Natural populations of *Galphimia* spp. attenuates peripheral and central inflammation

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Abstract

The genus *Galphimia* is widely distributed in Mexico, and is represented by 22 species, including medicinal species. The sedative and anti-inflammatory effects of galphimines produced by the species *Galphimia glauca* have been documented. Formerly, molecular studies using DNA barcodes demonstrated that nine populations botanically classified as *Galphimia glauca* belong to four different species of the genus *Galphimia*, and that only one exhibited the sedative properties; however, all the collected species showed anti-inflammatory activity. Other bioactive compounds like quercetin, galphins, galphimidins and glaucacetalins have been identified from methanolic extracts of plants botanically classified as *Galphimia glauca*. The aim of this work was to determine the anti-inflammatory activity of methanolic extracts of nine collected *Galphimia* spp. populations grown in Mexico. The possible modes of action were analyzed by evaluating the inhibition of LPS-induced inflammation processes both *in vitro* and *in vivo*. The nine populations were evaluated by an *in vitro* model using RAW 264.7 murine macrophage cells, and two populations (a galphimine-producing and a non-galphimine-producing population) were selected for the *in vivo* experiments of systemic inflammation and neuroinflammation in mice. Results suggest that an anti-inflammatory *in vitro* effect was present in all the studied populations, evidenced by the inhibition of nitrite production. An inhibitory systemic inflammation in mice was exerted by the two analyzed populations. In the neuroinflammation model, the anti-inflammatory effect was demonstrated in methanolic extract of the non-galphimine-producing population. For the populations of *Galphimia* spp. studied herein, the anti-inflammatory effect could not be correlated to the presence of galphimines.

Introduction

The genus *Galphimia* (Malpighiaceae) is represented in Mexico by 22 of the 26 existing species worldwide (Anderson 2007). At present, the taxonomic classification of species belonging to the genus *Galphimia* has been challenging and confused. The misunderstanding has been caused by the similarity in the morphology of various of the species, or by mistakes in labelling all specimens from Mexico as *G. gracilis* and *G. glauca* (Anderson 2007). It is reported that plants botanically classified as *G. glauca* have been used since pre-Hispanic times to treat different illnesses, including inflammation and central nervous disorders (Estrada 1985). Many investigations have been conducted to understand the phytochemical and pharmacological properties of *G. glauca* (Dorsch et al. 1992; Tortoriello and Ortega 1993; Müller et al. 1998; del Rayo et al. 2002; Cardoso-Taketa et al. 2004, 2008; Herrera-Ruiz et al. 2006; Náder et al. 2006; Ortíz et al., 2010; Sharma et al., 2012a; Abarca et al., 2014). In Mexico, Doctor Mora, Guanajuato is the locality where the first studies in a natural population botanically classified as *G. glauca* were carried out (Tortoriello and Lozoya 1992; Tortoriello and Ortega 1993; Toscano et al. 1993; Osuna et al. 1999). Subsequent studies showed that plants from this locality have anxiolytic and sedative activities in both, mice (Cardoso-Taketa et al. 2008; Sharma et al. 2012a) and humans (Herrera-Arellano et al. 2007, 2012; Romero-Cerecero et al. 2018). It has been demonstrated that galphimines are the bioactive compounds with the anxiolytic and sedative effects (Tortoriello and Lozoya, 1992; Cardoso-

Taketa et al., 2008; Sharma et al., 2012a). Furthermore, anti-inflammatory effects of these metabolites have been proposed using an *in vivo* model in mice (González-Cortazar et al. 2014). Galphimines constitute a family of 15 (named from A to O) *nor*-secofriedelane-type triterpenes (Toscano et al. 1993; Cardoso-Taketa et al. 2004; Ortega et al. 2020). Additionally, two investigations developed by our group in seven natural populations collected in the states of Chiapas, Guanajuato, Jalisco, Morelos and Querétaro, and botanically classified as *G. glauca*, showed that only two populations produce galphimines (Cardoso-Taketa et al. 2008; Sharma et al. 2012a), exhibiting anxiolytic and sedatives activities in mice; however, all of them had anti-inflammatory activity, using the tetradecanoylphorbol acetate-induced mouse ear inflammation model (TPA) (Sharma et al. 2012a). Other studies have documented the anti-inflammatory activity of *G. glauca* (Müller et al. 1998; González-Cortazar et al. 2014), in extracts and with pure galphimines (González-Cortazar et al. 2014). Other bioactive compounds as methyl gallate, gallic acid, quercetin, tetragalloylquinic acid, ellagic acid, galphins A-C, galphimidin, galphimidin B and glaucacetalins A, D and E, have been isolated from plants botanically classified as *G. glauca* (Dorsch et al. 1992; Neszmélyi et al. 1993; Müller et al. 1998; del Rayo-Camacho et al. 2002; Ortíz et al. 2010; Rios et al. 2020).

In order to clarify the identity of *Galphimia* species, we performed two molecular studies, using DNA barcoding analysis of natural populations botanically classified as *G. glauca*, including the populations studied in the present investigation. These studies suggest the presence of four species of the genus *Galphimia* among the collected populations (Sharma et al. 2012b; Gesto-Borroto et al. 2019). In consequence, it was considered to use the term *Galphimia* spp. to refer to the populations here studied.

In the present investigation, individuals (six per population) from nine natural populations of *Galphimia* spp. were collected in different geographical locations in Mexico; five of these populations were studied for the first time. To determine the anti-inflammatory activity of methanolic extracts of all of these populations, one *in vitro* and two *in vivo* bioassays were used. These bioassays were employed for the first time to evaluate the anti-inflammatory activity from plants of *Galphimia* spp.

Material And Methods

Plant material and extraction

Collection of plant material was made during August and September of 2015 and 2016 in nine different locations of six states of Mexico (Table 1): Doctor Mora, Guanajuato (GM); Jalpan de Serra (QJ) and Cadereyta (QC), Querétaro; Zimapán, Hidalgo (HZ); Cuernavaca (MC), Miacatlán (MM) and Santa Catarina (MS), Morelos; Ciudad Valles, San Luis Potosí (SV) and Valparaíso (ZV), Zacatecas. Six individuals per population were collected, and specimens were deposited at the HUMO Herbarium, CIBYC (Centro de Investigación en Biodiversidad y Conservación), UAEM, Mexico, and at the HGOM Herbarium, CIB (Centro de Investigaciones Biológicas), UAEH, México. The samples (leaves) were dried for 3–4 days in a cool and dry place without direct sunlight, and then powdered by mortar and pestle. Samples were kept under −70°C before to extract preparation. The methanolic extracts were prepared from all nine

populations to evaluate the *in vitro* anti-inflammatory effect of *Galphimia* spp. Pulverized dried material (100 mg) for each sample was mixed with 1 mL MeOH. Samples were vortexed for 2 min, sonicated for 15 min and then centrifuged at 10,000 rpm for 15 min. The material residue containing the pellet was reprocessed four times to achieve exhaustive extraction. The four supernatants were collected, mixed and dried at room temperature. For both *in vivo* anti-inflammatory assays, four samples (5g each) of the pulverized dried material of QC and MS populations were mixed with 50 mL MeOH. Samples were vortexed for 2 min, sonicated for 30 min and then centrifuged at 4,000 rpm for 30 min. Further steps of the procedure were followed as describe above.

Table 1
General data for the studied *Galphimia* spp. populations.

Population	Voucher (No.)	Locality	Date and time of collection	Curator
GM	15189*	Doctor Mora, Guanajuato	August 14, 2015, 11:00–12:00h	Gabriel Flores Franco
QC	35894*	Cadereyta, Querétaro	September 10, 2015, 9:00–10:00h	Gabriel Flores Franco
QC	35894*	Cadereyta, Querétaro	September 10, 2015, 9:00–10:00h N	Gabriel Flores Franco
MC	15011*	Cuernavaca, Morelos	August 24, 2015, 10:00–11:00h	Gabriel Flores Franco
MS	35896*	Santa Catarina, Morelos	August 24, 2015, 12:00–13:00h	Gabriel Flores Franco
MM	15426*	Miacatlán, Morelos	August 31, 2015, 11:00–12:00h	Gabriel Flores Franco
HZ	1483**	Zimapán, Hidalgo	August 20, 2016, 12:00–13:00h	Claudia Hornung Leoni
SV	35895*	Ciudad Valles, San Luis Potosí	September 17, 2016, 10:00–11:00h	Gabriel Flores Franco
ZV	38870*	Valparaíso, Zacatecas	September 17, 2015, 10:00–11:00h	Gabriel Flores Franco

*HUMO Herbarium, CIByC (Centro de Investigación en Biodiversidad y Conservación), UAEM, México.
**HGOM Herbarium, CIB (Centro de Investigaciones Biológicas), UAEH, México.

