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Research Paper

Anti-inflammatory activity and possible mechanism of extract from *Mikania laevigata* in carrageenan-induced peritonitis

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Abstract

Objectives The aim was to test the potential use of an extract of *Mikania laevigata* (popularly known in Brazil as guaco), made from leaves harvested in different months of the year, on neutrophil migration after an inflammatory stimulus and investigate the underlying molecular mechanisms.

Methods We examined the effect of guaco on vascular permeability and leucocyte function in carrageenan-induced peritonitis in mice.

Key findings Our results demonstrated that guaco extract administered subcutaneously (3 mg/kg) decreased the vascular permeability and also leucocyte rolling and adhesion to the inflamed tissues by a mechanism dependent on nitric oxide. Specifically, inhibitors of nitric oxide synthase remarkably abrogated the guaco extract-mediated suppression of neutrophil migration to the inflammatory site. In addition, guaco extract-mediated suppression of neutrophil migration appeared to be dependent on the production of the cytokines interleukin-1β and tumour necrosis factor-α. One of the major constituents of the guaco extract, coumarin, was able to inhibit the neutrophil migration towards the inflammatory focus.

Conclusions In conclusion the anti-inflammatory effect induced by guaco extract may be by inhibition of pro-inflammatory cytokine production at the inflammatory site.

Keywords coumarin; inflammation; *Mikania laevigata*; neutrophil

Introduction

Neutrophils are the main leucocyte subtype participating in organism defence, and their migration from blood vessels into tissue is a crucial process in the host response against microorganism infections.[1] Although they have a protective role in inflammation, tissue damage is a deleterious consequence of the intense neutrophil migration as observed in immune inflammatory diseases.[2]

Neutrophils express abundant adhesion molecules for rapid binding to inflammation-induced counter-receptors on activated endothelial cells.[3] The interaction of recruited neutrophils, at the site of inflammation, with resident cells, local inflammatory mediators or extracellular matrix may lead to the production of several other mediators, including cytokines/chemokines, degrading enzymes, oxygen and nitrogen species and metalloproteases that may further amplify the inflammatory response and injure surrounding tissue.[4–7] Thus, it is now apparent that inhibiting leucocyte trafficking is a very effective strategy for treating inflammatory diseases.[8]

Our group recently demonstrated that nitric oxide (NO) inhibits neutrophil migration by a mechanism dependent on the expression of intercellular adhesion molecule (ICAM)-1 on mesenteric microcirculation vessels of mice subjected to experimental acute peritonitis by an injection of either lipopolysaccharide, carrageenan or *N*-formyl peptide (fMLP).[9] Treatment of experimental peritonitis in mice with chemical inhibitors for NO synthase (NOS) increased the migration of neutrophils into venular endothelium and enhanced the expression of ICAM-1 on the endothelium.[9] Constitutively produced NO normally regulates leucocyte recruitment, and its inhibition increases leucocyte rolling

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and adhesion.[10] Low levels of NO would be expected from activation of the constitutive NOS isoform, and in general decrease vascular permeability.[11] In fact, early nonselective inhibition of NOS after endotoxin challenge increased vascular permeability in a rat model.[12]

In Brazil, the leaves of species of *Mikania laevigata* Schultz Bip. ex Baker, popularly known as 'guaco',[13] have been widely used as infusions or plasters, while the crude extract of this species is commonly commercialized as phytomedicine. Among the few pharmacological and phytochemical studies published, preparations obtained from aerial parts of *Mikania laevigata* have been described as anti-ulcerogenic,[14] antimicrobial,[15,16] antispasmodic, bronchodilatory[17] and anti-inflammatory,[18] possibly accounted for by the presence of several chemical constituents.[16]

It has been demonstrated that the chemical composition of several plants is dependent upon the hour or season of collection, which could negatively influence the development of a commercial drug. Thus, in this study, we assessed the pharmacological properties and the underlying molecular mechanisms of guaco extract, as a putative anti-inflammatory drug, and also its possible toxicological effects.

Material and Methods

Preparation of the guaco extract

Leaves of *Mikania laevigata* were collected at the Farm School of the University of Uberaba (Triângulo Mineiro, MG, Brazil). A voucher specimen (HUFU 54.748) was deposited at the Herbarium of the Federal University of Uberlândia, Brazil. The samples were collected before 0100 h, from December (2006) to November (2007) between days 15 and 17 of each month and allowed to dry at 30°C in an oven with air circulation for 15 days. The dry plant, triturated by knife mill, was extracted by maceration with 70% ethanol–water under continuous agitation (shaker) three times over 7 days, totalling 21 days (the end ratio between plant and solvent was 1:8 (w/v)), to obtain a crude extract. The crude extract was dried, filtered using filter paper and concentrated in an air-forced chamber at 30°C until dry crude extract was obtained.

