Safflower oil: an integrated assessment of phytochemistry, antiulcerogenic activity, and rodent and environmental toxicity


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ABSTRACT

Gastric ulcers are a significant medical problem and the development of complications lead to significant mortality rates worldwide. In Brazil, Carthamus tinctorius L., Asteraceae, seeds essential oil, the safflower oil, is currently used as a thermogenic compound and as treatment for problems related to the cardiovascular system. In this study, by Raman spectroscopy, it was shown that oleic and linoleic acids are the compounds present in higher concentrations in the safflower oil. We demonstrated that safflower oil (750 mg/kg, p.o.) decrease the ulcerogenic lesions in mice after the administration of hydrochloric acid-ethanol. The gastric ulcers induced by non-steroidal anti-inflammatory drug (NSAID) in mice treated with cholinomimetics were treated with four different doses of safflower oil, of which, the dose of 187.5 mg/kg (p.o.) showed significant antiulcerogenic properties (**p < 0.01). Moreover, the safflower oil at doses of 187.5 mg/kg (i.d.) increased the pH levels, gastric volume (**p < 0.01) and gastric mucus production (**p < 0.001), and decreased the total gastric acid secretion (**p < 0.001). The acute toxicity tests showed that safflower oil (5.000 mg/kg, p.o.) had no effect on mortality or any other physiological parameter. Ecotoxicological tests performed using Daphnia similis showed an EC50 at 223.17 mg/l, and therefore safflower oil can be considered "non-toxic" based on the directive 93/67/EEC on risk assessment for new notified substances by European legislation. These results indicate that the antiulcer activity of Safflower oil may be due to cytoprotective effects, which serve as support for new scientific studies related to this pathology.
Introduction

The development of peptic ulcers is one of the world’s major gastro-intestinal disorders, including both gastric and duodenal ulcers, which affects 10% of the global population (Zapata-Colindres et al., 2006). The pathophysiology of peptic disease is attributed to an imbalance between aggressive factors like acid, pepsin, and an Helicobacter infection, and local mucosal defenses like bicarbonate, mucus and prostaglandin secretion (Jain et al., 2007). Helicobacter pylori infection, the use of non-steroidal anti-inflammatory drugs (NSAID), emotional stress, alcohol abuse, and smoking are the principal etiological factors associated with peptic ulcer disease (Malfertheiner et al., 2009).

There are several drug treatments used to treat gastric ulcer. These include gastric acid neutralizing therapeutic drugs (e.g. aluminum hydroxide and magnesium), H2 receptor antagonists (e.g. ranitidine), and proton pump inhibitors (e.g. omeprazole). However, despite the large range of treatments gastric ulcer recurrence has long been thought to be an unavoidable feature of peptic ulcer disease, and therefore, maintenance treatment has been necessary to prevent recurrence (Arakawa et al., 2012).

During the past decade, complementary and alternative medicines have become a topic of global importance. Current estimates suggest that in many developing countries, a large portion of the population relies heavily on traditional practitioners and medicinal plants to meet primary healthcare needs. Although modern medicine may be available in these countries, herbal medicines (phytomedicines) have often maintained their popularity for historical and cultural reasons (Saraf and Saraf, 2012).

Carthamus tinctorius L. is a member of the family Asteraceae, it possibly originated in Southern Asia and is known to have been cultivated in China, India, Iran and Egypt almost from prehistoric times. During the middle ages it was grown in Italy, France, and Spain, and afterwards it was introduced into North and South America from the Mediterranean region (Bae et al., 2002).

Carthamus tinctorius seed are rich in edible oil (safflower oil), with similar content to olive, sunflower, and peanut oils (40% dry matter weight). This oil is composed of typically linoleic acid (63-72%), oleic acid (16-25%) and linolenic acid (1-6%) (Kim et al., 2000).

