Tephrosia sinapou extract reduces inflammatory leukocyte recruitment in mice: effect on oxidative stress, nitric oxide and cytokine production

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Abstract: *Tephrosia toxicaria* (Sw.) Pers., which is currently known as *T. sinapou* (Buc'hoz) A. Chev., Fabaceae, is a source of compounds such as flavonoids, however, few studies addressed the anti-inflammatory and antioxidant effects of *T. sinapou*. Therefore, we evaluated the antioxidant mechanisms of the *T. sinapou* ethyl acetate extract *in vitro*, and whether the extract affects leukocyte recruitment in four models of inflammation and the involvement of nitric oxide and cytokines in its mechanism. In vitro, it was observed that the extract presented hydrogen donating ability to 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH·), 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS+), and also efficiently inhibited iron-dependent and independent lipid peroxidation and iron chelation assays. *In vivo*, it inhibited the recruitment of total leukocytes and neutrophil induced by carrageenin, zymosan, glycogen and lipopolysaccharide in the peritoneal cavity of mice. Two mechanisms were detected: 1) *T. sinapou* effect on leukocyte recruitment depends on nitric oxide since was dose-dependently inhibited by treatment with L-NAME (nitric oxide synthase inhibitor), and 2) the extract also inhibited the production of crucial cytokines for the leukocyte recruitment; tumor necrosis factor α and interleukin-1β. Concluding, *T. sinapou* ethyl acetate extract reduces oxidative stress *in vitro,* and inflammatory leukocyte recruitment by a mechanism related to inhibition of cytokine production, and in a nitric oxide dependent manner *in vivo*.

Introduction

Although not completely understood, there is a link between oxidative stress and inflammation (Bowie & O'Neill, 2000; Valério et al., 2009). A conceivable explanation for such relationship is that free radicals activate the transcription factor NFκB to induce the production of inflammatory mediators, and are also responsible for tissue lesions by activated phagocytes (Gloire et al., 2006). The opposite route seems also to be truth since inflammatory mediators such as cytokines (e.g. tumor necrosis factor α [TNFα]) activate macrophage NADPH oxidase to produce superoxide anion, which in turn generates hydroxyl radical and peroxynitrite (Bowie & O'Neill, 2000; Valério et al., 2009; Vicentini et al., 2011). Thus, there is a two way route linking inflammatory mediators and free radicals, and therefore, the interference with this route by antioxidants such as flavonoids explains their anti-inflammatory activity, and suggests their application or even of extracts containing these molecules as anti-inflammatory agents (Andrade et al., 2010; Valério et al., 2009; Vieira et al., 2011; Vicentini et al., 2011).

In this sense, evidence demonstrates that

flavonoids inhibit inflammation-induced production of free radicals (Casagrande et al., 2006), cytokines (Valério et al., 2009), prostaglandin E (Morikawa et al., 2003), inducible nitric oxide synthase expression and NFκB activation (Vicentini et al., 2011). The modulation of the production of those molecules leads to reduced inflammatory pain (Anjaneyulu & Chopra, 2003; Anjaneyulu & Chopra, 2004; Filho et al., 2008; Valério et al., 2009), edema (Valério et al., 2009), and leukocyte recruitment (Souto et al., 2011). Furthermore, there is evidence that inhibition of superoxide anion production reduces neutrophil recruitment (Hattori et al., 2010). Thus, these data support the anti-inflammatory effects of flavonoids and other antioxidants.

An important component of the inflammatory response is the recruitment of leukocytes (*e.g.* neutrophils) to the inflammatory foci (Favacho et al., 2011; Souto et al., 2011; Valério et al., 2007; Verri at al., 2007; Verri et al., 2010). Many models are used to study leukocyte recruitment, of which the carrageenin stimulus is a widely used model to trigger cytokine-dependent inflammation (Neto et al., 2010). In fact, the carrageenin-induced inflammation is a seminal model in the description of the activity and mechanisms of the many of clinically available drugs (Verri et al., 2006). Furthermore, other models are also used such as the glycogen stimulus, which is used to recruit neutrophils *in vivo* for further *in vitro* analysis. Zymosan is a stimulus that activates toll-like receptor 2 (Guerrero et al., 2006; Guerrero et al., 2008), and LPS is an agonist of toll-like receptor 4 (Alves-Filho et al., 2010). Activation of these receptors induces cytokine-dependent neutrophil recruitment (Alves-Filho et al., 2010; Guerrero et al., 2006; Guerrero et al., 2008).