The relation between the weights of the dry leaves with their respective dry crude extract was calculated. The concentration of coumarin (1,2-benzopyran) in the dry crude extract was measured by spectrophotometry at $\lambda = 320$ nm[19] as well by GCMS.

Pharmacologic assays were carried out using dry crude extract dissolved in saline solution.

GC-MS analysis of the constituents of *M. laevigata*

The sample of the hydroalcoholic guaco extract was diluted in methanol (20 mg/ml). GC-MS analyses were carried out using a gas chromatograph (Hewlett-Packard 6890), equipped with a mass selective detector (Hewlett-Packard 5975) with an HP-5 MS capillary column (25 m × 0.25 mm i.d. × 1.0 μm d.f.), under the following conditions: injection temperature 250°C; detector temperature 280°C; column temperature 110°C, 2 min to 300°C, 10 min; carrier gas He (1.0 ml/min); and split injection. The MS was operated in the EI mode at 70 eV in the m/z range of 42–350. Compounds were identified by comparing the mass spectra according to mass spectra of the standard compounds isolated from *M. laevigata* and *M. glomerata* (dihydrocoumarin, coumarin, spathulenol, kaurenoic acid and ent-beyer15-en-19-oic-acid) and the mass spectral library database (NIST-05). The relative proportions of the constituents were expressed as percentage obtained by peak area normalization; all relative response factors were taken as one.

Animals

Male Swiss mice, 30–35 g, were housed in temperature-controlled rooms (22–25°C) with free access to water and food. All experiments were conducted in accordance with the National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the University of Uberaba (protocol No. 004/2008). The mice were used only in a single experimental group.

Experimental procedure to evaluate neutrophil migration

For the determination of neutrophil migration to the peritoneal cavity, the guaco extract (0.3, 1 or 3 mg/kg) or 0.5 mg/kg of coumarin was administered subcutaneously 30 min before the inflammatory stimulus (intraperitoneal injection of carrageenan (500 μg/cavity)) in mice. In a different set of guaco extract-treated mice (3 mg/kg), aminoguanidine (selective inhibitor of iNOS; 50 mg/kg; Sigma) was administered followed by an intraperitoneal injection of carrageenan (500 μg/cavity; Sigma) 30 min later.

Mice were sacrificed 4 h after carrageenan administration and the peritoneal cavity cells were harvested by washing the cavity with 3 ml of phosphate-buffered saline (PBS) containing EDTA 1 mM. The volume recovered was similar in all experimental groups and equated to approximately 95% of the injected volume. Total counts were performed in a cell counter (COULTER A CT; Coulter, Miami, USA) and differential cell counts (100 cells in total) were carried out on cytocentrifuge (Fanem, São Paulo, Brazil) slides stained with Rosenfeld. The results are presented as the number of neutrophils per cavity.

Determination of serum nitrite concentration

The nitrite concentration in serum samples was determined by enzymatic reduction of nitrate with nitrate reductase, as previously described.[20] Briefly, 50 μl of undiluted serum was incubated with the same volume of reductase buffer (0.1 mol/l potassium phosphate, pH 7.5, containing 1 mmol/l nicotinamide adenine dinucleotide phosphate, 10 mmol/l flavin adenine dinucleotide, and 4 U of nitrate reductase per ml) for 20 h at 37°C. A standard nitrate curve was obtained by incubating sodium nitrate (10–200 μgmol/l) with the reductase buffer. The nitrite concentration was determined using the Griess method.[21] Briefly, 50 μgl of supernatant was incubated with an equal volume of the Griess reagent at

room temperature. The absorbance was measured on a plate scanner (Spectra Max 250; Molecular Devices, Menlo Park, USA) at 540 nm. The NO_2 concentration was determined using a standard curve for 1–200 μgmol/l $NaNO_2$.

Vascular permeability

The vascular permeability was analysed by Evans Blue test, as described previously.[22] Thirty minutes before carrageenan administration, Evans Blue (50 mg/kg) was injected with 100 μgl of saline intravenously into the ocular plexus.

Mice were sacrificed 4 h after carrageenan administration and the peritoneal cavity was washed with 3 ml of PBS. The Evans Blue content was measured at 620 nm using a spectrophotometer (Genesys).