In vitro anti-inflammatory effect of *Galphimia* spp. in LPS-stimulated macrophages

Cells

RAW 264.7 (ATCC) murine macrophage cells were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F12) (Biowest) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO) without

antibiotics, in a 25 cm^2 flask, at 37°C, and 5% CO_2 atmosphere in a humidified incubator.

Treatment of macrophages with lipopolysaccharide

RAW 264.7 cells (2x10^5 cells/well) were plated into 96-well plates in 180 µL DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated 1 h at 37ºC and 5% CO_2 atmosphere in a humidified incubator (Flores-Vallejo et al. 2020). After that, cells were treated with 20 µL of methanolic extracts from *Galphimia* spp. at four concentration (100, 75, 50 y 25 µg/mL), using dimethyl sulfoxide (Sigma Aldrich) at 0.5 % v/v as vehicle; and aminoguanidine hydrochloride (Sigma Aldrich) at four concentrations (100, 75, 50 y 25 µg/mL) as positive control; and they were incubated 1 h at the same conditions mentioned above. Macrophages were then stimulated with lipopolysaccharide (LPS, 1 µg/mL) from *Escherichia coli* serotype 0111:B4 (Sigma Aldrich) and incubated 24 h to induce the nitric oxide (NO) production (Flores-Vallejo et al. 2020).

Determination of nitrite concentration

Nitrite is a stable product of the NO oxidation and its concentration was determined in the cultured medium via the Griess reaction as an indicator of NO production. Specifically, 100 µL of supernatant from each well was mixed with 100 µL of Griess reagent in separates 96-well plate. After an incubation of 10 min at room temperature, the optical density was determined at 540 nm with a microplate reader SpectraMax® iD3.

Cell viability

Cell viability was assessed using the rezasurin assay (Ahmed et al. 1994). The cell viability assay was performed after the NO determination. Briefly, 180 µL of DMEM/F12 medium supplemented with 10% heat-inactivated FBS and 20 µL of rezasurin 2 mM (Sigma Aldrich) were added to each well, and further incubated for 12 h at 37ºC in a 5% CO_2 humidified incubator. The fluorescence was recorded using an excitation and emission wavelength of 544 and 590 nm, respectively, with a microplate reader SpectraMax® iD3.

In vivo anti-inflammatory effects of *Galphimia* spp. in LPS-treated mice

Mice

Male C57BL/6 mice, 7 to 8 weeks-old (25 g approx.), from a breeding colony of the Instituto de Investigaciones Biomédicas (IIB) at Universidad Nacional Autónoma de México (UNAM) were employed. Mice were divided into groups of five to six animals, and kept in polysulfon boxes with food and water *ad libitum* before and during the experiments. The holding room was maintained at 22 ± 3°C with a 12:12 h light-dark cycle.

All housing and experimental procedures were approved and conducted under the guidelines established by the Institutional Committee on the Care and Use of Experimental Animals of the IIB at UNAM (approval number ID 232).

In vivo anti-inflammatory effect of *Galphimia* spp. extracts in the periphery

A model for systemic LPS-induced inflammation was employed (Qin et al. 2007; Meneses et al. 2016, 2017). Mice received 1 mg/kg of LPS or and equivalent volume of the vehicle (0.9% NaCl; endotoxin-free isotonic saline solution (ISS); PiSA, Guadalajara, Mexico) administered intraperitoneally (i.p.).

Treatments

The peripheral anti-inflammatory activity of methanolic extracts of *Galphimia* spp. was evaluated in LPS treated mice (Fig. 1A). The anti-inflammatory effect of methanolic extracts of *Galphimia* spp. was evaluated employing a galphimine-producer population (QJ) and a non-galphimine-producer population (MS). Two doses of extracts from each population (200 and 600 mg/kg) were injected i.p. after the administration of LPS dose. Two hours later mice were sacrificed by cervical dislocation, and peritoneal fluid was collected to obtain macrophages and dendritic cells.

Flow cytometry

Isolated peritoneal cells were treated following the methodology reported by Meneses et al. (2016, 2017). For analysis, cells were distinguished using antibodies against CD86 and MHC-II, for macrophages, as well as CD86 and CD11b for dendritic cells. Macrophage and dendritic cells activation status was assessed by examining the medium fluorescence intensity of the membrane receptors mentioned above.

Anti-inflammatory effect of *Galphimia* spp. extracts in the LPS-induced neuroinflammation

Mice received 5 mg/kg of LPS or and equivalent volume of the vehicle (0.9% NaCl; endotoxin-free isotonic saline solution (ISS); PiSA, Guadalajara, Mexico) administered i.p.

Treatments

The anti-inflammatory effect of methanolic extracts of *Galphimia* spp. was evaluated employing a galphimine-producer population (QJ) and a non-galphimine-producer population (MS). One dose of each extract (600 mg/kg) was injected i.p. 48 h after the administration of LPS dose. Mice were sacrificed 24 h later by cervical dislocation, and brains were extracted. The expression of ionized calcium binding adaptor molecule (Iba1) and glial fibrillary acidic protein (GFAP), which are expressed specifically in microglia and astrocytes, respectively, was performed by immunofluorescence analysis (Fig. 1B).

Immunofluorescence analysis

For the immunofluorescence analysis, each brain was processed according to the methodology reported by Meneses et al. (2016, 2017). Brain sections were labeled with rabbit anti-GFAP (Invitrogen, Carlsbad, CA, USA), anti-Iba1 (Wako Chemicals, Inc., Richmond, VA, USA) and 4’,6-diamidino-2-phenylindole (DAPI), to detect astrocytes, microglia and nuclei imaging, respectively. Photographs were obtained using a digital camera attached to a light microscope (Nikon Digital Sight DS-Ri1). Regions of the hippocampus (*cornu ammonis* (CA1 and CA2)) and the cortex (CR1 and CR2) were selected and processed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistical analysis

For the analysis of nitrite concentration, and for the anti-inflammatory effect in the systemic inflammation and neuroinflammation models, data were reported as mean ± standard deviation. For nitrite concentration determinations, and for the anti-inflammatory effect in the systemic inflammation model, statistical analysis was done by one-way ANOVA, followed by Dunnett's t-test, p value 0.05 was considered to show significant difference among groups. In the neuroinflammatory experiment, statistical analysis was done by a non-parametric test (Kruskal-Wallis followed by Mann-Whitney U-test), p value 0.05 was considered to show significant differences among groups. All statistical analyses were carried out using the program GraphPad Prism 6.01 (GraphPad Software Inc.).

Results

In vitro inhibition of macrophage activation

Determination of nitrite concentration and cell viability

A significant decrease using one way ANOVA in the production of nitrite (stable product of the NO oxidation) was observed in the cells treated with the four concentrations (25, 50, 75 and 100 µg/mL) of the methanolic extracts of all studied populations of *G. glauca*, in comparison with the cells that were only stimulated with LPS (Fig. 2). A significant difference among the galphimines-producer and non-galphimines producer populations was not demonstrated. The value of nitrite production of macrophages that only were LPS-treated was 9.74 ± 2.21 µM. The highest effect in the inhibition of nitrite production (1.71 ± 0.83 µM) was observed with the HZ population extract at 75 µg/mL. Five populations showed the better results inhibiting the nitrite production in the LPS-stimulated macrophages at 100 µg/mL and 75 µg/mL, from which two (HZ and QJ) are galphimines-producer populations, and three (MM, MS and ZV) are non-galphimines producer populations. At the concentration mentioned above, the methanolic extracts of these five populations, did not showed significant differences in the production of nitrite, in comparison with the cells treated with aminoguanidine (100 µg/mL), or with the control macrophages without LPS (Fig. 2).

The four concentrations of the methanolic extracts were assessed to determinate its effect on the viability of the cell line RAW 264.7. None of the methanolic extracts, at any evaluated concentration showed a significant reduction on viability of the macrophages, in comparison with the cells that did not received any treatment (Fig. 3).