Neutrophils are recruited by cytokines such as TNFα and Interleukin-1β (IL-1β) and are required to phagocyte and eliminate infectious agents in the inflammatory foci (Lemos et al., 2009; Verri at al., 2007; Verri et al., 2010). These cytokines are crucial in inflammation in a manner that they are virtually involved in every type of inflammation (Lemos et al., 2009; Valério et al., 2007; Verri et al., 2006; Verri et al., 2007; Verri et al., 2008; Verri et al., 2010; Vieira et al., 2009). Moreover, TNFα and IL-1β are clinically-proved therapeutic targets to diminish inflammation as observed in rheumatoid arthritis with infliximab, etanercept, anakinra and other anti-cytokine antibodies, soluble receptors and receptor antagonists (Verri et al., 2006; Verri et al., 2010).

Neutrophil recruitment is also regulated by other molecules such as nitric oxide (NO), which diminishes the expression of adhesion molecules by endothelial cell, therefore, reducing the rolling, adhesion and transmigration of leukocytes (Tsao et al., 1996). NO also induces the internalization of receptors for chemotatic mediators (Paula-Neto et al., 2011). Nevertheless,

excessive inflammation causes tissue damage dependent on metalloproteinases and free radicals produced by neutrophils (Lemos et al., 2009; Verri et al., 2010; Paula-Neto et al., 2011).

The genus *Tephrosia* involves approximately 400 species distributed in warm regions of both hemispheres (Allen & Allen, 1981). Species of this genus are known by the presence of compounds with larvicidal, antiviral, anti-cancer and antioxidant activities (Andel, 2000; Andrei et al., 1997; Andrei et al., 2000; Jang et al., 2003; Niassy et al., 2005; Pezzuto et al., 1999; Saleem et al., 2001; Vasconcelos et al., 2009; Yenesew et al., 2006). Preliminary data on the composition analysis of ethyl acetate extract of *T. sinapou*, also known as *T. toxicaria*, revealed the presence of flavonoids, including novel compounds. Therefore, it was investigated *in vitro* whether *T. sinapou* ethyl acetate extract inhibits oxidative stress and its antioxidant mechanism, and *in vivo* the effect of the extract on leukocyte recruitment and the role of cytokines and nitric oxide in its mechanisms in mice.

Material and Methods

Plant material

Tephrosia sinapou (Buc'hoz) A. Chev., Fabaceae, was cultivated and collected at the Instituto Agronômico de Campinas (IAC), São Paulo, Brazil by S. Myasaka. The identification was performed by A. K. Pastorek in June 2005, and a voucher specimen was deposited at IAC under the number IAC 17211.

Preparation and phytochemical characterization of the extract

The roots of *T. sinapou* were dried and ground with a knife mill and then the powder was submitted to cool exhaustive extraction with ethyl acetate during ten days with cycles of 48 h. The ethyl acetate extract was exhaustively washed with methanol followed by evaporation. NMR and mass spectral analysis were used for preliminary phytochemical characterization of *T. sinapou* ethyl acetate extract.

Drugs and reagents

Drugs and reagents were obtained from the following sources: prednisolone from Ouro fino (Cravinhos-SP, Brazil), carrageenin from FMC Corporation (Philadelphia, PA, USA), ELISA kits to determined murine TNFα and IL-1β from eBioscience (San Diego, CA, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH·), thiobarbituric acid (TBA), linoleic acid, 2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (ABTS+), DMSO, L-NAME (*N*ω-Nitro-L-arginine methyl

ester hydrochloride), and zymosan from Sigma Chemical Co. (St. Lowis, MO, USA). Bathophenanthroline and quercetin at 95% purity from Acros (Pittsburgh, PA, USA). Glycogen from Merk (Whitehouse Station, NJ, USA). Lipopolysaccharide from Cayman (Ann Arbor, MI, USA). KH_2PO_4, Tween 20 and $(NH_4)_2Fe(SO_4)_2$ from Labsynth (Diadema-SP, Brazil). NaOH, sodium citrate, H_2O_2 and methanol from Biotec (Curitiba-PR, Brazil). H_3PO_4, KOH and $Cl_3Fe_6H_2O$ from Vetec (Rio de Janeir-RJ, Brazil). KCl and ethanol from Dinâmica (Diadema-SP, Brazil). Propylene glycol, *n*-buthanol from Nuclear (Diadema-SP, Brazil). Potassium persulfate from Neon (São Paulo-SP, Brazil). EDTA from CAAL (São Paulo-SP, Brazil). HCl from F. Maia (Cotia-SP, Brazil).

Animals

The experiments were performed on male Swiss mice (20-25 g) and Wistar rats (200-250 g) from Universidade Estadual de Londrina, Londrina-PR, Brazil housed in standard clear plastic cages for mice or rats (five per cage) with free access to food and water. All testing was performed between 9 am and 5 pm in a temperature-controlled room. Animals' care and handling procedures were performed in accordance with National Institute of Health Guide for Care and Use of Laboratory Animals, and with the approval of the Ethics Committee of the Universidade Estadual de Londrina (Of. Circ. CEEA No 147/2010 in November 29, 2010, registered under the number CEEA 80/10, process no 31468.2010.84). All efforts were made to minimize the number of animals used and their suffering.