Real time in-situ microscopic analysis for rolling and adhesion events of neutrophils in the mesenteric microcirculation

Two hours after carrageenan injection, leucocyte rolling was assessed as previously described.[23,24] Briefly, mice were anaesthetized and the mesenteric tissue exposed for microscopic examination *in situ*. The mice were maintained on a special board thermostatically controlled at 37°C, keeping the tissue moist and warm by irrigating with Ringer Locke's solution, pH 7.2–7.4, containing 1% gelatin. The postcapillary venules, with a diameter of 10–18 μm were chosen and the interaction of leucocytes with the luminal surface of the venular endothelium was evaluated, counting the number of rolling leucocytes after 10 min. A leucocyte was considered to be adherent to the venular endothelium if it remained stationary for >30 s. Cells were counted in the recorded image using five different fields for each mouse to avoid variability due to sampling. Data were then averaged for each mouse.

Detection of cytokines by ELISA

Mice received guaco extract (3 mg/kg, s.c.) and after 2 h of carrageenan stimulus (500 μg per cavity), peritoneal exudates were recovered for cytokine measurement. Levels of tumour necrosis factor (TNF)-α and interleukin (IL)-1β were determined by ELISA using protocols supplied by the manufacturer (R&D Systems, Minneapolis, USA) for both experiments. The results are expressed as pg/ml.

Statistical analysis

Data were expressed as mean ± SEM. Statistical comparisons between groups were made using analysis of variance followed by the Bonferroni's test. Significance was accepted when $P = 0.05$.

Results

GC-MS analyses

The hydroalcoholic extract of guaco was analysed by GC-MS (Figure 1) and the major compounds identified were coumarin (36.90%) and dihydrocoumarin (32.30%). The relative percentages of identified and isolated compounds in the hydroalcoholic extract are listed in Table 1.

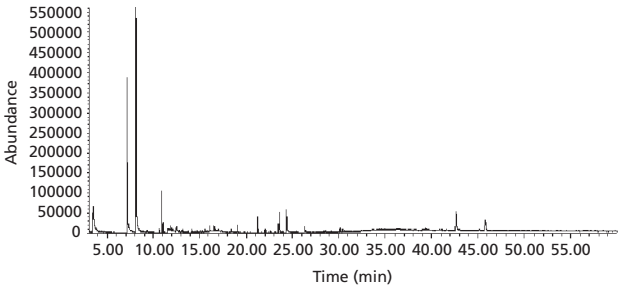

Figure 1 GC-MS chromatogram of the hydroalcoholic extract of *M. laevigata*

Table 1 Relative percentage of the compounds in the *Mikania laevigata* ethanolic extract identified by gas chromatography (GC–MS)

Compound	Retention time (min)	Relative %
Dihydrocoumarin	7.11	32.30
Coumarin	8.14	36.92
Spathulenol	10.92	4.48
Phytol	21.21	2.40
Ent-beyer-15-en-19-oic-acid	24.39	3.32
Lupeol	42.65	5.91
Lupeol acetate	45.80	3.34

Determination of coumarin concentration in the extract from *M. laevigata* collected in each month

The dry residue concentration did not show significant differences among months (2.72 ± 0.38 mg). Besides, after the extraction procedures, the dry crude extract was weighed and no statistical difference detected (4.61 ± 0.64 mg). The coumarin concentration was determined since this substance is a known marker used as reference. The mean concentration of coumarin in the analysed months was 2.08 ± 0.27 mg/ml.

Effect of extract from *M. laevigata* on neutrophil migration and exudate volume induced by carrageenan

Compared with PBS administration, the injection of carrageenan (500 μg/cavity, i.p.) significantly increased neutrophil migration and exudate volume in mice. On the other hand, pretreatment with guaco extract (0.3, 1 or 3 mg/kg, s.c.) decreased neutrophil migration (Figure 2a) in a dose-dependent manner as well as decreasing the exudate volume (Figure 2b) ($P < 0.05$), induced by intraperitoneal injection of carrageenan determined 4 h later.

As coumarin was one of the major constituents found by GC-MS analyses, we investigated whether coumarin may be involved in the anti-inflammatory effect of guaco extract. Mice were pretreated subcutaneously with coumarin (0.5 mg/kg) and neutrophil migration to the inflammatory focus was analysed. Figure 2c shows that coumarin significantly inhibited neutrophil migration to the peritoneal cavity as well as the guaco