The oral administration of safflower oil at doses of 750 mg/kg in rodents has been proven to have thermogenic properties and may thus contribute to the treatment of obesity (Takeuchi et al., 1995). Other reports show that this oil has the capacity to relieve constipation and ease rheumatic pains, and that it has laxative and antifungal activities (Pintão and da Silva, 2008). However, despite the widespread use of safflower flowers for their pharmaceutical, cosmetic, and medicinal properties; there have been no studies regarding the use of this oil for the treatment of gastric ulcers, or tests to determine its rodent toxicity (side effects) and environmental risks of this oil.

Medicinal plant species that are being used to treat particular diseases on a large scale can have serious side effects (George, 2011). The likelihood of side effects increases when the production and sale of such products are largely uncontrolled and/or unregulated and the consumer is not adequately informed about their proper uses. Regulatory controls are therefore considered necessary to safeguard drug interactions with herbal drugs (WHO, 1997; 2002).

The monitoring of pharmaceutical compounds in environmental matrices has been addressed in several studies since the late 1990s. Drugs excreted by patients can contaminate rivers, even after treatment in wastewater-processing facilities. In addition, there is mounting evidence that effluents from pharmaceutical factories could also be carrying drugs into the rivers (Gilbert, 2012). The effects are already evident: they include the feminization of fish by residues of contraceptive pills, and the death of millions of vultures on the Indian subcontinent following the ingestion of the anti-inflammatory drug diclofenac. Antibiotic overuse has led to the emergence of resistant pathogenic bacteria in the environment, and not just in medical settings (Deplege, 2011).

Given the need for new therapeutic approaches to treat gastric ulcer with a low probability of toxicological effects; added to concerns regarding the ecological risks of chemical compounds with therapeutic properties, this study aimed to evaluate the phytochemistry, antiulcer activity, and rodent and environmental toxicity of safflower oil. Our results will serve as the basis for new pharmacological assays involving the Safflower oil treatment for the gastric ulcer diseases.

Material and methods

Drugs and reagents

Carboxymethylcellulose (CMC), lansoprazole and safflower oil from Carthamus tinctorius L. Asteraceae, seed were purchased from Sigma-Aldrich, USA. Safflower oil (test substance) was used at doses of 93.75; 187.5; 375 and 750 mg/kg; whereas lansoprazole (positive control) was administered at a dose of 30 mg/kg. Both substances were diluted in 0.5% CMC and the resulting solutions were administered at a concentration of 10 ml/kg by the oral route (p.o.).

Analysis of safflower oil by dispersive Raman spectroscopy

Safflower oil has been evaluated using dispersive Raman spectroscopy. For that, a portable near-infrared, dispersive Raman system (Dimension P-1 Raman system, Lambda Solutions, Inc., MA, USA), with an 830 nm excitation, adjustable laser of 230 mW, and spectral resolution of about 2 cm^{-1} in the range of 400 to 1800 cm^{-1} was used. The spectrometer was connected to a Raman probe (Vector probe, Lambda Solutions, Inc., MA, USA) of about 3 m long, with band pass and rejection pass filters. The 1320×100 pixel, back-thinned, deep-depleted CCD detector was cooled down (Peltier) to -75°C to decrease thermal noise. For spectral collection, the safflower oil sample was placed in an aluminum sample holder with wells of 5 mm in diameter and 100 μl capacity; then the probe was placed at a 10 mm distance perpendicular to the sample surface (probe’s focal length). The sample spectra of oleic and linoleic acids (Sigma-Aldrich) were also obtained. The signal scattered by the sample spectra was collected in 5 and 10 s scans.
for all samples. Finally, the gross spectra were calibrated, pre-processed and stored for further analysis using Microsoft Excel software (Microsoft Office 2003). For calibration a Raman spectrum of naphthalene was measured and the known Raman shift of selected bands were compared with the respective Raman shifts found in the measurement. When needed, the x-axis was displaced to match the correct band shift. For pre-processing, cosmic rays spikes were manually removed and the background fluorescence was removed by fitting and subtracting a 5th order polynomial to the gross spectrum. No normalization was applied to the data.