Antioxidant tests in vitro

Determination of DPPH· scavenging activity

The reduction of DPPH· radical was determined by the change in the absorbance at 517 nm (Asuntha et al., 2010; Blois 1958; Casagrande et al., 2007). The *T. sinapou* ethyl acetate extract was diluted with ethanol/propilenoglicol solution (50:50, v/v). Samples of *T. sinapou* ethyl acetate extract (25-800 µg/mL) were added to reaction medium containing 1 mL of acetate buffer 0.1 M (pH 5.5), 1 mL of ethanol and 0.5 mL of ethanolic solution of DPPH· 250 µM. The absorbance values were determined after 20 min of incubation at room temperature. Samples were analysed in triplicate. The positive control was prepared with sample vehicle, and blank was 1 mL of acetate buffer 0.1 M (pH 5.5) and 1.5mL of ethanol. The ability of scavenging DPPH· was calculated by the following equation: Equation I: % of activity = (1- sample absorbance/control absorbance) x 100.

ABTS+ [2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid)] free radical scavenging assay

The antioxidant capacity by free radical ABTS+ was determined by decrease of absorvance at 730 nm (Sánchez et al., 2005). The *T. sinapou* ethyl acetate extract was diluted with dimethylsulfoxide (DMSO). The ABTS+ cation was formed with 7 mM ABTS+ solution and 2,45 mM potassium persufate. The mixture was stored in amber flask and room temperature for 16 h before use. The ABTS+ solution was diluted in phosphate buffer 20 mM (pH 7.4) to obtain an absorvance of 0,7 at 730 nm. Different concentrations of *T. sinapou* ethyl acetate extract (12.5-250 µg/mL) were added in 4 mL of ABTS+ solution. The absorvance was determined after 6 min of incubated at room temperature. The positive control was prepared with 100 µL of DMSO and 4 mL ABTS+ solution and blank prepared with phosphate buffer 20 mM (pH 7.4). Samples were analysed in triplicate. The results were expressed as by the equation I.

Iron-independent lipid peroxidation

In vitro iron-independent lipid peroxidation was determined by the production of lipid hidroperoxides (Lingnert et al., 1979). Firstly, linoleic acid 10 mM was prepared in phosphate buffer 0.1 M (pH 6.5) containing tween 20, and in 2 mL of this solution samples of T. sinapou ethyl acette extract (0.5-160 µg/mL) were added. After 8 h incubation at 37 ºC or without incubation, 0.2 mL of the linoleic acid emulsion containing or not extract sample were added 2 mL of methanol and 6 mL of methanol 60%. The absorbance was determined at 234 nm. The positive control was prepared with sample vehicle (DMSO and ethanol/propilenoglicol [50:50, v/v]) and blank was phosphate buffer 0.1 M (pH 6.5) with tween 20. All measurements were performed in triplicate. The following equation was used: equação II: % activity = 1- (absA after incubation - absA without incubation)/(absC after incubation - absC without incubation) x 100. AbsA is the absorbance of sample, and absC is the absorbance of the control.

Iron-induced lipid peroxidation

The ability of the *T. sinapou* ethyl acetate extract to inhibit *in vitro* lipid peroxidation was evaluated by diminish in the malondialdehide (MDA) formation, which is a secondary product of lipid peroxidation (Buege & Aust, 1978; Casagrande et al., 2006; Rodrigues et al., 2002). Samples of *T. sinapou* ethyl acetate extract (1-160 µg/mL) were added to reaction medium containing 1 mL of reaction medium (KCl 130 mM and tris-HCl 10 mM, pH 7.4) and 2 mM sodium citrate, mitochondria (1 mg of protein) and amonical ferrous sulphate 50 µM. Reaction was incubated at 37 °C

for 30 min. After, MDA formation was determined using 1 mL of thiobarbituric acid (TBA) 1% prepared in NaOH 50 mM, 0.1 mL of NaOH 10 M and 0.5 mL of H_3PO_4 20% followed by incubation for 20 min at 85 °C. The MDA-TBA complex was extracted with 2 mL of n-butanol, centrifuged at 3000 x *g* for 15 min and the absorbance determined at 535 nm. The following controls were used: positive control with sample vehicle (DMSO in ethanol/propilenoglicol; 50:50, v/v), and negative control without sample and iron. Blank was prepared with the reaction medium without mitochondria. The mitochondria were obtained from male Wistar rats and their isolation was performed by differential centrifugation (Pedersen et al., 1978). The mitochondrial protein content was determined by the biuret reaction (Gornal et al., 1949). All measurements were made in triplicate. The inhibition of iron-dependent lipoperoxidation was calculated by the equation I.