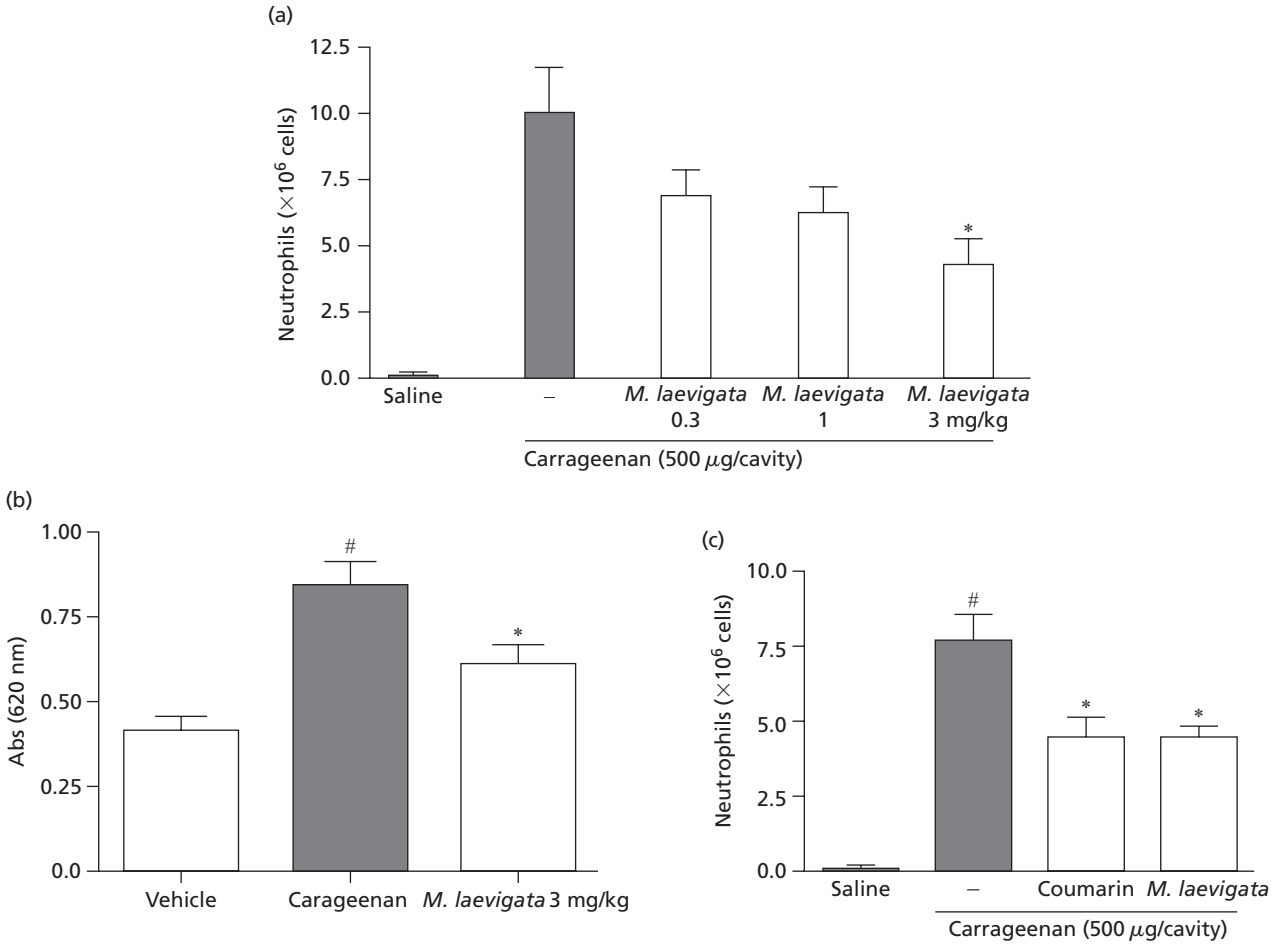

Figure 2 Anti-inflammatory effect of extract from *M. laevigata* on carrageenan-induced peritonitis in mice. Guaco extract reduces neutrophil migration (a) and vascular permeability (b) in carrageenan-induced peritonitis in mice. The mice were treated with saline (0.2 ml, s.c.) or guaco extract (0.3, 1 or 3 mg/kg, s.c., 30 min before) and then injected intraperitoneally with carrageenan at a dose of 500 μg/cavity. (c) Coumarin reduces the neutrophil migration in carrageenan-induced peritonitis in mice. Mice were pretreated subcutaneously (30 min) with saline (Control), coumarin (0.5 mg/kg) or guaco extract (3 mg/kg) and received an intraperitoneal injection of carrageenan (500 μg/cavity). The neutrophil migration was evaluated 4 h later. The values are means ± SEM of eight mice per group. $^{*}P < 0.05$, compared with carrageenan group; $^{\#}P < 0.05$ compared with saline group (analysis of variance followed by Bonferroni's *t*-test).

extract. These experiments strongly suggest that coumarin may be involved in the inhibitory effect of guaco extract.

