Received 17 September 2007; Accepted 2 February 2008

Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
18(1): 11-15, Jan./Mar. 2008

Artigo

Immunomodulatory activity of extracts from *Cordia superba* Cham. and *Cordia rufescens* A. DC. (Boraginaceae), plant species native from Brazilian Semi-arid

José Fernando Oliveira Costa,[1] Juceni P. L. David,[2] Jorge M. David,[3] Ana M. Giulietti,[4] Luciano P. Queiroz,[4] Ricardo R. Santos,[1] Milena Botelho P. Soares*,[1]

[1]Laboratório de Engenharia Tecidual e Imunofarmacologia, Centro de Pesquisas Gonçalo Moniz, FIOCRUZ/BA, Rua Waldemar Falcão, 121, 40296-750 Salvador-BA, Brazil,
[2]Departamento do Medicamento, Faculdade de Farmácia, Universidade Federal da Bahia, 40170-280 Salvador-BA, Brazil,
[3]Departamento de Química Orgânica, Instituto de Química, Universidade Federal da Bahia, 40170-280 Salvador-BA, Brazi,
[4]Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana, 44031-060 Feira de Santana-BA, Brazil

RESUMO: "Atividade imunomoduladora de extratos de *Cordia superba* Cham. and *Cordia rufescens* A. DC. (Boraginaceae), espécies de plantas nativas do semi-árido brasileiro". A família Boraginaceae é amplamente distribuída no Brasil e na região nordeste algumas espécies são usadas popularmente no tratamento de reumatismo, dores menstruais e dispepsias. Neste trabalho foram estudadas as espécies *Cordia superba* Cham. and *C. rufescens* A. DC., nativas da região semi-árida brasileira, objetivando investigar a atividade imunomoduladora. Seis extratos foram preparados a partir de partes aéreas das espécies. A citotoxicidade foi avaliada usando culturas de esplenócitos de camundongos BALB/c. A atividade imunomoduladora foi determinada por ensaios *in vitro* usando macrófagos e linfócitos murinos ativados. Macrófagos peritoneais obtidos de camundongos BALB/c foram estimulados com IFN-γ and LPS na presença/ausência das amostras. A produção de NO foi medida indiretamente através do método de Griess. Três amostras inibiram a produção de NO em valores próximos a 50% (100 µg/mL). Os efeitos das amostras sobre os linfócitos foram avaliados cultivando esplenócitos de camundongos BALB/c em presença destas amostras e de concanavalina A. A proliferação foi determinada pela análise da incorporação de ^{3}H-tritiada. Amostras de duas espécies apresentaram uma forte atividade inibidora sobre a proliferação de linfócitos e sobre a produção de IL-2. Dois extratos clorofórmicos (partes aéreas de *C. rufescens*) tiveram os menores valores de IC_{50} (7,6 and 11,0 µg/mL).

Unitermos: *Cordia*, Boraginaceae, atividade imunomoduladora.

ABSTRACT: The family Boraginaceae is widely distributed in Brazil and in the Northeastern region some species are popularly used to treat symptoms of rheumatism, painful menstruation and dyspepsia. In this work we studied *Cordia superba* Cham. and *C. rufescens* A. DC., native from Brazilian Semi-arid region, in order to investigate their immunomodulatory activity. Six extracts were prepared from aerial parts of *C. superba* and *C. rufescens*. The cytotoxicity was evaluated using splenocytes from BALB/c mice. The immunomodulatory activity was determined by *in vitro* assays using activated mouse macrophages and lymphocytes. Peritoneal macrophages obtained from BALB/c mice were stimulated with IFN-γ and LPS in the presence/absence of the samples. The NO production was measured indirectly through Griess method. Three samples inhibited the production of nitric oxide in values near 50% at a concentration of 100 µg/mL. To evaluate the effects of the extracts on lymphocytes, splenocytes from BALB/c mice were incubated with the samples and concanavalin A. Proliferation inhibition was determined by analysis of ^{3}H-thymidine uptake. Samples from the two species had a strong inhibitory activity on lymphocyte proliferation and IL-2 production. Two chloroform extracts prepared from aerial parts of *C. rufescens* had the lowest IC_{50} values (7.6 and 11.0 µg/mL).