Determination of iron-chelating activity using the bathophenanthroline (BPS) assay

The iron-chelating activity was determined using the bathophenanthroline (BPS) assay (Bolanm & Ulvik, 1987). Briefly, $(NH_4)_2Fe(SO_4)_2$ (50 µmol/L), *T. sinapou* ethyl acetate extract (0.125-2 mg/mL diluted in DMSO, and after in a solution of ethanol:propileneglicol; v:v), and BPS (0.2 mmol/L) were added to 2 mL of a reaction medium containing 130 mmol/L de KCl e 10 mmol/L tris-HCl pH 7.4. Fe^{2+} and *T. sinapou* ethyl acetate extract were left in contact for 15 min in the reaction medium prior to the addition of BPS, and after that, all reagents were incubated for 15 min at room temperature. The iron-chelating activity was monitored through the absence of the $Fe_2(BPS)_3$ complex formation. Measurements were performed at 530 to 700 nm. The positive control was prepared with sample vehicle (DMSO and ethanol/propilenoglicol [50:50, v/v]) and blank was prepared with the reaction medium and iron. All measurements were made in triplicate. The iron-chelating activity was calculated by the equation I.

Leukocyte recruitment in mice

Mice were treated with vehicle (20% tween 80 in saline), *T. sinapou* ethyl acetate extract (10-100 mg/kg, *i.p.*) or prednisolone (4 µmol/kg, *p.o.*) 30 min before carrageenin (500 mg/cavity) stimulus. In another set, mice were treated with L-NAME (nitric oxide synthase inhibitor; 10-100 mg/kg, *s.c.*) 30 min before treatment with *T. sinapou* (100 mg/kg, *i.p.*). After 30 min mice received carrageenin (500 µg/mice, *i.p.*) stimulus. It was also evaluated whether *T. sinapou* inhibits the leukocyte recruitment induced by other stimulus: zymosan (100 µg/mice, *i.p.*), glycogen (500 µL of 5% glycogen), and lipopolysaccharide (LPS, 100 ng/mice, *i.p.*). The negative

control of inflammation was saline (200 µL) injection. Six hours after the injection of carrageenin, zymosan, glycogen or LPS, mice were terminally anesthetized, and the peritoneal exudates were harvested by introducing 2 mL of phosphate-buffered saline (PBS) containing 1 mM of EDTA. Total leukocytes counts were performed with a Newbauer chamber, and differential cell counts were carried out on cytocentrifuge slides (Cytospin 3; Shandon Southern Products, Astmoore, UK) stained using the Diff-Quik system (Dade Behring). The results were expressed as the number of total leukocytes or neutrophils (Valério et al., 2007).

Cytokine production

Mice were treated with vehicle (20% tween 80 in saline) or *T. sinapou* ethyl acetate extract (100 mg/kg, *i.p.)* 30 min before carrageenin (500 mg/cavity) stimulus. Two hours after the injection of carrageenin, mice were terminally anesthetized, and the peritoneal exudates were harvested by introducing 1 mL of phosphate-buffered saline (PBS) containing 1 mM of EDTA. TNFα and IL-1b levels were determined as described previously (Verri et al., 2007) by an enzyme-linked immunosorbent assay (ELISA) according to manufacture's instructions (eBioscience).

Statistical analysis

The *in vitro* concentration of *T. sinapou* ethyl acetate extract necessary to inhibit the oxidative process in 50% (IC50) was determined by GraphPad Prism® software, version 3.02, using hyperbolic curve (one site binding and two site binding hyperbole). *In vivo* results are presented as means±SEM. of measurements made on 6 animals in each group, and are representative of 2 separated experiments. *In vitro* results are presented as means±SEM of measurements made on triplicate at each concentration, and are representative of 3 separated experiments. The differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were considered to be significant at $p<0.05$.

Results

Phytochemical characterization of the extract

Preliminary phytochemical analysis of *T. sinapou* ethyl acetate extract by NMR and mass spectral analysis showed the presence of flavonoids: 1) a novel biflavonoid named toxicarine, constituted by glabranine and 5-*O*-methylnitenin units; 2) flavanone: 7-*O*-methylglabranine; 3) rotenoids: tephrosin, rotenolone, deguelin, 6-oxo-6a,12a-dehydrodeguelin, 6-oxo-

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6a,12a-dehydro-α-toxicarol, 6a,12a-dehydrorotenone, rotenonone and villosone; 4) flavanols: tephrowatsin A and quercetol B. Other identified compounds were: 1) pterocarpan: flamichapparin B; 2) coumarins: 2,3-dihydro-*p*-coumaric acid. Two novel compounds were also identified, a substituted benzaldehyde and a chalcone.