Influence of month in which the leaves of *M. laevigata* were harvested on neutrophil migration induced by carrageenan in mice

We established that the effective dose of guaco extract to inhibit neutrophil migration is 3 mg/kg, as previously demonstrated (Figure 2a). Thus, in this experiment, this was the concentration used for all guaco extract prepared monthly. As we observe in Figure 3, all monthly harvested guaco extracts similarly inhibited neutrophil migration as compared with carrageenan-injected mice ($P < 0.05$), and no statistical significance was detected between the analysed months ($P > 0.05$).

Effect of the extract from *M. laevigata* on neutrophil rolling and adhesion induced by carrageenan in mice

To clarify the mechanisms by which guaco extract activity modulates neutrophil migration to the inflammatory site, we also investigated its effect on leucocyte–endothelium interactions (rolling and adhesion) in mesenteric postcapillary venules. Carrageenan administration increased rolling (Figure 4a) and adhesion (Figure 4b) of leucocytes to the endothelium, whereas pretreatment with guaco extract significantly decreased this phenomenon. Finally, guaco extract inhibited carrageenan-induced neutrophil migration and adherence (Figures 4a, b). These results suggest that guaco extract activity down regulates neutrophil–endothelium interactions and, consequently, neutrophil migration during the inflammatory process.

Effect of the extract from *M. laevigata* on carrageenan-induced release of neutrophil chemotactic cytokines in mice

Next, we investigated the possible interference of guaco extract on the release of TNF-α and IL-1β cytokines, in an attempt to clarify if the down-regulation of neutrophil migration promoted by guaco extract metabolites was related to a decrease in the release of neutrophil chemotactic mediators. As shown in Figure 5, mice pretreated with guaco

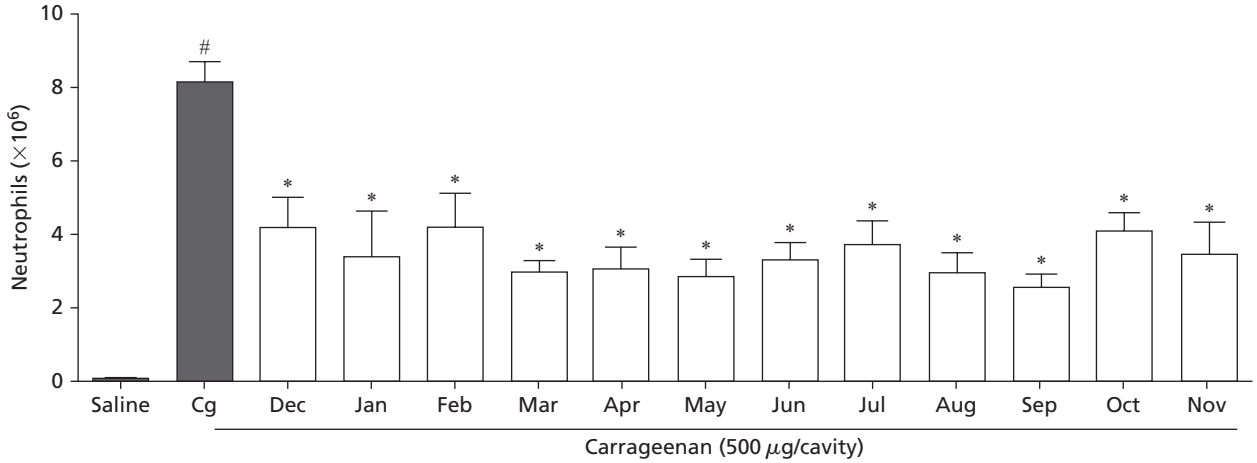

Figure 3 Anti-inflammatory effect of monthly extract of *M. laevigata* in mice. The mice were treated with saline (0.2 ml, s.c.) or guaco extract prepared from *M. laevigata* collected in different months (3 mg/kg, s.c., 30 min before) and then injected intraperitoneally with carrageenan at a dose of 500 μg/cavity. The neutrophil migration was evaluated 4 h later. The values are means ± SEM of eight mice per group. No difference among the months was observed. $^{*}P < 0.05$ compared with carrageenan group (Cg); $^{\#}P < 0.05$ compared with saline group (analysis of variance followed by Bonferroni's *t*-test).