Keywords: *Cordia*, Boraginaceae, immunomodulatory activity.

** E-mail: milena@bahia.fiocruz.br, Tel. +55-71-31762260, Fax +55-71-31762292*

INTRODUCTION

The family Boraginaceae comprises about 2740 species and 148 genera distributed in temperate and tropical zones of Europe, Asia, Africa, Australia, and Americas (Langstrom and Chase, 2002). *Cordia*, with more than 200 species, is one of the largest genera (Gottschling et al., 2005). It was recorded 46 species of Boraginaceae in the Brazilian semi-arid, 26 belonging to *Cordia* (Stapf, 2006).

Species of *Cordia* are largely employed in folk medicine worldwide. In Northeastern Brazil these plants are used to treat symptoms of rheumatism, painful menstruation and dyspepsia (Silva et al., 2004; Agra et al., 2007; Biavatti et al., 2007). *Cordia rufescens* is a shrub popularly known in the same region as "rameladevelho" and along with other species from genus *Cordia* is used in popular medicine as abortive, anti-inflammatory and to treat dysmenorrhea (Silva et al., 2004).

Immunopathologies such as autoimmune and allergic processes are diseases with high incidence and to which the currently available drugs, although efficient in most cases, cause undesirable side effects, resulting in complications to patients, especially after long term use. The search for new bioactive molecules in plants is of great interest, considering the diversity of chemical entities produced by them (Gottlieb et al., 1996) and the need of new medicines for more effective treatment of pathologies with lower toxic effects. In this report we evaluated the immunomodulatory activity of *C. rufescens* and *C. superba* extracts on inhibition of lymphoproliferation and nitric oxide production.

MATERIAL AND METHODS

Preparation of extracts

Plant specimens were collected in semi-arid areas of the Bahia State, Brazil (Table 1) in authorized areas by IBAMA (Brazilian Institute for the Environment and Natural Resources), dried at 40 ºC and received botanic identity. Vouchers were prepared and stored at the Universidade Estadual de Feira de Santana Herbarium (HUEFS). Material from one individual of *C. superba* and from two individuals of *C. rufescens* were studied. Dried stem and leaves of *C. superba* (200 and 90 g, respectively) and *C. rufescens* (183 and 235 g of stems; 100 and 57 g of leaves) were individually extracted with MeOH. Thus, 1.32 g (0.66%) of MeOH extract was obtained from 200 g of stem and 10.5 g (11.67%) from 90 g of leaves of *C. superba*. Also, 891 mg (0.49%) and 8.5 g (8.5%) of MeOH extract were obtained from 183 g and 100 g of stems and 6.1 g (2.6%) and 6.0 g (10.53%) from 235 g and 57 g of leaves of *C. rufescens* specimens. Separately, the crude extracts obtained were partitioned with $CHCl_3$/MeOH:H_2O (6:4). These procedures allowed the production of 538.2 mg (40.8%) and 7.4 g (70.5 %) of $CHCl_3$ extract from *C. superba* (stem and leaves) and 83.2 mg (9.3%) and 1.8 g (21.1 %) of one sample and 108.7 mg (1.8 %) and 1.2 g (20 %) from the other sample of *C. rufescens*. The hydroalcoholic fraction, after the evaporation of MeOH under vacuum, gave rise to the aqueous phases and was further partitioned with EtOAc:H_2O. In the latter procedure 121.4 mg (9.2%) and 838.5 mg (8%) of *C. superba* were obtained (stem and leaves, respectively) and 215.3 mg (24.2%) and 2.2 g (25.9%) of *C. rufescens* and 82 mg (1.34%) and 138.0 mg (2.3%)from the other sample of the same specie. The final extracts were dissolved using dimethyl sulfoxide (Sigma, St. Louis, MO) adjusting a final concentration for 10 mg/mL. The samples were sterilized with 60,000 rad of gamma radiation (CIS International IBL437C, France) for use in biological assays.