T. sinapou *ethyl acetate extract inhibits oxidative stress* in vitro

The *T. sinapou* ethyl acetate extract concentration-dependently scavenged the negatively charged DPPH· radical (Figure 1A) and the positively charged ABTS+ radical (Figure 1B). The IC50 for DPPH· and ABTS+ assays were 142 µg/mL and 54.51 µg/mL, respectively (Figure 1A and 1B). Control quercetin exhibited IC50 of 1.17 μg/mL and 0.82 µg/mL in the DPPH· and ABTS+ tests, respectively.

The extract also concentration-dependently inhibited *in vitro* the lipid peroxidation in the linoleic acid iron-independent assay (Figure 2A), in the iron-dependent peroxidation (Figure 2B) and batophenantroline (Figure 2C), assays with IC50 of 8.53 µg/mL, 20.10 µg/mL and 1.34 mg/mL, respectively. Control quercetin exhibited with IC50 of 0.51 µg/mL, 0.34 µg/mL and 4 µg/mL in the linoleic acid iron-independent lipid peroxidation, iron-dependent peroxidation, and iron chelation assays, respectively.
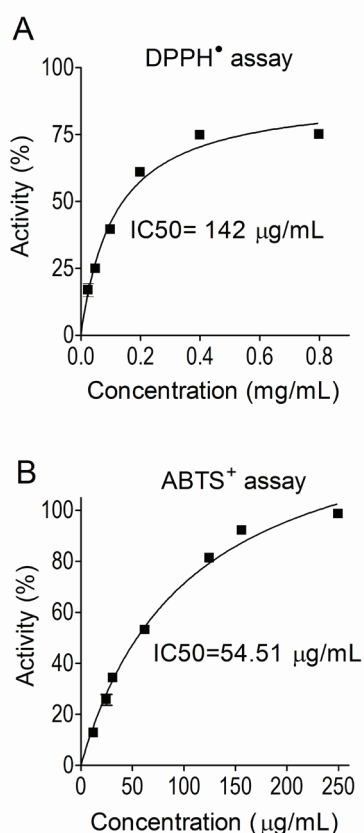

Figure 1. Free radical scavenging activity of *Tephrosia sinapou* ethyl acetate extract. *T. sinapou* ethyl acetate extract was added at indicated concentration and assayed for scavenging negatively (DPPH•; Panel A) and positively (ABTS+, Panel B) assays. Data are present as percentage of inhibition relative to control. Results represent means±SEM of triplicate values representative of three separated experiments.
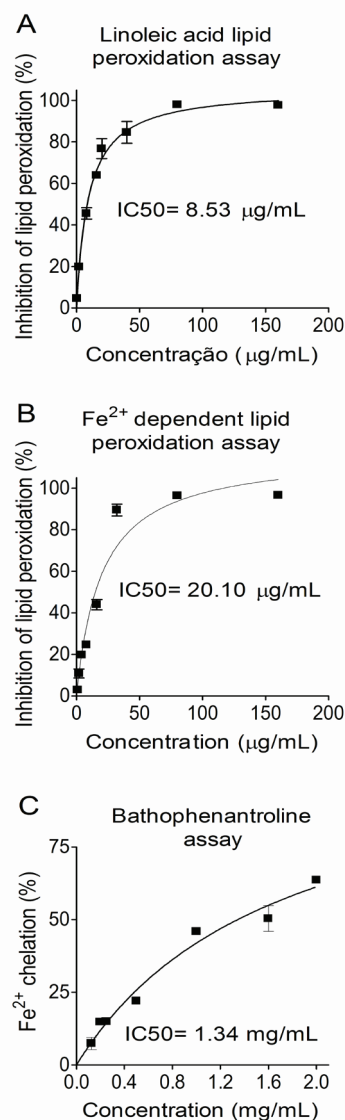

Figure 2. *Tephrosia sinapou* ethyl acetate extract inhibits lipid peroxidation *in vitro* at initiation, propagation, termination stages and iron chelating. *T. sinapou* ethyl acetate extract was added at indicated concentration and assayed for linoleic acid peroxidation (Fe^{2+}-independent peroxidation; Panel A), Fe^{2+}-dependent peroxidation (Panel B) and bathophenatroline assay to determine Fe^{2+} chelation (Panel C). Data are present as percentage of inhibition relative to control. Results represent means±SEM of triplicate values representative of three separated experiments.

T. sinapou *ethyl acetate extract inhibits in a dose-dependent manner the carrageenin-induced leukocyte recruitment in mice.*

There was a significant increase of total leukocytes and neutrophils by carrageenin (500 µg/cavity) stimulus, which was dose-dependently inhibited by *T. sinapou* ethyl acetate extract. The dose of 10 mg/kg of extract did not alter carrageenin-induced cell recruitment. On the other hand, the dose of 30 mg/kg of extract significantly inhibited the cell recruitment (total leukocytes and neutrophils) induced by carrageenin (Figure 3A and 3B, respectively). The inhibition by 100 mg/kg of extract was significant compared to the 10 and

30 mg/kg dose of extract regarding total leukocytes and neutrophil recruitment (Figure 3A and 3B). Therefore, the dose of 100 mg/kg of extract was selected for the next experiment. The control drug prednisolone (4 µmol/kg, corticosteroid) inhibited carrageenin-induced leukocyte and neutrophil recruitment (Figure 3A and 3B).