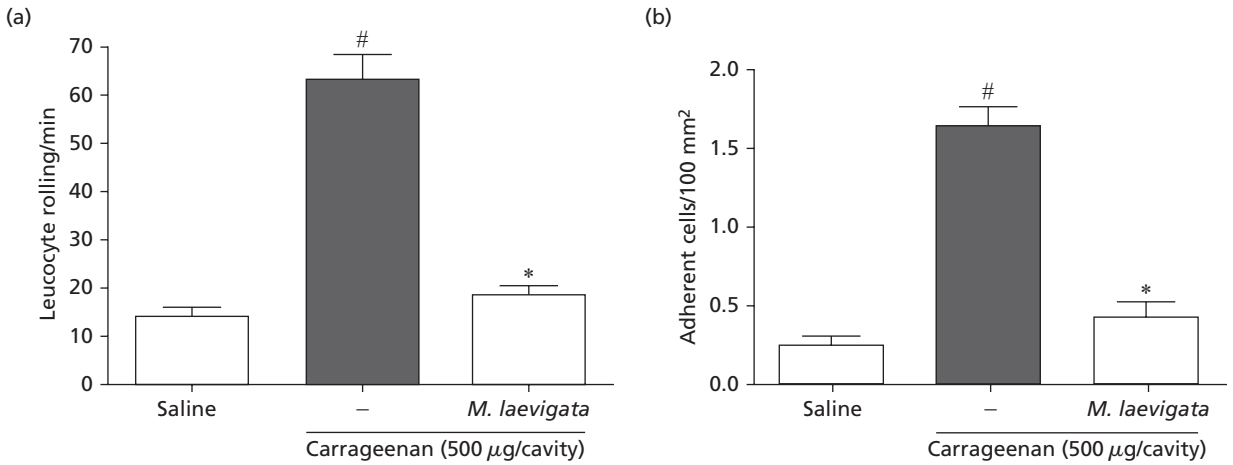

Figure 4 The effect of extract from *M. laevigata* on leucocyte rolling and adhesion to venular endothelial cells of mice. The mice were treated with saline (0.1 ml, s.c.) or guaco extract (3 mg/kg, s.c., 30 min before) and then injected with carrageenan (500 μg/cavity). The leucocyte rolling (a) and adhesion (b) were evaluated by intravital microscopy in the mesentery 4 h after carrageenan injection. The values are means ± SEM. $^{*}P < 0.05$ compared with carrageenan-injected group; $^{\#}P < 0.05$ compared with saline group (analysis of variance followed by Bonferroni's *t*-test).

extract and challenged with carrageenan presented lower levels of cytokines in the peritoneal exudate, when compared with mice pretreated with PBS and injected with carrageenan (Figure 5a, b).

Anti-inflammatory response to extract from *M. laevigata* depends on nitric oxide pathway

To test our hypothesis that guaco induces down-regulation of neutrophil migration by increasing NO production which, in turn, suppresses ICAM-1 expression on micro-vessels, we used an NOS inhibitor (aminoguanidine). First, injection of carrageenan (500 μg/cavity, i.p.) caused a significant increase in leucocyte migration, when compared with injection of PBS (i.p.) in mice (Figure 6a). Pretreatment of the carrageenan-injected mice with aminoguanidine (selective inhibitor of iNOS; 50 mg/kg) completely abrogated the inhibitory effect of guaco extract on neutrophil migration (Figure 6a), whereas aminoguanidine alone had no effect on the increased neutrophil migration induced by carrageenan injection.

To confirm the role of NO on guaco extract-mediated anti-inflammatory activity, we measured the serum nitrite content in the blood of mice injected with carrageenan. The results clearly demonstrated that the mice pretreated with guaco extract had increased nitrite content as compared with PBS-injected mice, confirming that guaco extract activity is modulated by the NO pathway (Figure 6b).