In vivo anti-inflammatory activity

Activation of peritoneal macrophages and dendritic cells

The activation of macrophages was evaluated identifying the CD86 and MHC-II molecules. The statistical analysis by one-way ANOVA allowed to identify a significant reduction in the percentage of macrophages CD86+, CD86+/MHC-II + and the medium fluorescence intensity (MFI), in mice treated with methanolic extracts of both populations; the one that produces galphimines (QJ) and the non-producer (MS), at both

evaluated doses (200 and 600 mg/kg), in comparison with mice that were only LPS-stimulated (Fig. 4C, D and E). The decrement in CD86 + macrophage activation was higher at 600 mg/kg with values of 44.00 ± 13.45% and 47.86 ± 10.78% for MS and QJ, respectively, in comparison with the group that only received LPS (74.16 ± 4.83 %). The same results were obtained in the analysis of macrophages CD86+/MHC-II+, the dose of 600 mg/kg was more effective in the inhibition of the macrophages activation for both populations (MS, 50.04 ± 13.61% and QJ, 53.90 ± 10.51%), in comparison with LPS-stimulated (76.80 ± 4.79%) (Fig. 4D). Besides, the MFI significantly decreased for CD86 + macrophages for the methanolic extracts of both populations at both analyzed doses (Fig. 4E). The reduction of the MFI was higher at 600 mg/kg with values of 986.80 ± 358.70 and 1031 ± 314.40, for MS and QJ populations, respectively.

Dendritic cells activation was analyzed by the detection of the constitutive membrane receptor CD11b and the costimulatory receptor CD86. In the analysis of CD11b+/CD86 + dendritic cells it is not possible to refer to an anti-inflammatory effect, since significant differences between the mice LPS-stimulated, the mice without any treatment, or the mice that only received ISS, were not present. However, the MFI of CD36 was significantly reduced in mice that received the methanolic extracts of both populations evaluated at both doses (Fig. 5C). The decrease of the MFI was higher at the dose of 600 mg/kg for MS (790.85 ± 462.3) and QJ (572.00 ± 252.40) populations, in comparison with mice LPS-treated only (1931.00 ± 440.10) (Fig. 5C). No significant differences was observed for macrophages MHC-II + or dendritic cells CD86 + or CD11b+ (data not shown).

Immunofluorescence analysis of the neuroinflammation model

The anti-inflammatory effect of two methanolic extracts of *Galphimia* spp. was evaluated in an LPS-induced neuroinflammation model. The samples corresponded to a galphimine-producer population (QJ) and a non-galphimine-producer population (MS). Considering the results arose in the experiment of systemic inflammation, only the dose of 600 mg/kg was injected i.p. 48 h after the administration of LPS (5 mg/kg).

The visualization of Iba1 and GFAP proteins, which are expressed specifically in microglia and astrocytes, respectively, was performed by immunofluorescence analysis (Fig. 6). The quantification of Iba1 (Fig. 7) and GFAP (Fig. 8) expression was developed in the hippocampus (CA1 and CA2) and cortex (CR1 and CR2) determining the MFI of two brain sections for each mouse, in order to calculate the area on every photomicrograph and to analyze the resulting data.

The immunofluorescence analysis demonstrated that LPS increased the expression of Iba1 in microglia in the hippocampus (Fig. 7). A statistically significant reduction of the expression of Iba1 in CA1 and CA2 was observed in mice that received the methanolic extract of MS population in comparison with the group in which LPS was only administered (Fig. 7). On the other hand, the ones which received the methanolic extract of QJ population, exhibited a reduction in the expression of Iba1, in CA1 and CA2 regions; however this decrease was not significant different with the MFI of sections from mice treated only with LPS (Fig. 7). In the cortex region, no increased of Iba1 expression was observed (Fig. 7).

In the analysis of the expression of GFAP; LPS was able to increase its presence in the CA1 region. The administration of the methanolic extracts of MS showed a reduction in the expression of GFAP, but was not significant in comparison with the group that received only LPS (Fig. 8). A reduction in the expression of GFAP in mice treated with the methanolic extract of QJ was not observed. For GFAP, neither in CR1 or CR2, an induction of inflammation was observed (Fig. 8).

Discussion

In a previous work carried out by our group, the tetradecanoylphorbol acetate (TPA)-induced mouse ear inflammation test, was performed to determine the anti-inflammatory effect of methanolic extracts of seven populations of the genus *Galphimia* (Sharma et al. 2012a). Four of these populations (GM, MC, MM and QJ) were included in this study, with other five new populations (HZ, QC, MS, SV and ZV) to further evaluate *in vitro*, their capacity to inhibit macrophage activation, and *in vivo* activity against LPS-induced peripheral and central inflammation.

In this study, we first evaluated the methanolic extracts of the nine collected populations of *Galphimia* spp. in an *in vitro* experimental model. Four of these populations (GM, HZ, QC and QJ) produce galphimines, while five (MC, MM, MS, SV and ZV) do not produce these metabolites (Gesto-Borroto et al. 2019). All the studied populations showed a similar inhibitory effect in the nitrite production. Subsequently, a galphimine-producer population (QJ) and a nongalphimine-producer population (MS) were selected to analyze the anti-inflammatory activity in an *in vivo* model of LPS-induced peripheral inflammation. Two doses (200 and 600 mg/kg) of the methanolic extracts were evaluated and the best results were obtained with the highest dose. According to the results of the peripheral inflammation model, the dose of 600 mg/kg was chosen to study the anti-inflammatory effect of the methanolic extracts in an *in vivo* neuroinflammation model.

For the *in vitro* nitrite concentration, a dose-response relationship was not demonstrated among the evaluated concentrations of the studied methanolic extracts. However, the inhibitory effect in the nitrite production increased with the highest concentration (100 µg/mL) of the methanolic extract, in comparison with the lowest one (25 µg/mL). The presence of galphimines in four (GM, HZ, QC and QJ) of the nine population here studied was demonstrated previously by means of TLC analysis (Gesto-Borroto et al. 2019). Even though the anti-inflammatory effect of galphimines have been demonstrated before in the TPA-induced mouse ear inflammation test (González-Cortazar et al. 2014), in the present work the methanolic extracts from the galphimines-producer (GM, HZ, QC, QJ) and non-galphimine-producer (MC, MM, MZ, SV and ZV) populations did not show significant differences in the inhibition of the NO synthesis. This probably indicates that other metabolites in addition to galphimines, are involved in the anti-inflammatory effect of the methanolic extracts of *Galphimia* spp. These results corroborates those formerly obtained by our group, in which similar anti-inflammatory effects from methanolic extracts of galphimines producer and non-producer populations were demonstrated in the TPA-induced mouse ear inflammation model (Sharma et al. 2012a).

Otherwise, the inhibition of the nitrite production at the highest concentrations (100 and 75 µg/mL) of five of the methanolic extracts (HZ, QC, MM, MS and ZV) is similar to the level of nitrite production of amininoguanidine-treated cells or macrophages without any treatment, indicating a reduction of the nitrite concentration to basal levels. Furthermore being aminoguanidine a selective inhibitor of the inducible nitric oxide synthase (iNOS) (Misko et al. 1993; Corbett and McDaniel 1996), these results suggest that methanolic extracts reduced the activity of this enzyme, which is activated in macrophages that differentiate to an M1 phenotype and take part of the inflammatory process (Murray and Wynn 2011; Martinez and Gordon 2014). The methanolic extracts of *Galphimia* spp. did not show a cytotoxic effect over the RAW 264.7 cells, suggesting that the decrease in the nitrite production was due to an inhibition of its synthesis and not to cell death.

Regarding to the *in vivo* model it is well known that CD86 and MHC-II are molecules involved in T cell activation, that act as costimulatory signals and in the presentation of antigens, respectively (Ashley et al. 2012). In the evaluation of macrophages activation, for both methanolic extracts (QJ and MS) at the dose of 600 mg/kg the level of expression of CD86 did not show significant differences with regard to the mice without treatment or to those which only received ISS, hence the methanolic extracts of the population QJ and MS were decreasing to basal levels the expression of CD86 in the membrane of the macrophages (Fig. 4D).