T. sinapou *ethyl acetate extract inhibits zymosan-, glycogen-, and LPS (lipopolysaccharide)-induced leukocyte recruitment in mice.*

Zymosan (100 µg/cavity), glycogen (500 µL of 5% solution/cavity) and LPS (100 ng/cavity) induced significant total leukocyte and neutrophil recruitment to the peritoneal cavity, which was inhibited by *T. sinapou* ethyl acetate extract (Figure 4A and 4B).
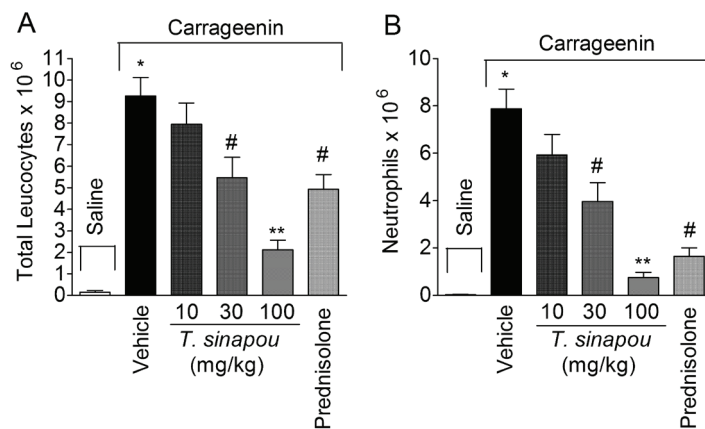

Figure 3. *Tephrosia sinapou* ethyl acetate extract inhibits in a dose-dependent manner the carrageenin-induced leukocyte recruitment. Mice were treated with vehicle (20% tween 80 in saline) or ethyl acetate extract of *T. sinapou* (10-100 mg/kg, intraperitoneal route) 30 min before carrageenin (500 µg/cavity) stimulus. The negative control of inflammation was saline (200 µL) injection, and control drug was prednisolone (4 µmol/kg, 30 min before carrageenin). Total leukocytes (Panel A), and neutrophils (Panel B) were determined 6 h after stimulus injection. Bars represent means±SEM of six mice per group, and are representative of two separated experiments. *$p<0.05$ compared to saline group, #$p<0.05$ compared to vehicle+carrageenin group, and **$p<0.05$ compared to the doses of 10 and 30 mg/kg of extract. One-way ANOVA followed by Tukey's t-test.
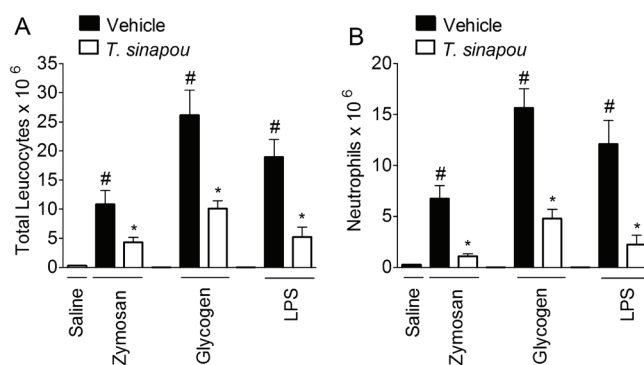

Figure 4. *Tephrosia sinapou* ethyl acetate extract inhibits zymosan-, glycogen-, and LPS-induced leukocyte recruitment. Mice were treated with vehicle (20% tween 80 in saline) or ethyl acetate extract of *T. sinapou* (100 mg/kg, *i.p.*) 30 min before zymosan (100 µg/cavity), glycogen (500 µL of 5% solution/cavity) or LPS (100 ng/cavity) stimulus. The negative control of inflammation was saline injection. Total leukocytes (Panel A), and neutrophils (Panel B) were determined 6 h after stimulus injection. Bars represent means±SEM of six mice per group, and are representative of two separated experiments. *$p<0.05$ compared to saline group, #$p<0.05$ compared to vehicle+stimulus group. One-way ANOVA followed by Tukey's t-test.

T. sinapou *ethyl acetate extract inhibition of carrageenin-induced inflammation depends on nitric oxide.*

The subcutaneous treatment with L-NAME dose-dependently prevented *T. sinapou* anti-inflammatory inhibition of total leukocytes and neutrophil recruitment induced by carrageenin (500 µg/cavity) (Figure 5A and 5B). The doses of 10 and 30 mg/kg of L-NAME presented no significant effect, but the dose of 30 mg/kg showed a tendency of prevention of *T. sinapou* activity. The dose of 100 mg/kg of L-NAME significantly prevented *T. sinapou* inhibition of total leukocytes and neutrophil recruitment compared to T. sinapou extract control and also to L-NAME 10 mg/kg group (Figure 5).