Cytotoxicity assay

To determine the cytotoxicity of the samples, splenocytes from BALB/c mice (6 x 10^5 cells/well) were cultured in 96 well plate in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (Cultilab, Campinas, SP, Brazil) and 50 µg/mL of gentamycin (Novafarma, Anápolis, GO, Brazil). Each extract was evaluated in five concentrations starting at 100 µg/mL, in triplicates. Cells were incubated in the presence of 1 µCi/well [*methyl*-^{3}H] thymidine (Amersham, Little Chalfont, England) during 24 h at 37 ºC and 5% CO_2. After this period, cultures were harvested using a cell harvester (Filtermate 196, Packard, Groningen, Netherlands) to determine the ^{3}H-thymidine incorporation using a beta radiation counter (β-matrix 9600, Packard, Groningen, Netherlands). The viability of the cells was determined by the thymidine incorporation and the cytotoxicity was calculated in relation to the ^{3}H-thymidine incorporation of untreated cultures.

Nitric oxide assay

Peritoneal exudate cells were obtained by washing the peritoneal cavity of mice with cold Hank's balanced salt solution (HBSS; Life Technologies, GIBCO-BRL, Gaithersburg, MD) five days after injection of 3% thioglycollate in saline (1.5 mL per mouse). Peritoneal cells were washed twice with HBSS and re-suspended in RPMI medium (GIBCO-BRL) supplemented with 10% fetal calf serum (Cultilab, Campinas, SP, Brazil), L-glutamine (2 mM), RPMI 1640 vitamins solution (1%) (Sigma), sodium pyruvate (1 mM), HEPES (10 mM), 2-mercaptoethanol (50 µM), and gentamycin (50 µg/mL) (Sigma, St. Louis, MO). Cells were plated (2 x 10^5 cells/well) in 96 well plates. After one hour of incubation at 37 ^{o}C, non-adherent cells

Table 1. Voucher of the sampled species of *Cordia.*

Sample	Species	Local of collect	Solvent used	Plant part	Voucher
CS1	*C. superba* Cham.	Rio de Contas/BA	Chloroform	Stem	A.M.Giulietti 2023 (HUEFS 59810)
CS2	*C. superba* Cham.	Rio de Contas/BA	Ethyl acetate	Stem	A.M.Giulietti 2023 (HUEFS 59810)
CR1	*C. rufescens* A. DC.	Sento Sé/BA	Chloroform	Aerial parts*	K.R.B.Leite et al. 155 (HUEFS 59982)
CR2	*C. rufescens* A. DC.	Sento Sé/BA	Ethyl acetate	Aerial parts	K.R.B.Leite et al. 155 (HUEFS 59982)
CR3	*C. rufescens* A. DC.	Morro do Chapéu/BA	Chloroform	Aerial parts	C. Correia et al. 93 (HUEFS 59087)
CR4	*C. rufescens* A. DC.	Morro do Chapéu/BA	Ethyl acetate	Aerial parts	C. Correia et al. 93 (HUEFS 59087)

* Branches and leaves

were removed by washing with complete RPMI. Cultures were then stimulated with 1 µg/mL lipopolysaccaride (LPS from *Escherichia coli* serotype 0111:B4, Sigma) in combination with 5 ng/mL interferon-γ (IFN-γ; PharMingem, S. Diego, CA) and treated with various concentrations of extracts, as described in figure legends, in a final volume of 200 µL. Cell-free supernatants were collected at 24 h of culture for determination of nitrite concentration using the Griess method, as described previously (Ding et al., 1988).