The analysis of dendritic cells activation was performed through the detection of the constitutive membrane receptor CD11b and the costimulatory receptor CD86. CD11b regulates cell adhesion and migration to mediate the inflammatory response (Tan et al. 2000). The statistical analysis by one-way ANOVA showed that the percentage of dendritic cells CD11b+/CD86 decreased significantly in mice that were treated with the methanolic extracts of both populations (QJ and MS) for the dose of 600 mg/kg (Fig. 5B). Nevertheless, it is not possible to clearly associate this phenomenon with an anti-inflammatory effect, since significant differences between the mice LPS-stimulated, the mice without any treatment, or the mice that only received ISS, is not present. However, the significant reduction of the MFI of CD86 in mice which received both doses (200 and 600 mg/kg) of the two methanolic extracts (QJ and MS), in comparison with the mice that only received LPS, could be correlated with an anti-inflammatory effect. The methanolic extracts inhibited the expression of CD86, being greater to the higher doses in both populations; even for the population of QJ was significantly different in comparison with the lower doses of QJ and MS.

The reduction in the activation of macrophages and dendritic cells as a consequence of the actions of the evaluated methanolic extracts (QJ and MS) at both doses (200 and 600 mg/kg) indicates that the LPS-induced inflammatory process was being controlled. This inhibitory effect is better with the higher doses, suggesting that higher proportion of the metabolites with an anti-inflammatory effect present in the methanolic extracts, is required in order to obtain a more effective results. Likewise in the *in vitro* assay, in most of the evaluations performed, no significant differences were found among the galphimine-producer population (QJ) and the non-galphimine-producer population (MS); consequently

the anti-inflammatory effect in *Galphimia* spp. populations is a complex response probably due to the presence of several metabolites.

The effect of *Galphimia* spp. in reducing microglia/macrophages activation is consistent with the macrophages activation in the periphery. Microglia are involved in the immune defense of the brain and also contribute maintaining homeostasis. These cells could change their phenotype to an activated form, when the homeostasis in central nervous system is disrupted by different kind of damages. The uncontrolled activation of microglia induces the production of pro-inflammatory cytokines and cytotoxic mediators, which are factors implicated in neuropathological conditions (Liu et al. 2011; Meneses et al. 2017; Kabba et al. 2018; Vainchtein and Molofsky 2020). The results of this work suggest that the methanolic extract of MS population control the LPS-induced excessive activation of microglia.

In this study, we confirmed the inhibition in the expression of Iba1 protein, in mice that received the methanolic extract of MS population, suggesting a decreased of microglia/macrophages activation. Nevertheless, an inhibitory effect of the methanolic extract of QJ population was not observed, neither in Iba1 or GFAP expression. Similarly to the evaluations previously shown, no significance differences were found among the galphimine-producer population (QJ) and the non-galphimine-producing population (MS); consequently the anti-inflammatory effect it is not potentiated by galphimines. Furthermore, in the neuroinflammation model, no anti-inflammatory effect was demonstrated for the methanolic extracts of the galphimine-producer population (QJ).

Conclusion

In this work we studied nine natural population of *Galphimia* spp., taking into account an integrative approach involving *in vitro* and *in vivo* anti-inflammatory analyses. It was possible to demonstrate the anti-inflammatory activity of crude extracts from all the collected populations of *Galphimia* spp. in the *in vitro* model, as well as from two selected populations in two *in vivo* models, which were used by the first time with the genus *Galphimia*. The results obtained in this study support the potential use of methanolic extracts of *Galphimia* spp., as an alternative to treat central nervous system inflammation. The current research enabled to enhance the knowledge concerning *Galphimia* species in the light of its pharmacological activity. For further investigations it is necessary to identify the presence of diverse secondary metabolites with anti-inflammatory activity in the methanolic extracts of the natural populations of *Galphimia* spp., in order to develop a comprehensive analysis on the important anti-inflammatory properties of crude extracts from plants of *Galphimia* species.

Declarations

Funding

Not applicable

Authors' contributions

RGB, GM, AEC, ACT, ES and MLV conceptualized the experimental procedures. RGB, GM, AEC, GG and JCT carried out the experiments. RGB, GM, AEC, ACT, ES and MLV performed the data analysis. RGB and MLV prepared the first draft of the manuscript. RGB, GM, AEC, ACT, ES and MLV edited and revised the final draft of the manuscript. All authors approved the final version for submission.

Ethics approval

All housing and experimental procedures were approved and conducted under the guidelines established by the Institutional Committee for the Care and Use of Experimental Animals of the Instituto de Investigaciones Biomédicas at Universidad Nacional Autónoma de México (approval number ID 232).

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Conflicts of interest/Competing interests

The authors report no conflict of interest.

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Figures

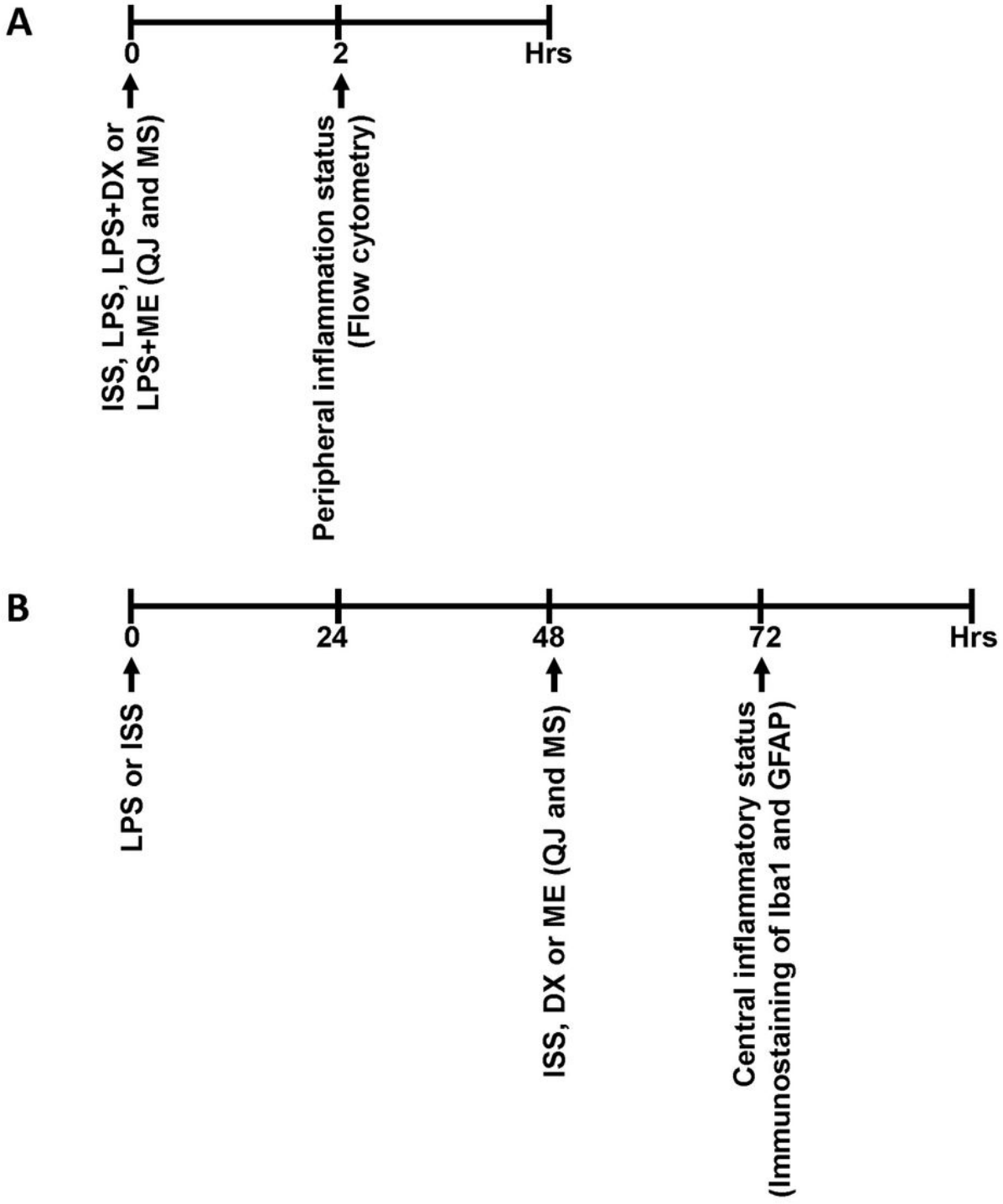


Figure 1

Experimental design. (A) Groups of five to six mice received lipopolysaccharide (LPS), isotonic saline solution (ISS) or LPS+methanolic extracts (ME) injected intraperitoneally (i.p.) (Hour 0). Two hours later, mice were sacrificed and peripheral inflammation status was evaluated. (B) Groups of five to six mice received LPS or ISS injected i.p (Hour 0). Twenty four hours later mice were treated with ISS or ME, i.p.