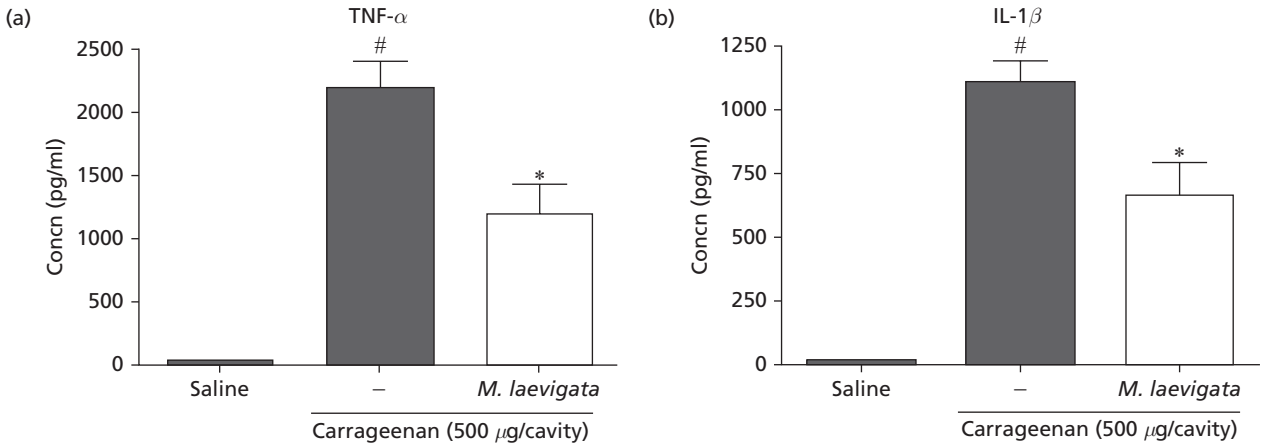

Figure 5 Effect of extract from *M. laevigata* pretreatment on cytokines TNF-α (a) and IL-1β (b) production in peritoneal exudates of mice. Mice were injected with saline (control) or guaco extract (3 mg/kg) and 30 min later injected with carrageenan. The concentrations of the tested cytokines were determined by ELISA. Results are reported as means ± SEM of five mice per group and are representative of two different experiments. $^{*}P < 0.05$ compared with carrageenan-injected group; $^{\#}P < 0.05$ compared with saline group (analysis of variance followed by Bonferroni's *t*-test).

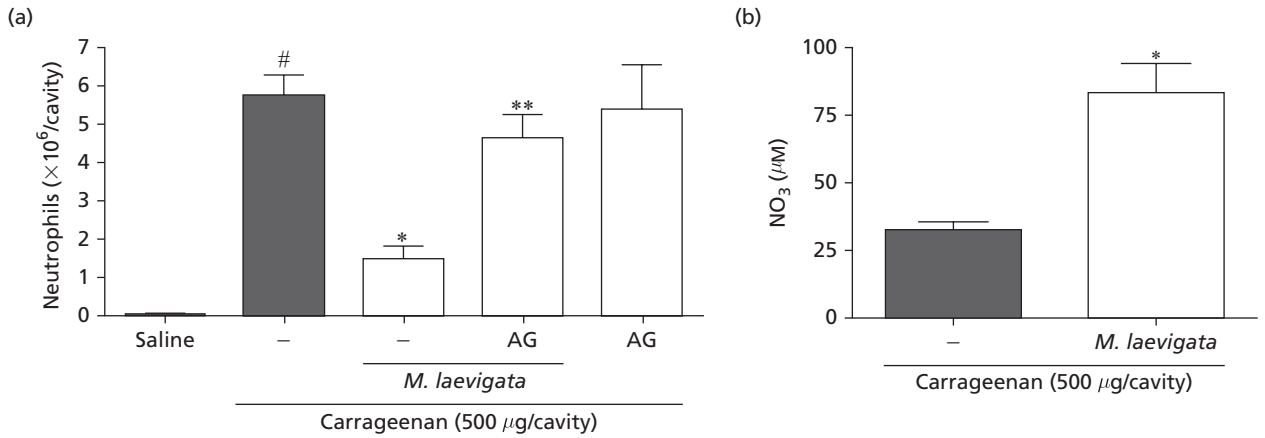

Figure 6 Requirement of nitric oxide generation in the *M. laevigata* extract-mediated suppression of neutrophil migration in response to carrageenan-triggered inflammation. (a) Mice were pretreated subcutaneously with vehicle or aminoguanidine (AG) 15 min before subcutaneous administration of vehicle or guaco extract. After 30 min, mice received an intraperitoneal injection of carrageenan (500 μg per cavity), and neutrophil migration was determined 4 h later. (b) Mice were injected with saline (control) or guaco extract (3 mg/kg) and 30 min later injected with carrageenan. The concentrations of nitrite (NO_3) were determined by Greiss assay. Results are expressed as mean ± SEM of 10 mice per group. $^{\#}P < 0.05$ compared with vehicle-treated mice; $^{*}P < 0.05$ compared with carrageenan-injected mice; $^{**}P < 0.05$ compared with guaco-treated mice (analysis of variance, followed by Bonferroni's test).

Discussion

It is recognized that most of the new drugs discovered in the last few decades have originated from nature.[25] Chemical constituents obtained from medicinal plants and other natural products have been increasingly used to treat many inflammatory diseases.