Lymphoproliferation assay

BALB/c splenocytes suspension were prepared in complete RPMI medium and cultured in 96 well plates at 6 x 10^5 cells/well in 200 µL, in triplicates, in the presence of concanavalin A (Con A; 2 µg/mL), alone or various concentrations of plant extracts, as described in figure legends. After 48 h, plates were pulsed with 1 µCi of [*methyl*-^{3}H] thymidine for 12 h, and proliferation was assessed by measurement of ^{3}H-thymidine uptake. The percentage of inhibition of lymphocyte proliferation by the extracts was determined in relation to untreated controls.

Myxed lymphocyte reaction (MRL)

BALB/c (H-2^d) mice were weekly immunized with C57BL/6 (H-2^b) splenocytes intraperitoneally (10^7 cells/mouse). After 3 weeks of immunization, mice were sacrificed for spleen cell preparation in DMEM medium supplemented as described above. Spleen cells were cultured in 96 well plates at 5 x 10^5 cells/well in the absence or in the presence of irradiated C57BL/6 splenocytes at 10^6cells/well (dose of 3000 rad) and extracts, in triplicates. After 72 h of culture, plates were pulsed with ^{3}H-thymidine for 12 h for proliferation assessment, as described above.

Table 2. LC50 and EC50 of samples tested.

Sample	LC_{50} (µg/mL)	EC_{50} lymphoproliferation (µg/mL)	EC_{50} Myxed lymphocyte reaction (µg/mL)
CS1	NC	21.6	20.7
CS2	NC	36.9	13.6
CR1	34.2	7.6	6.0
CR2	NC	40.3	15.5
CR3	32.8	11.0	7.9
CR4	NC	36.9	8.8

NC = not calculated

EC_{50} and LC_{50} calculations and statistical analyses

The lethal concentration for 50% of cells (LC_{50}), the effective concentrations for 50% (EC_{50}) of lymphoproliferation and MLR were calculated based in a nonlinear regression (curve fit). The statistical analyses were made by one-way ANOVA with Dunnett's post test using Graph Pad Prism version 4.0 (Graph Pad Software, San Diego, CA, USA). Differences were considered significant when p values were < 0.05.

RESULTS

Samples and cytotoxicity values

Two species of the genus *Cordia* were analyzed, *C. superba* and *C. rufescens*. The extracts were obtained using chloroform and ethyl acetate as solvents. Six different extracts were analyzed in different concentrations. Stem and aerial parts of the plants were used and, from *C. rufescens*, two collects were done in different localities, generating two extracts of the same part and with the same solvent (Table 1). Samples were assayed in five different concentrations, starting at 100 µg/mL. As shown in Table 2, the lethal concentration for 50% (LC_{50}) could be determined only for two samples, CR1 and CR3 obtained from *C. rufescens* (34.2 and 32.8 µg/mL, respectively) The other four samples did not present cytotoxicity to allow the calculation the LC_{50}. The cytotoxicity of the samples is shown in Figure 1a.

Immunomodulatory activity assays

First we tested the activity of the extracts on cultures of activated macrophages. The samples tested did not have a high inhibitory activity on the nitric oxide production. The maximum inhibition was achieved with CS1, CR1 and CR3, which had, respectively, 54.8, 52.9 and 44.9% when assayed at 100 µg/mL (Figure 1b; $p < 0.05$).

The extracts had strong inhibitory activity in lymphocyte cultures, CR1 and CR3 have the lowest