T. sinapou *ethyl acetate extract inhibited carrageenin-induced cytokine production.*

There was a significant increase of TNFα (Figure 6A) and IL-1β (Figure 6B) production in the peritoneal exudates of mice comparing saline control group and carrageenin (500 µg/cavity) stimulus 2 h after injection. On the other hand, the treatment with *T. sinapou* extract (100 mg/kg) significantly inhibited the production of TNFα (Figure 6A) and IL-1β (Figure 6B). It is noteworthy to mention that the carrageenin-induced production of TNFα and IL-1β was almost abolished by *T. sinapou* extract.
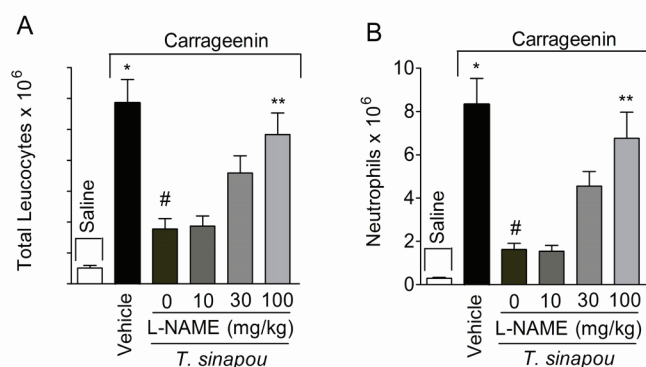

Figure 5. *Tephrosia sinapou* ethyl acetate extract inhibition of leukocyte recruitment depends on nitric oxide. Mice were treated with L-NAME (10-100 mg/kg, *s.c.*, nitric oxide synthase inhibitor) 30 min before *T. sinapou* ethyl acetate extract (100 mg/kg, intraperitoneal route) treatment. After additional 30 min, mice received *i.p.* stimulus with carrageenin (500 µg/cavity). The negative control of inflammation was saline (200 µL) injection. Total leukocytes (Panel A), and neutrophils (Panel B) were determined 6 h after stimulus injection. Bars represent means±SEM of six mice per group, and are representative of two separated experiments. *$p<0.05$ compared to saline group, #$p<0.05$ compared to vehicle+carrageenin group, and **$p<0.05$ compared to extract and lower dose of L-NAME tested. One-way ANOVA followed by Tukey's t-test.
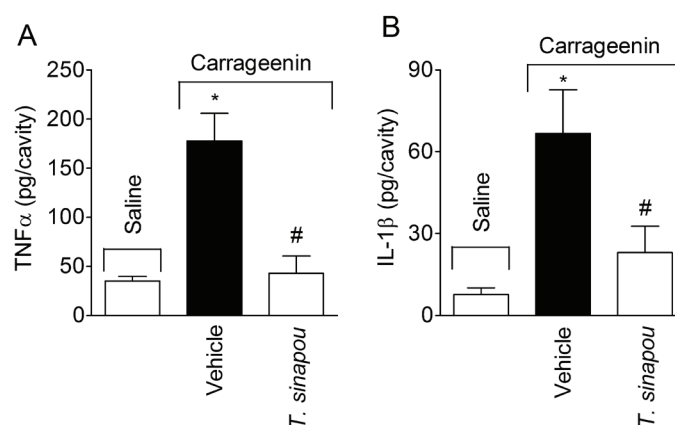

Figure 6. *Tephrosia sinapou* ethyl acetate extract inhibits carrageenin-induced cytokine production. Mice were treated with vehicle (20% tween 80 in saline) or ethyl acetate extract of *T. sinapou* (100 mg/kg, intraperitoneal route) 30 min before carrageenin (500 µg/cavity) stimulus. The negative control of inflammation was saline (200 µL) injection. The production of the cytokines TNFα (Panel A) and IL-1β (Panel B) were determined 2 h after stimulus injection. Bars represent mean±SEM of five mice per group, and are representative of two separated experiments. *$p<0.05$ compared to saline group, and #$p<0.05$ compared to vehicle+carrageenin group. One-way ANOVA followed by Tukey's t-test.

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Discussion

In the present study, it was demonstrated that the ethyl acetate extract of *Tephrosia sinapou* (Buc'hoz) A. Chev., Fabaceae, inhibits inflammatory total leukocytes and neutrophil recruitment induced by a variety of inflammatory stimuli corroborating its wide applicability. Furthermore, the mechanisms of *T. sinapou* involve the inhibition of leukocyte recruitment in a nitric oxide (NO)-dependent manner and also by inhibition of carrageenin-induced production of the chemotatic cytokines TNFα and IL-1β.