**Animals**

We used male albino Swiss mice with an average weight of 30-45 g. They were obtained from the Animal Facility of the Federal University of São Paulo. The animals were acclimated to the vivarium conditions of Santa Cecília University at a temperature of 23 ± 2°C and a 12 h light-dark cycle. Mice had access to Nuivital and water ad libitum. The animals were kept in cages with raised floors of wide mesh to prevent coprophagy. All protocols employed were approved by the Unisanta Institutional Animal Care and Use Committee, following the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993) under reference number 001/2012.

**Hydrochloric acid (HCl)/ethanol-induced gastric ulcer**

The methodology used was as previously described by Mizui and Doteuchi (1983). After a 12 h fasting the different groups of mice were treated orally with 0.5% CMC (10 ml/kg, p.o.) (negative control) (n = 5), lansoprazole 30 mg/kg (10 ml/kg, p.o.) (positive control) (n = 5), or safflower oil at a dose of 750 mg/kg (10 ml/kg, p.o.) (n = 5). One hour after administration of the treatments, gastric damage was induced in animals by oral administration (0.1 ml/20 g body weight) of a solution of 150 mM HCl in 98% ethanol. After one hour the animals were sacrificed and the stomachs removed for s lesion counts, according to Szelenyi and Thiemer (1978). The number of gastric lesions and the stomachs removed for s lesion counts, according to Szelenyi and Thiemer (1978). Statistical differences between experimental groups were determined by analysis of variance (ANOVA) followed by Dunnet’s and Tukey’s tests, with a significance level of $p < 0.05$ for all analyses.

**Determination of gastric secretion**

The assay was performed by the method of Shay (1945). All groups of mice fasted for 24 h, with free access to water. Immediately after pylorus ligature, safflower oil at a dose of 187.5 mg/kg (10 ml/kg, p.o.) (n = 8); Lansoprazole 30 mg/kg (10 ml/kg, p.o.) (n = 8) as positive control or 0.5% CMC (10 ml/kg, p.o.) (negative control) (n = 8) were administered intraduodenally. The animals were killed 4 h later; the abdomen was opened and another ligature was placed around the esophagus, close to the diaphragm. The stomachs were removed and the gastric content collected to determine the total amount of gastric acid (ml) and pH values. Distilled water (5 ml) was added, and the solution obtained was centrifuged at 2000×$g$ for 10 min. Total acid in the gastric secretion was determined in the supernatant volume by titration to pH 7.0 with 0.01 N NaOH.

**Determination of mucus in gastric content**

This assay was performed following the methodology described previously by Sun et al. (1991). Mice fasted for 24 h, under anesthesia, the abdomen incised and the pylorus ligated. The safflower oil at a dose of 187.5 mg/kg (10 ml/kg, p.o.) (n = 8); Lansoprazole 30 mg/kg (10 ml/kg, p.o.) (n = 8) as positive control or 0.5% CMC (10 ml/kg, p.o.) (negative control) (n = 8) were administered intraduodenally (i.d.) after the pylorus ligature. The animals were killed 4 h after the drug treatments. The stomach content was immersed in 10 ml 0.02% alcian blue in 0.16 M sucrose – 0.05 M sodium acetate, pH 5.8, and incubated for 24 h at 20°C. The alcian blue binding extract was centrifuged at 2000×$g$ for 10 min. The absorbance of the supernatant was measured at 615 nm using a light spectrophotometer U/2000 (Hitachi, Japan). The free mucous in the gastric content was calculated from the amount of alcian blue binding (mg/wet tissue [g]).

