leprosy and inflammatory processes. The methanolic extract of Melia azedarach (500 mg/kg) administered in rats for seven days, followed by the paracetamol administration on the 5th day showed a hepatoprotective activity, reducing levels of AST, ALT and alkaline phosphatase and increasing enzyme activity(22). The hepatoprotective potential in our study was due to the MEIS pretreatment at 50 mg/kg, which showed restoration of normal physiology of serum enzyme levels.

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the methanolic extract of *I. suffruticosa* leaves may be related to the antitumor activity found by Vieira et al.\(^{(11)}\). Other studies have reported the preservation of structural organization of renal tubules and hepatocyte nuclei of mice following treatment with *I. suffruticosa*\(^{(20,22)}\).

Muthulingam et al., using paracetamol as acute hepatotoxicity model, reported that the treatment of animals with aqueous extract of *Indigofera tinctoria* showed marked reduction of necrosis and liver tissue degeneration\(^{(30)}\). The efficacy of any hepatoprotective drug is essentially dependent on its ability to reduce harmful effects or maintain the normal hepatic physiology that has been disrupted by a hepatotoxin\(^{(11,32)}\). Silymarin extracted from seeds of Marian thistle (*Silybum marianum*) consists of many polyphenolic flavonoids, including silibinin (the main one), isosilybine, silercistin and silydianin\(^{(33)}\). Silymarin has been used for centuries because of its known hepatoprotective effects and several pharmacological properties\(^{(34)}\).

Lipid peroxidation is a chain reaction of polyunsaturated fatty acids of cell membranes generating free radicals that change their permeability, fluidity and integrity\(^{(35)}\). Antioxidant activity provides health benefits by inhibiting lipid peroxidation\(^{(36)}\). The percentage of antioxidant activity is proportional to the amount of DPPH consumed by the sample\(^{(35,36)}\). Thus, the higher the DPPH consumption per sample, the lower its absorbance and the higher its antioxidant activity.

Our study on the antioxidant activity of MEIS (1,000 µg/mL and 500 µg/mL) suggests the presence of substances capable of donating electrons and thus reducing free radicals. These results suggest that MEIS have a great antioxidant potential directly related to the concentration increase. These results are similar to those found by Renukadevi et al. for methanolic extracts of *Indigofera tinctoria*, which showed significant antioxidant activity at the concentrations tested\(^{(37)}\). The hepatoprotective activity of MEIS found in this study is probably related to chemical compounds capable of donating electrons and reducing free radicals\(^{(38)}\); a fact confirmed by the antioxidant activity of this plant that induces the inhibition of the tissue injury according to studies reported in the pertinent literature\(^{(39)}\). Also, other studies are recommended to evaluate the curative efficacy on hepatic tissue of mice in experimental models of paracetamol-induced hepatic injury subjected to post-treatment with MEIS. In conclusion, this study suggests that hepatic tissue of mice in experimental models of paracetamol-induced hepatic injury subjected to pretreatment with MEIS showed restoration of hepatic physiology at serum enzyme levels, reorganization of tissue structural units, hepatic sinusoidal cells and capillaries, besides antioxidant potential, indicating reduced damage to the hepatic tissue and increased organ regeneration rate. These data suggest that MEIS has hepatoprotective activity.

**Authors’ contribution**

Professor Leite SP: author responsible for the integrity of the work. All authors Lima IR, Silva IB, Lima RML, Silva TMS were involved in the development of the research, collection of plant material preparation of the extract as well as histopathology and histomorphometry of hepatic tissue and the antioxidant potential. Professor Maia MBS: contributed to the experimental design and treatment schedule and to the analysis of enzymatic dosages.

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