Seventy two hours later mice were sacrificed and the expression of ionized calcium binding adaptor molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP) was studied by immunostaining.

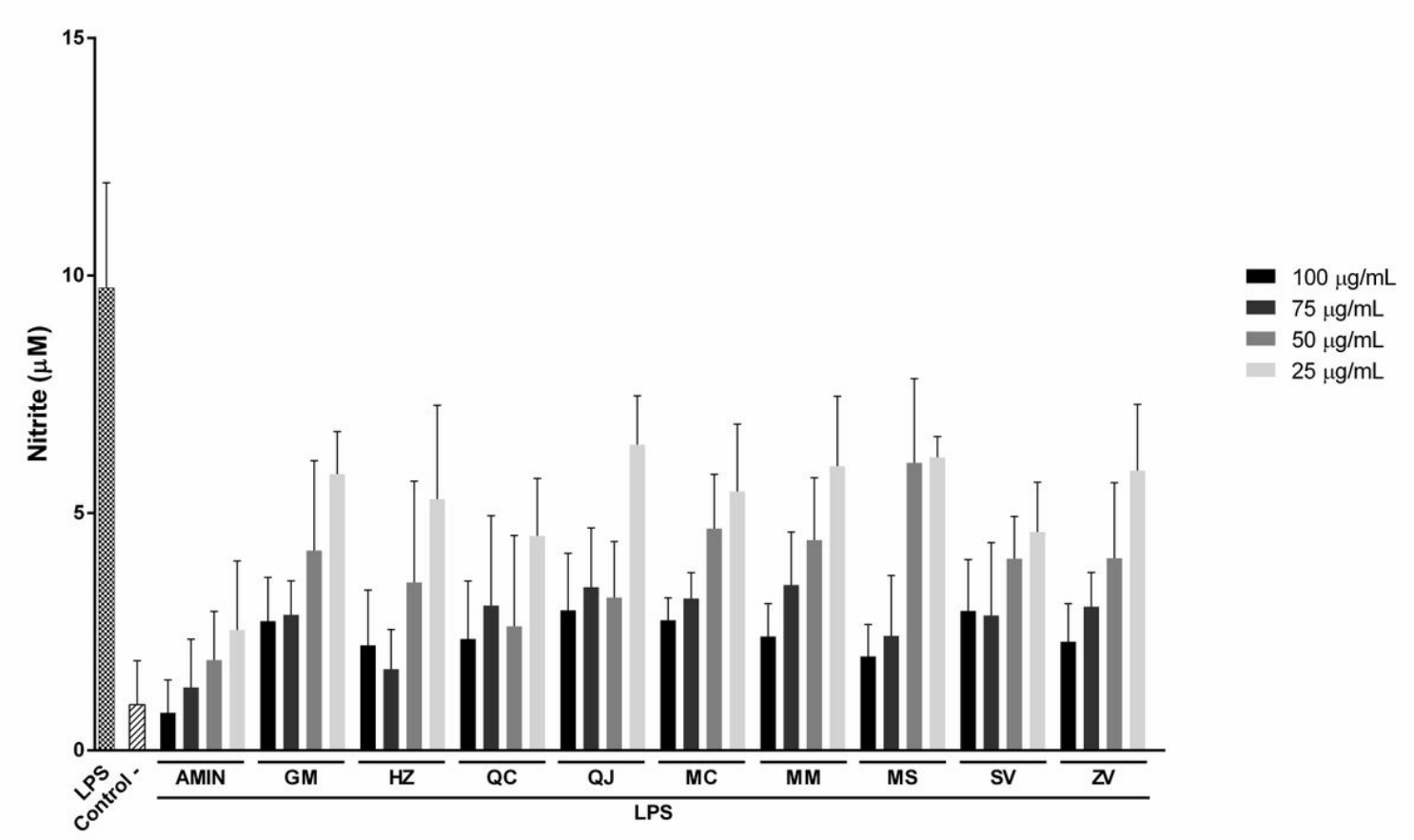


Figure 2

Effect of the methanolic extracts (100, 75, 50 and 25 μg/mL) of Galphimia spp. on nitrite production of the LPS-treated RAW 264.7 murine macrophage cell line. A statistically significant decrease in the production of nitrite was observed in the cells treated with the four concentrations of the methanolic extracts of all populations studied, in comparison with the cells that only were stimulated with LPS. Dr. Mora, Guanajuato (GM); Zimapán, Hidalgo (HZ); Cadereyta (QC) y Jalpan de Serra (QJ), Querétaro; Cuernavaca (MC), Santa Catarina (MS) y Miacatlán (MM), Morelos; Ciudad Valles (SV), San Luis Potosí y Valparaíso, Zacatecas (ZV). AMIN: aminoguanidine hydrochloride. LPS: lipopolysaccharide. Control -: non-treated. The nitrite concentration values correspond to the mean ± standard deviation. Significance was determined by one-way ANOVA ($p<0.05$, compared to LPS).