**Acute toxicity assay**

For an acute toxicity test by single dose, the animals were divided into two groups with $n = 10$ for each group. The negative control group received 0.5% CMC (10 ml/kg, p.o.) and the test group received safflower oil at a dose of 5 g/kg, p.o. diluted in 0.5% CMC at a concentration of 10 ml/kg. Water and food consumption, the mass of waste products (feces and urine), and the body weight of the animals were assessed daily for seven days. At the end of the experimental protocol, the animals were sacrificed and their organs (heart, lung, liver and kidney) were macroscopically analysis (Brito, 1994).

**Ecotoxicity assays with Daphnia similllis**

This assay was performed according to US EPA (2002) guideline, with adaptations proposed by the Brazilian standardization protocol (ABNT, 2004). According to this protocol, neonates...
of Daphnia similis were exposed to several concentrations of safflower oil diluted in 0.5% CMC for a period of 48 h. Four replicates for each concentration and controls were employed, with five organisms being added to each replicate, giving a total of twenty organisms per test concentration. In order to assess the possible effects of the solvent on the test organisms, the highest CMC concentration used for each experiment was simultaneously tested as the solvent control. At the end of 48 h of exposure, immobility and mortality were analyzed and the maximal effective concentration (EC<sub>50</sub>) was calculated using the Trimmed Spearman-Karber statistical method.

### Results and discussion

Dispersive Raman spectroscopy was employed for chemical composition analysis of safflower oil, as this analytical methodology allows a rapid and direct analysis without previous preparation of the samples, and with less waste generation when compared with conventional analytical techniques. The Raman spectra (Fig. 1) showed peak intensities at 1265 and 1655 cm<sup>-1</sup> attributed to in-plane C-H ethylene deformation and C=C alkyl stretching, respectively, characteristic bands of unsaturated fatty acids, and a band with peak at 1300 cm<sup>-1</sup> attributed to in-phase methylene twisting deformation vibration of fatty acids (Baeten et al., 2001). The band at 1442 cm<sup>-1</sup> is attributed to C-H deformation of CH2 of lipids. The ratio of peaks at 1265 and 1301 cm<sup>-1</sup> and at 1442 and 1655 cm<sup>-1</sup> also show a strong relationship with double bond content (Baeten et al., 2001; Silveira et al., 2010), which can be used to characterize the level of unsaturation of vegetable oils. Figure 2 shows a mixture of oleic and linoleic acids in a proportion that match the spectral features of these two compounds and the spectra of safflower oil (35% of oleic acid and 65% of linoleic acid). Moreover, the four peaks observed in the Raman spectral region of 800-1000 cm<sup>-1</sup> clearly showed the presence of linoleic acid in the safflower oil.

According to Rudolph et al. (2012), the seed oil of safflower (Carthamus tinctorius L., Asteraceae) has a high content of monounsaturated oleic and polyunsaturated linoleic acids, and is produced for nutritional as well as medicinal uses, due to its pharmacological properties. Linoleic acid is not synthesized by the human body and should therefore be consumed in the diet. In this study, the high concentrations of linoleic and oleic acids could be verified by the coincidence of the Raman peaks of these compounds found in the spectrum of safflower oil (Fig. 2).

Currently, there are several pharmacological options for the treatment of gastric ulcer disease. Some classes of therapeutic drugs include those that neutralize gastric acid (e.g. aluminum hydroxide and magnesiam), H<sub>2</sub> receptor antagonists (e.g. ranitidine, famotidine, nizatidine) and proton pump inhibitors (PPIs) (e.g. omeprazole, lansoprazole, pantoprazole). However, the use of acid-suppressing medications (H2 antagonists or proton pump inhibitors) was associated with an increased risk of hip fracture in a large, general population (Corley et al., 2010). In addition, Madanick (2011) reported that the US Food and Drug Administration has issued alerts mentioning that PPI may increase the rate of osteoporosis-related fractures, as well as decrease the effectiveness of clopidogrel for preventing serious cardiovascular events. Other concerns include increased rates of pneumonia, Clostridium difficile infection, and others. These data reinforce the need for ongoing studies that concern the development of new molecules that promote improvement in patients with gastric ulcer.