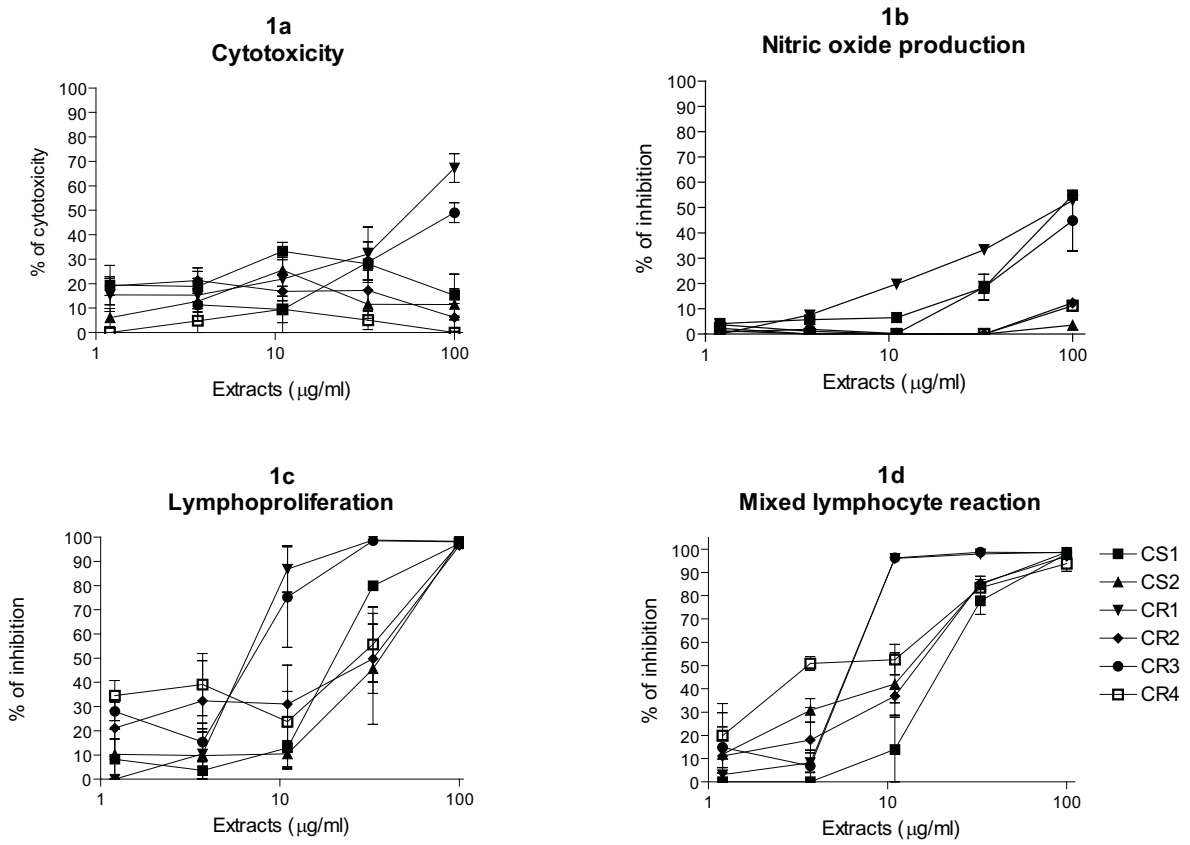

Figure 1. Cytotoxicity and immunomodulatory activity of extracts obtained from two species of *Cordia*. (1a) Mouse splenocytes (6 x 105 cells/well) were cultured during 24 h in DMEM, in presence or absence of extracts and 3H-thymidine to determine the cytotoxicity. (1b) Mouse macrophages obtained from peritoneal exudate were stimulated with IFN-γ and LPS and cultivated in presence or absence of the samples. The nitric oxide production was estimated through nitrite dosage by Griess method. The inhibition of lymphoproliferation was determined by culturing splenocytes from naïve mice during 48 h with Con A (1c) or BALB/c (H-2d) splenocytes with C57BL/6 (H-2b) irradiated splenocytes for 72 h (1d), in triplicates, as described in Materials and methods. Proliferation was measured by ^{3}H-thymidine incorporation. Data represent the mean ± SD 2-4 independent experiments.

EC_{50} values for proliferation inhibition of lymphocytes stimulated with concanavalin A (Table 2). These samples had high inhibitory activity even when assayed at concentrations below 100 μg/mL. CR2 and CR4, samples extracted from the same specie (*C. rufescens*) but using different parts of the specimen, had higher values of EC_{50}. CS1 and CS2, extracts from *C. superba*, presented values of inhibition up to 70% (Figure 1c). All the samples inhibited IL-2 production higher than 50% when assayed at 100 μg/mL, being CR2 and CR4 the most potent samples (80.7 and 84.8%, respectively; $p < 0.05$).