In this study we demonstrated that the month in which guaco extract is prepared did not influence its anti-inflammatory properties. We also partially elucidated the molecular mechanism underlying guaco-mediated suppression of neutrophil migration toward inflammatory stimuli using a mouse peritonitis model, in which neutrophil migration is a key event. Furthermore, we also showed that this decreased neutrophil migration is associated with guaco-mediated suppression of rolling and adhesion of leucocytes to endothelium and is linked to decreased cytokine production. Moreover, its action is dependent on the NO signalling pathway.

The inflammatory response is orchestrated by a large range of mediators able to promote vascular events, oedema and recruitment of inflammatory cells.[26] Previous studies demonstrated that extracts of *M. laevigata* display marked activity against the mouse allergic pneumonitis model.[27] In this study, we demonstrated that guaco extract was effective in reducing, in a dose-related manner, the neutrophil influx into the peritoneal cavity and this anti-inflammatory activity is related to the NO pathway.

It has been reported that the chemical composition of medicinal plants varies according to the time and the season the plant is collected.[28] The current study demonstrated

that temporal variation (months in which leaves were collected), has no influence on coumarin concentration, and does not substantially alter the basic pharmacological activity of guaco extract. To our knowledge this is the first study evaluating the influence of the month in which guaco extracts were made correlating with its anti-inflammatory activity.

In response to injury or infection, the body mobilizes cells of the immune system to initiate an inflammatory response at the site of damage. A critical step in this response is the adhesion of circulating leucocytes to the endothelial cells lining the blood vessels, allowing their subsequent migration across the endothelial cell barrier to access the insult.[3,29] Here, we demonstrated that guaco extract could inhibit neutrophil migration by different means, such as leucocyte–endothelium interaction (rolling and adhesion) or neutrophil transmigration, and also decreasing vascular permeability. It has become well established that the expression of surface molecules on the vascular endothelium is altered at sites of pathological inflammation.[30] The expression of these molecules is influenced by the cytokine milieu in which the endothelial cells reside. Herein, we sought to determine whether the anti-inflammatory effect of the guaco extract could be associated with the inhibition of pro-inflammatory cytokines (TNF-α and IL-1β). Our findings clearly demonstrated that treatment with guaco extract strikingly prevented the release of both TNF-α and IL-1β in response to carrageenan injection. The inhibition of cytokine expression contributed to a reduction in leucocyte adhesion and transmigration across the endothelium as observed by intravital microscopy.

Many of the effects of inflammatory cytokines elaborated during inflammation are mediated through nitric oxide (NO), which is an important regulator of vascular tone, leucocyte adhesion to microvascular endothelium, and capillary leakage. Activation of the cytokine-inducible NO synthase isoform (iNOS), with consequent over-production of NO has been well documented in animal models.[9] NO synthases are classified into three isoforms: inducible NOS (iNOS); endothelial NOS (eNOS); and neuronal NOS (nNOS).[31] While iNOS gene expression is induced by inflammatory stimuli, eNOS and nNOS genes are constitutively expressed irrespective of stimulation. Therefore, the latter two are also termed constitutive NOS (cNOS). It is now well established that NO plays a key regulatory role in numerous, disparate, physiological and (patho)physiological processes.[32] Since the mouse model of peritonitis induces an acute inflammation, we examined the NO-mediated neutrophil migration suppression, using pharmacological tools. Very importantly, pretreatment of mice with aminoguanidine followed by guaco administration completely abrogated the suppression of neutrophil migration into mesenteric postcapillary venules (Figure 6a) and increase in the nitrite content. These findings indicated that NO produced via iNOS activation is associated with the suppression of neutrophil migration caused by guaco extract.

The absence of effects on body weight gain and behavioral patterns in the mice subjected to the repeated-dose 14-, 28- or 60-day treatment was indicative of no relevant toxicity induced by guaco in mice. There were no alterations in haematological parameters or serum aminotransferases (AST and ALT), indicative of a normal hepatic and biliary function and lack of liver cell injury, as well in urea, indicative of absence of alterations in the kidney.

Thus, we showed that the pharmacological concentration used here presented no toxicity. Also, LD50 (concentration 50% that of lethal dose) was found to be almost 75 times higher than the pharmacological dose tested (data not shown).

Conclusions

In conclusion, we demonstrated that the anti-neutrophil migration effects of extract from *M. laevigata* are associated with NO expression dependent on iNOS activation and also inhibition of the production of cytokines and consequently neutrophil migration. In this context, it is important to strengthen the therapeutic potential of drugs that target leucocyte migration to the inflammatory focus to control either acute or chronic inflammatory diseases. Taken together, our results suggest that use of the medicinal guaco extract may be able to suppress the development of acute inflammatory lesions which are initiated by neutrophil recruitment.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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