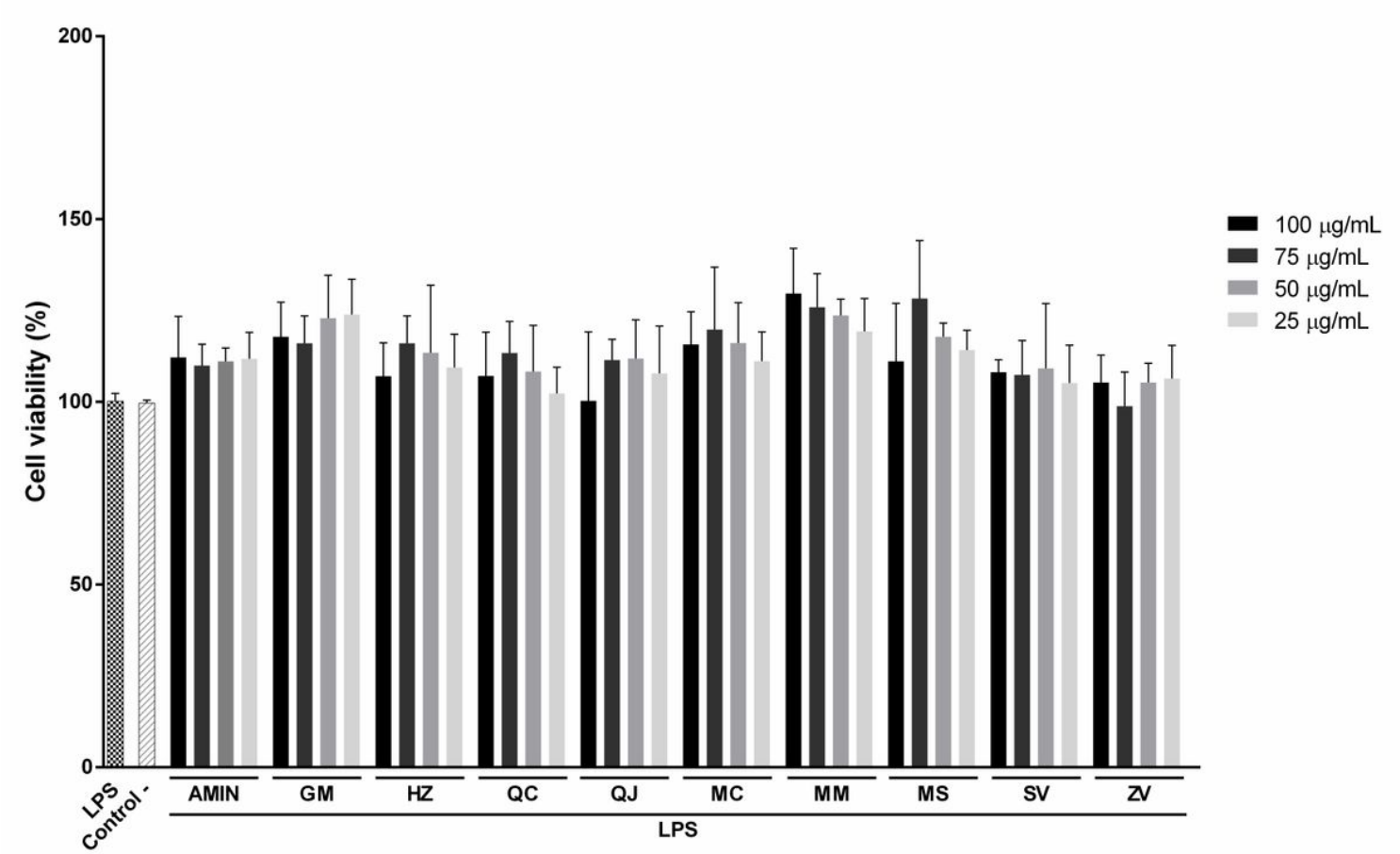


Figure 3

Effect of the methanolic extracts (100, 75, 50 and 25 µg/mL) of Galphimia spp. on the viability of the LPS-treated RAW 264.7 murine macrophage cell line. None of the methanolic extracts show a significant reduction on viability. Dr. Mora, Guanajuato (GM); Zimapán, Hidalgo (HZ); Cadereyta (QC) y Jalpan de Serra (QJ), Querétaro; Cuernavaca (MC), Santa Catarina (MS) y Miacatlán (MM), Morelos; Ciudad Valles (SV), San Luis Potosí y Valparaíso, Zacatecas (ZV). AMIN: aminoguanidine hydrochloride. LPS: lipopolysaccharide. Control -: non-treated. The cell viability values correspond to the mean ± standard deviation. Significance was determined by one-way ANOVA ($p<0.05$, compared to LPS).

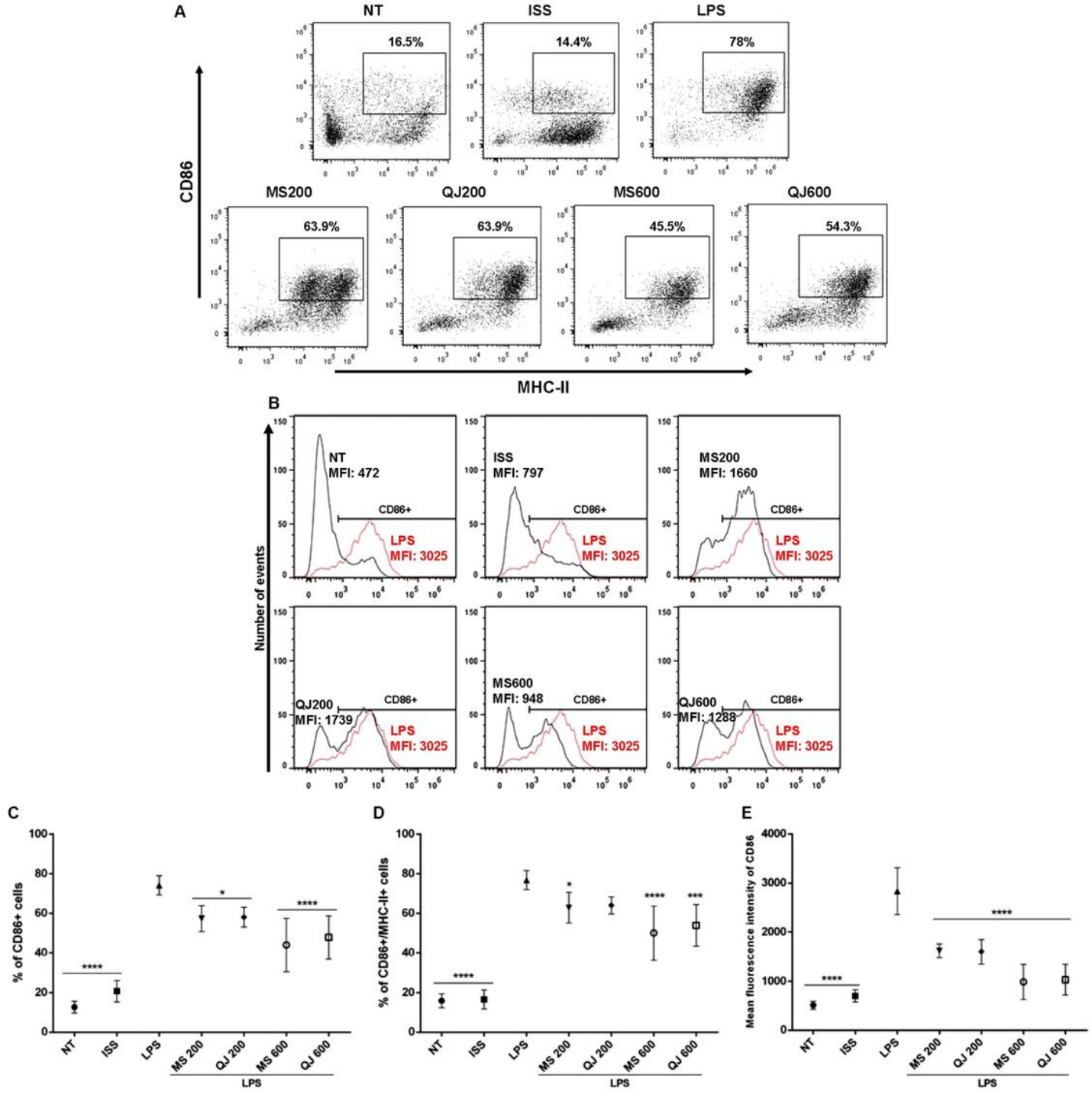


Figure 4

(A) Representative dot plot of peritoneal macrophages analyzed by flow cytometry, (B) representative histograms of medium fluorescence intensity (MFI) of CD86 of each treatment in comparison with lipopolysaccharide (LPS) treated mice, (C) percentage of CD86+ and (D) CD86+/MHC-II+, (E) MFI of CD86. NT: non-treated; ISS: isotonic saline solution; MS200 y MS600: methanolic extracts of the population of Santa Catarina, Morelos, 200 and 600 mg/kg, respectively; QJ200 y QJ600: methanolic

extracts of the population of Jalpan de Serra, Querétaro, 200 y 600 mg/kg, respectively. The percentage and MFI values correspond to the mean ± standard deviation. Significance was determined by one-way ANOVA ($p<0.05$, compared to LPS).