There is evidence of pharmacological activity of extracts obtained from plants of *Tephosia* genus including toxic and lavicidal effects (Arriaga et al., 2009a; Lapointe et al., 2003), antifungal (Vijayan et al., 2008), anti-*Helicobacter pylori* (Chinniah et al., 2009), wound healing potential (Lodhi et al., 2006), hepatoprotective against thioacetamide-induced hepatotoxicity (Khatri et al., 2009), antihyperglycemic and antihyperlipidemic in streptozotocin induced diabetic rats (Pavana et al., 2007), alleviates phorbol ester-induced tumor promotion response in murine skin (Saleem et al., 2001), ameliorates *N*-diethylnitrosamine and potassium bromate-mediated renal oxidative stress and toxicity in rats (Khan et al., 2001), reduction in the severity of lithium-pilocarpine induced status epilepticus (Asuntha et al., 2010) and a flavonoidal fraction of *T. purpurea* inhibits sheep red blood cells-induced delayed-type hypersensitivity reactions (Damre et al., 2003). Thus, there are some pharmacological studies on the *Tephosia* genus activities. One report demonstrated the cancer chemopreventive effect of flavonoids from *T. sinapou* (Jang et al., 2003).

Preliminary results detected the presence of flavonoids including novel compounds in *T. sinapou* ethyl acetate extract. Flavonoids are phenolic antioxidant compounds, and this activity explains, at least in part, their other effects on modulation of inflammation. It is consensus that there is a relation between oxidative stress and the production of inflammatory mediators including cytokines. In fact, oxidative stress activates the transcription factor NFκB to induce cytokine production (Vicentini et al., 2011). The present data demonstrates that *T. sinapou* extract inhibited oxidative stress and its antioxidant mechanisms. *T. sinapou* components act as free radical scavengers of negatively and positively charged free radicals, DPPH· and ABTS+, respectively. Furthermore, *T. sinapou* also inhibited *in vitro* the iron-independent lipid peroxidation as observed in the linoleic acid assay, which is a step in the initiation of lipid peroxidation as well as iron-dependent lipid peroxidation that involves propagation and termination events of lipid peroxidation as observed in the malondialdehyde (MDA, product of lipid peroxidation) and iron chelating assays. Herein, we provide evidence that *T. sinapou* ethyl acetate extract dose-dependently inhibited total leukocytes and neutrophil recruitment to the inflammatory foci in the classic model of carrageenin-induced peritonitis. Furthermore, *T. sinapou* also inhibited the recruitment of leukocytes induced by zymosan, glycogen and LPS. Therefore, *T. sinapou* inhibited the leukocyte recruitment induced by a variety of stimuli supporting its possible usefulness.

Mechanistically, *T. sinapou* inhibition of leukocyte recruitment was dependent on NO since was prevented by L-NAME treatment, a NO synthase (NOS) inhibitor. NO is a gas with multiple functions, including the reduction of adhesion molecules expression by endothelial cell during inflammatory conditions (Tsao et al., 1996) and internalization of chemotatic receptors in neutrophil (Paula -Neto et al., 2011).

Cytokines are also important mediators in inflammation responsible for leukocyte recruitment. *T. sinapou* almost abolished carrageenin-induced TNFα and IL-1β production in the peritoneal exudates. Thus, the *T. sinapou* extract inhibited the recruitment of leukocytes by reducing the production of those clinically relevant pro-inflammatory cytokines, TNFα and IL-1β.

At least in part, the pharmacological property of *T. sinapou* extract to inhibit inflammation and oxidative stress might be related to its flavonoidal content as shown for other plants of *Tephrosia* genus (Arriaga et al., 2009b; Hegazy et al., 2009; Khalivulla et al., 2008; Reddy et al., 2009) since flavonoids inhibit oxidative stress, inflammation and/or cytokine production, and present NO-dependent effects (Casagrande et al., 2006; Filho et al., 2008; Georgetti et al., 2008; Souto et al., 2011; Valério et al., 2009; Vicentini et al., 2011).

This study provides pre-clinical evidence of anti-inflammatory activity of an extract of *Tephrosia sinapou*, and demonstrates that the anti-inflammatory mechanisms of *T. sinapou* ethyl acetate extract are related to inhibition of pro-inflammatory cytokine (TNFα and IL-1β) production and NO-dependent inhibition of leukocyte recruitment in mice. Furthermore, *in vitro* data demonstrated that the extract inhibits of oxidative stress by scavenging free radicals, iron chelating activity and inhibition of iron-dependent and iron-independent lipoperoxidation. The extension of these antioxidant mechanisms remains to be determined *in vivo*. Considering that preliminary data detected novel flavonoids in *T. sinapou* extract, we speculate that isolated compounds from Tephrosia genus plants, including *T. sinapou*, are conceivable sources of novel anti-inflammatory compounds worthy of further investigation.

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