Similar results were obtained in cell cultures of mixed lymphocyte reaction (MLR). CR1 and CR3 had also a low EC_{50} values (6.0 and 7.9 μg/mL) in this assay. In addition, CR4 had the third lowest EC_{50} (8.8 μg/mL) in MLR assay (Table 2). All samples inhibited the proliferation in MLR in percentuals above 90%, when assayed at 100 μg/mL (Figure 1d).

DISCUSSION

A great variety of uses in ethnopharmacology for species of *Cordia* sp. has been reported (Ficarra et al., 1995; Reddy et al., 2002; Agra et al., 2007). *C. globosa* and *C. verbenacea* have been used in the folk medicine for treatment of rheumatism, painful menstruation and gastric ulcer (Reddy et al., 2002; Al-Awadi, 2001). Some pharmacologically active metabolites with analgesic, antiinflammatory, antiarthritic and larvicidal activities were already purified from *Cordia* sp., emphasizing an immunomodulatory activity of this genus (Sertié et al., 1991; Silva et al., 2003; Arrebola et al., 2004; Santiago et al., 2005). The species *Cordia superba* Cham. and *Cordia rufescens* A. DC (Boraginaceae), endemic in the Brazilian semi-arid, were investigated for the first time regarding their biological activities.

In general the samples had low cytotoxicity. Only two samples had their LC_{50} calculated (Table 2). The others could not be determined because the results obtained did not permit the calculation of the lethal doses, indicating that LC_{50} values for these samples

are superior to 100 μg/mL. It is interesting to note that LC_{50} values of CR1 and CR3 extracts are similar (Table 2). The samples were extracted from the same specie, prepared with aerial parts of different specimens, collected in different localities (Sento Sé and Morro do Chapéu) and the same solvent was used (Table 1).

Three samples (CS1, CR1 and CR3) inhibited NO production next to 50% when assayed at 100 μg/mL. However, CR1 and CR3 had high cytotoxicity at 100 μg/mL, and thus only CS1 activity on NO production can be considered.

In addition to the effect on the NO production, the immunomodulatory potential for the samples was also measured in lymphoproliferation assay using concanavalin A as stimulus for the splenocytes or allogenic stimulation in mixed lymphocyte reaction. CR1 and CR3 had the highest values of lymphocyte proliferation inhibition (up to 100%), when assayed at 100 and 33 μg/mL in both assays (Figures 1c and 1d). Consequently, these samples had the lowest EC_{50} values for these parameters. These data are relevant if observed that LC_{50} for them were very superior to these values (Table 2), suggesting an inhibition of proliferation and not toxicity-induced cell death. These data reinforce again the hypothesis of production of bioactive metabolites soluble in chloroform by *Cordia rufescens*.

CS1, CS2 and CR2, in spite of having the most elevated EC_{50} values, had not a detectable LC_{50} or they are superior to 100 μg/mL (Table 2). Two samples had a high percentual of lymphocyte proliferation inhibition with low EC_{50}, CR1 and CR3 (Table 2). IL-2, an important cytokine produced upon lymphocyte activation, was also inhibited. Corroborating the immunosuppressive potential of these two samples, both of them had higher values of LC_{50} when compared with EC_{50} values obtained. Thus, these results indicate a real effect on inhibition of proliferation and not due cell toxicity.

The results confirm the basis of the utilization of some *Cordia* species by traditional medicine in pathologies mediated by the immune system. Studies should be continued with the development of more specific assays and *in vivo* evaluation models for immune-mediated pathologies, followed by fractionation of extracts in order to identify the active compounds.

ACKNOWLEDGMENT

This work was supported by Millenium Institute of Semi-Arid (MCT), National Council on Scientific and Technological Development (CNPq), FAPESB and FIOCRUZ.

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