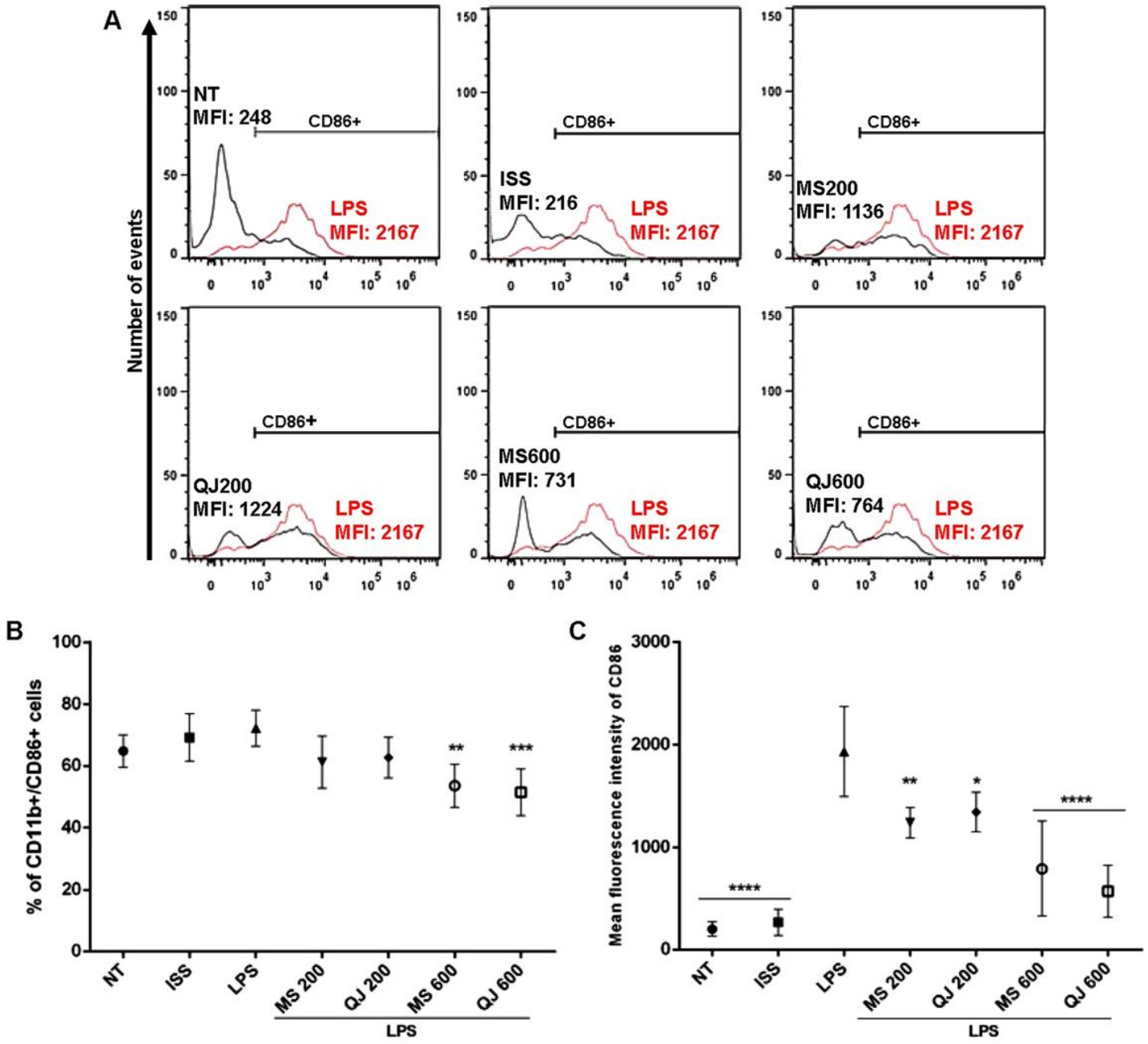


Figure 5

(A) Representative histograms of medium fluorescence intensity (MFI) of CD86 (peritoneal dendritic cells) of each treatment in comparison with lipopolysaccharide (LPS) treated mice, (B) percentage of CD11b+/CD86+ peritoneal dendritic cells, (C) MFI of CD86. NT: non-treated; ISS: isotonic saline solution; MS200 y MS600: methanolic extracts of the population of Santa Catarina, Morelos, 200 and 600 mg/kg, respectively; QJ200 y QJ600: methanolic extracts of the population of Jalpan de Serra, Querétaro, 200 y

600 mg/kg, respectively. The percentage and MFI values correspond to the mean ± standard deviation. Significance was determined by one-way ANOVA ($p<0.05$, compared to LPS).

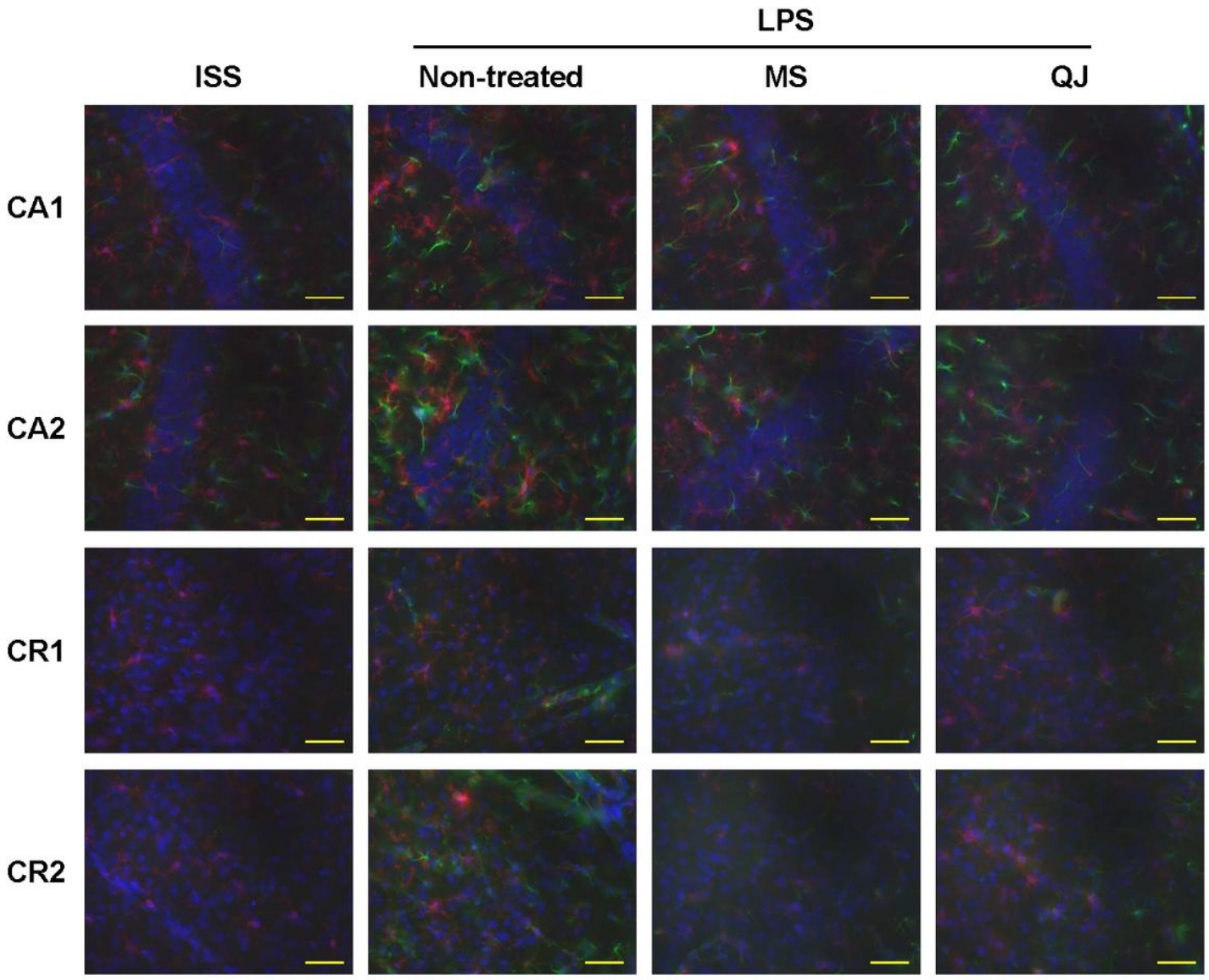
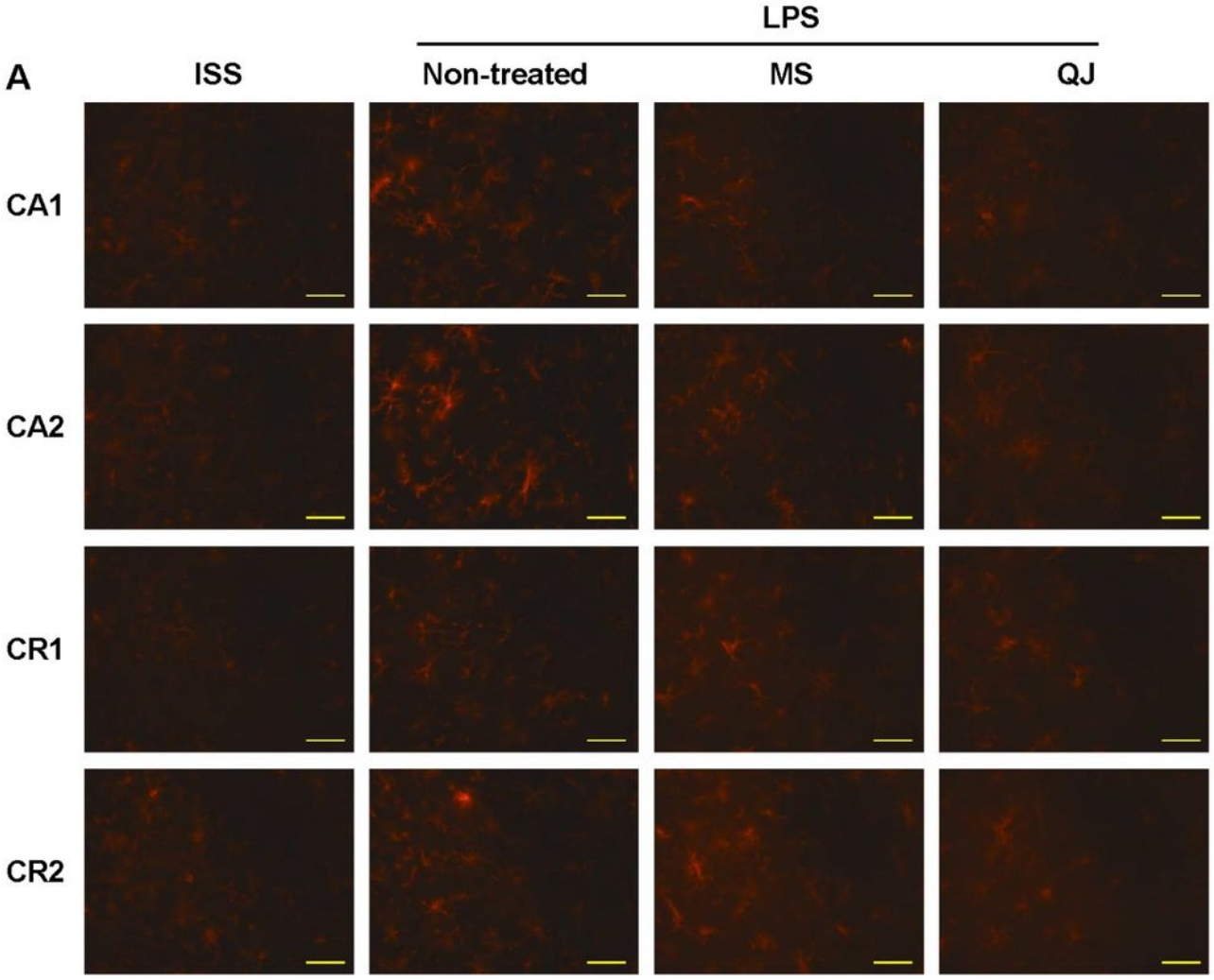