The classical model of gastric ulcer induced by the administration of an ethanol-HCl solution in mice is often used as the initial source for the study of probable substances with gastric mucosal protective properties. It is known that this experimental protocol is capable of reducing the cytoprotection of the gastric mucosa, particularly by reducing the concentration of prostaglandins (PG<sub>E</sub>(2)), which facilitates damage by hydrochloric acid, thereby promoting ulcerogenesis (Amandeep et al., 2012).

The results obtained following the administration of safflower oil (750 mg/kg, p.o.) in the ulcerogenic HCl-ethanol model showed a reduced ulcerogenic lesion index (ILU) (**p < 0.01) in comparison to the negative control group (0.5% CMC) (Fig. 3).
The results of this experimental protocol served as an important evidence to establish gastric ulcer induction of cholinomimetic-treated mice with anti-inflammatory drugs (NSAID)-treatment with four different doses (93.75, 187.5, 375, and 750 mg/kg, p.o.) of safflower oil. In this protocol the safflower oil at doses of 187.5, 350, and 750 mg/kg demonstrated to significantly reduce damage to the gastric lesions when compared to the respective control. Statistical analyzes also show no significant differences between the doses of 750 and 187.5 mg/kg (Fig. 4), a fact crucial to use the dose of 187.5 following the pharmacological tests.

According to Stachowska et al. (2009), unsaturated fatty acids such as linoleic acid can be incorporated into cellular membranes in the form of phospholipids, free fatty acids or cholesterol esters. These are hydrolyzed by the enzyme phospholipase A2, which contributes to the synthesis of eicosanoids such as prostaglandins (PGE$_2$). These PGE$_2$ in turn stimulate the secretion of mucus and mucosal bicarbonate, accelerate cell proliferation, enhance mucosal blood flow, and increase sulfhydryl groups in the mucosa, promoting lysosomal stability as well as the formation of mucosal phospholipids (Amandeep et al., 2012). All these cellular events mean that PGE$_2$ are a large endogenous cytoprotective component of the gastric mucosa. We observed in the pylorus ligature protocols significant effects after administration of Safflower oil (187.5 mg/kg, i.d.), such as the increases of pH values (**$p < 0.01$), gastric juice volume (**$p < 0.01$) and reduction of total gastric acid (**$p < 0.001$), when compared to the negative control value group (Table 1).

Moreover, the safflower oil (187.5 mg/kg, i.d.) administration promotes the increase of mucous (**$p < 0.001$) when compared to the negative control group (Fig. 4). Since safflower contains large amounts of linoleic acid, it is therefore hypothesized that this is the cytoprotective mechanism involved in the pharmacological response observed in the pharmacological assays.

As important as the pharmacological evaluations are, it is also of great importance the evaluation of the toxicological properties of new compounds. Given the positive results obtained following the use of safflower oil in the experimental protocol to induce ulcerogenic lesions, an acute toxicity assay (single dose) was performed to determine the probable toxicological activity of the substance in question, thus providing information about the health risks resulting from short-term exposure through a chosen route. The acute toxicity assay may also provide initial information about the modes of action of a toxic substance (Brito, 1994). In this case, the oral administration of safflower oil at a dose of 5 g/kg (p.o.) did not have any effect on mortality, body weight, water consumption, feeding or excreta volume, when compared to the control group (CMC 0.5%).

**Table 1**  
Effects of safflower oil and lansoprazole administered intraduodenally (i.d.) on the biochemical parameters of gastric juice obtained from pylorus–ligature mice.