Figure 6

Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule (Iba1) expression in the four studied groups (mice that received an i.p. administration of ISS, LPS, or methanolic extracts of MS and QJ populations) of the neuroinflammation model experiment. Representative immunofluorescence of 50 µm coronal sections of mouse brain of the different groups stained with anti-Iba 1 (red) and anti-GFAP antibodies (green) and 4’,6-diamidino-2-phenylindole (DAPI) (blue nuclei). Scale bar 200 µm



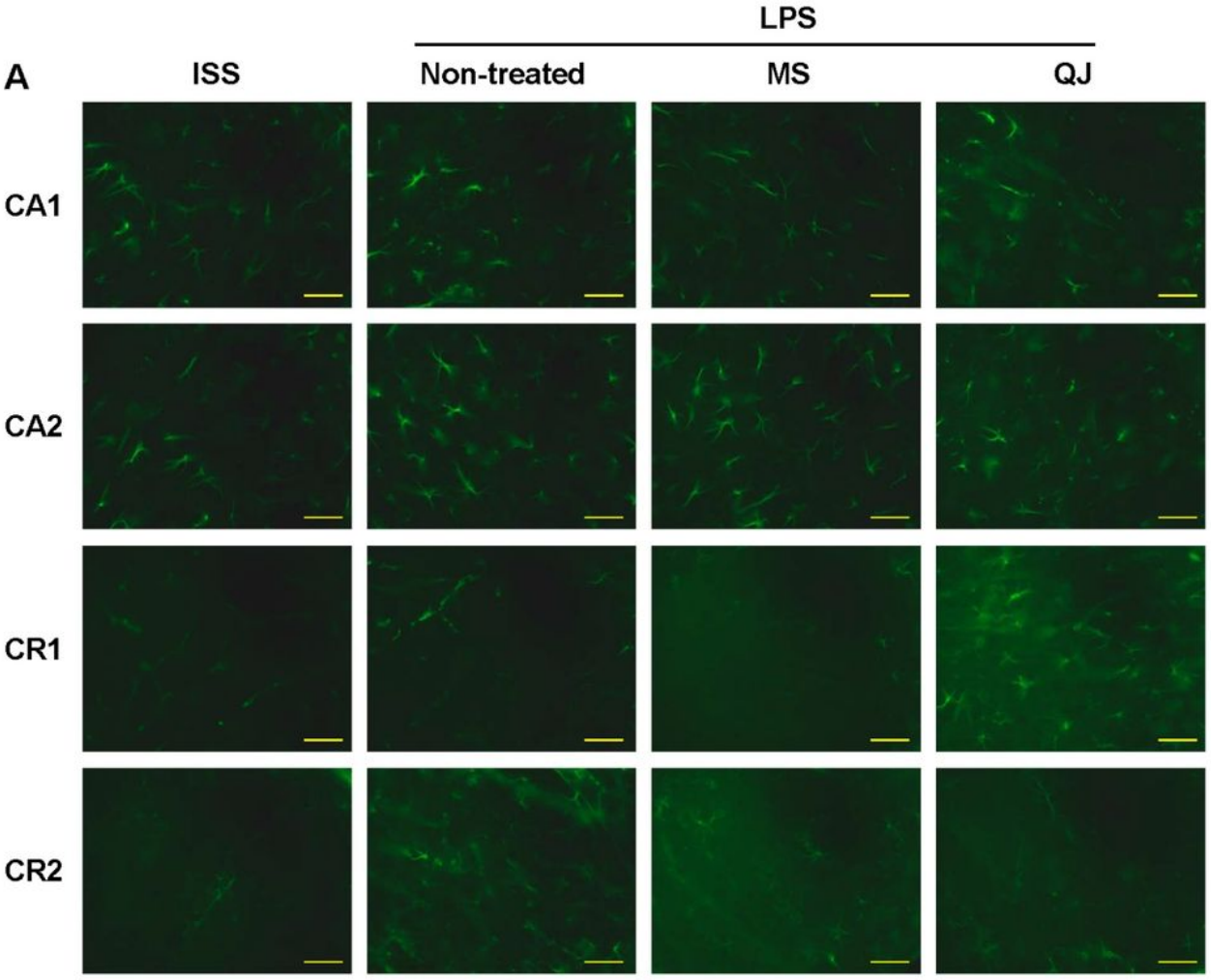
B

Marked area	ISS	LPS		
		Non-treated	MS	QJ
Hippocampus				
CA1	8.73±1.14 b	11.50±0.76 a	8.75±1.09 b	9.24±1.47 ab
CA2	8.61±1.04 b	11.50±0.65 a	8.82±1.21 b	9.16±1.42 ab
Cortex				
CR1	7.93±1.02 a	8.69±1.07 a	7.55±0.84 a	7.37±0.83 a
CR2	8.32±0.39 a	8.13±0.59 a	7.11±0.39 a	6.53±0.36 a

Figure 7

Immunohistological staining and quantification ionized calcium binding adaptor molecule (Iba1). (A) Representative immunofluorescence of 50 μm coronal sections stained with anti-Iba1 antibodies (red) in two cornu ammonis (CA1 and CA2) regions and the cortex (CR1 and CR2). The pictures derive from mice that received ISS, LPS, or the methanolic extracts of MS and QJ populations. (B) Table shows the mean±standard deviation of the medium fluorescence intensity. The effects of the different treatment in

Iba1 expression in each region were compared. Data labeled with different letters are significantly different. Significance was determined by Kruskal-Wallis test (p value 0.05, compared to LPS). 200 μm



B

Marked area	ISS	LPS		
		Non-treated	MS	QJ
Hippocampus				
CA1	7.44±1.42 b	9.99±0.50 a	8.73±1.83 ab	9.17±0.85 a
CA2	8.33±0.96 a	9.77±0.67 a	9.23±1.38 a	8.73±1.08 a
Cortex				
CR1	7.15±3.00 a	8.13±2.14 a	5.81±1.21 a	7.70±1.83 a
CR2	6.67±1.84 a	7.26±1.54 a	7.30±2.02 a	6.97±1.83 a

Figure 8

Immunohistological staining and quantification of glial fibrillary acidic protein (GFAP). (A) Representative immunofluorescence of 50 μm coronal sections stained with anti-GFAP antibodies (green) in two cornu ammonis (CA1 and CA2) regions and the cortex (CR1 and CR2). The pictures derive from mice that

received ISS, LPS, or the methanolic extracts of MS and QJ populations. (B) Table shows the mean±standard deviation of the medium fluorescence intensity. The effects of the different treatment in GFAP expression in each region were compared. Data labeled with different letters are significantly different. Significance was determined by Kruskal-Wallis test (p value 0.05, compared to LPS). 200 μm