<table>
<thead>
<tr>
<th>Treatments (i.d.)</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>pH (units)</th>
<th>Volume gastric juice (ml)</th>
<th>Total gastric acid (m Eq/4 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC 0.5 %</td>
<td>10 ml/kg</td>
<td>08</td>
<td>2.8 ± 0.4</td>
<td>1.44 ± 0.17</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>Lansoprazole</td>
<td>30</td>
<td>08</td>
<td>4.8 ± 0.95***</td>
<td>0.85 ± 0.18***</td>
<td>1.5 ± 0.2***</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>187.5</td>
<td>08</td>
<td>4.4 ± 1.2**</td>
<td>1.78 ± 0.26**</td>
<td>2.0 ± 0.25***</td>
</tr>
</tbody>
</table>

The results are expressed as mean ± SD. ANOVA for pH Units, Volume gastric juice and Total acid gastric with Dunnett’s test: **$p < 0.01$ and ***$p < 0.001$ compared to the negative control group.
The presence of pharmaceuticals in the environment is now being reported worldwide. New data on the source, fate and effects of pharmaceuticals in the environment seem to indicate the possibility of a negative impact on different ecosystems, and also implies a threat to public health. As a result, data from acute and chronic toxicity tests on species belonging to different trophic levels such as bacteria, algae, crustaceans and fish, among others, are required in order to illustrate the adverse effects of environmental exposure to measured concentrations of these contaminants (Santos et al., 2010). Several drugs are detectable in aquatic systems at concentrations in the order of ng/l up to μg/l, and these values may vary under treatment practice, market basis and prevalence of disease (Isidori et al., 2009).

Taking into account the potential environmental risk of safflower oil discharges into aquatic environments, a toxicity assay was performed using the freshwater cladoceran Daphnia similis. The EC50 calculated by Trimmed Spearman Karber was 223.17 mg/l (confidence limits 180.19 - 276.41) (Fig. 6). The European directive 93/67/EEC on risk assessment for new notified substances classifies the substances according to accurate results of toxicity (values of EC50, IC50), namely, substances with EC50 < 0.1 mg/l are classified as “extremely toxic”; between 0.1 and 1 mg/l are classified as “very toxic”; between 1 and 10 mg/l are classified as “toxic”; between 10 and 100 mg/l are classified as “harmful”; and higher than 100 mg/l are considered to be “non-toxic” (CEC, 1996). Based on this directive, safflower oil can be classified as a “non-toxic” substance.

Antiulcerogenic compounds may have environmental toxicity. Ranitidine (an antiulcerogenic, antagonist of H2 receptors) is in the top fifteen of prescribed drugs in various European countries (Fent et al., 2006), and the toxicological and eco-toxicological data of ranitidine and its photoproducts are known. Webb (2001), assessed the toxicity of ranitidine on Daphnia magna and reported an EC50 value for ranitidine of 650 mg/l. Isidori et al. (2009) showed that ranitidine did not show any acute toxicity at the highest concentration tested (100 mg/l) for Brachionus calyciflorus and Ceriodaphnia dubia. However, overall ranitidine toxicity could be linked to its photoproducts. In fact, the EC 50 of the two photoproducts for Brachionus calyciflorus were 0.248 and 0.830 mg/l, while for Ceriodaphnia dubia, the EC50 values were 0.007 and 0.510 mg/l (Isidori et al., 2009).

Based on the data reported herein, we can conclude that safflower oil shows significant antiulcer activity when administered at a dose of 187.5 mg/kg orally and intraduodenally. Its antiulcerogenic activity may be attributed to the presence of linoleic acid, and the probable mechanism might involve its cytoprotective activity in the gastric mucosa. Likewise, safflower oil at a dose of 5 g/kg had no toxic effects on rodent models and according EC50 obtained from Daphnia similis protocols The European directive 93/67/EEC classified the safflower oil as “non-toxic” substance. We conclude that this study provided important data regarding the biochemical, pharmacological, toxicological and environmental effects of safflower oil.

Authors’ contributions

WT, LLG, ARMSB and CDSP designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. WT, LLG and ARMSB contributed in the pharmacological and toxicological assays on rodents. ARS, FSC, FHP, AC and CDSP contributed to the ecotoxicological studies. LS Jr and MTTP contributed to the Raman spectral analyses. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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