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Artigo

Immunomodulatory and antibacterial activities of extracts from Rutaceae species

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RESUMO: "Atividade imunomoduladora e antibacteriana de extratos de espécies da família Rutaceae." A família Rutaceae apresenta espécies vegetais muito bem distribuídas no Semi-Árido Brasileiro e comumente usadas em medicina popular. Espécies dessa família tem diversas atividades biológicas descritas na literatura. Neste trabalho, atividades imunomoduladora e bactericida são descritas para o extrato acetato de etila e clorofórmico de três espécies da família (*Esenbeckia grandiflora* Mart., *Pilocarpus spicatus* A.St.-Hil. e *Galipea simplicifolia* Schult.). Todas as amostras foram inicialmente avaliadas quanto à sua citotoxicidade, com objetivo de determinar a LC50. O potencial imunomodulador foi avaliado em culturas de esplenócitos murinos estimulados ou não com concanavalina A e também em reação mista linfocitária (RML),usando também esplenócitos de camundongos BALB/c (H-2^d) imunizados com esplenócitos de camundongos C57Bl/6 (H-2^b). Quatro amostras tiveram os mais elevados valores percentuais de inibição da proliferação de linfócitos ativados pela concanavalina A e foram avaliados em RML. A atividade antibacteriana dos extratos foi também avaliada e a concentração mínima inibitória (CMI) para duas amostras ativas foi de 1.0 e 5.0 mg/mL, respectivamente para as espécies *Esenbeckia grandiflora* Mart. and *Galipea simplicifolia* Schult. Assim, os dados aqui apresentados reforçam informações da literatura científica relacionados à atividade biológica para muitas espécies da família Rutaceae e incentivam outros estudos com estas visando descobrir substâncias ativas, potenciais candidatas a novos fármacos.

Unitermos: Atividade imunomoduladora, Rutaceae, atividade antibacteriana.

ABSTRACT: Rutaceae is a taxon with species very well distributed in Brazilian semi-arid area, commonly used in folk medicine. Species from this genus have diverse biological activity described in literature. In this work, immunomodulatory and bactericidal activity are described for chloroform and ethyl acetate extracts of three of them (*Esenbeckia grandiflora* Mart., *Pilocarpus spicatus* A.St.-Hil. and *Galipea simplicifolia* Schult.). Initially all the samples had their cytotoxicity evaluated, aiming to determine the LC50. The immunomodulatory potential was evaluated in cultures of murine splenocytes stimulated or not with concanavalin A and in a mixed lymphocyte reaction (MLR) using splenocytes from BALB/c (H-2^d) mice immunized with splenocytes from C57Bl/6 (H-2^b) mice. Four samples had higher values of lymphoproliferation inhibition in concanavalin A-stimulated cultures and were evaluated in MLR. The antibacterial activity of extracts was also evaluated and the minimal inhibitory concentrations (MIC) for two active samples were 1.0 and 5.0 mg/ml for extracts from *Esenbeckia grandiflora* Mart. and *Galipea simplicifolia* Schult., respectively. Thus, our results reinforce data of literature relating biological activity for many species of the Rutaceae family and encourage studies with these species aiming to discover active compounds, candidates to new medicines.

Keywords: Immunomodulatory activity, Rutaceae, antibacterial activity.

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INTRODUCTION

The Rutaceae family is constituted of about 1800 species distributed in approximately 156 genera found in tropical and subtropical regions of the world. In Brazil, 28 genera were identified so far, including 182 species. Many species from *Ruta* and *Citrus* genus are commonly used in folk medicine (Joly, 1985; Cortez, 2006). In the Caatinga, a typical vegetation from the semi-arid region in Northeastern Brazil, a checklist of biodiversity identified twenty genera of Rutaceae family with 88 species (Barbosa, 2006).

The Rutaceae family has been found to contain many secondary metabolites such as alkaloids, coumarins and lignans with a large spectrum of biological activities, such as antiprotozoal activity against *Leishmania* parasites, peripheral stimulants of parasympathetic nervous system and apoptosis inductor (Cortez, 2006; Roy, 2005). Terpenoids are other common compounds identified in this family (Mafezoli, 2000). Little is known, however, about the pharmacological potential of Rutaceae species found in the Caatinga. This work aimed to study the immunomodulatory and antibacterial activities of eight extracts prepared from three species of Rutaceae native from the semi-arid area of Northeastern Brazil: *Esenbeckia grandiflora* Mart., *Pilocarpus spicatus* A.St.-Hil. and *Galipea simplicifolia* Schult.

MATERIALS AND METHODS

Preparation of extracts

Plant specimens were collected in the municipality of Rio de Contas, State of Bahia, Brazil (Table 1) and dried at 40º C. Vouchers were prepared, identified and deposited at the Herbarium from the State University of Feira de Santana (HUEFS). Dried aerial parts of plants were used to prepare an hydroalcoholic extract through maceration in methanol and water (MeOH:H_2O 6:4). The extracts were then partitioned using chloroform and ethyl acetate, successively. The final product was concentrated, dried and dissolved using dimethyl sulfoxide (Sigma, St. Louis, MO) adjusting to a final concentration of 10 mg/mL for use in biological assays. Samples were sterilized with gamma radiation (dose of 60,000 rads in a ^{137}Cs source irradiator purchased from CisBio International, France).

Animals

Male and female 4-6 weeks old BALB/c mice were used as spleen cell donors. All mice were raised and maintained at the animal facilities at Gonçalo Moniz Research Center, Fiocruz (Salvador, Brazil). Mice were provided rodent diet and water *ad libitum*. All mice were sacrificed and treated according to the Oswaldo Cruz Foundation guidelines for laboratory animals. The work was approved by the institutional ethics Committee.

Cytotoxicity assay (lethal concentration for 50% of cells - LC50)

The cytotoxicity of the extracts was established using BALB/c mice splenocytes ($5x10^6$ cells/well) cultured in 96 plate-well in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% of fetal calf serum (FBS; Cultilab, Campinas, SP, Brazil) and gentamycin (Novafarma, Anápolis, GO, Brazil) at 50 µg/mL. Each extract was evaluated in three concentrations (1, 10, and 100 µg/mL), in triplicates. A positive control was made with cells treated with a 1% saponin solution. Cultures were incubated in the presence of ^3H-thymidine (1 µCi/well) during 24 h at 37 ºC and 5% CO_2. After this period, the content of the plate was harvested to determine the ^3H-thymidine incorporation using a Beta Radiation Counter (β-matrix 9600, Packard, Meriden, CT). The viability of the cells was determined by ^3H-thymidine incorporation and the cytotoxicity was calculated in relation to untreated cultures.

Lymphoproliferation assay

BALB/c splenocytes suspensions were prepared in RPMI medium (Life Technologies, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2 mM of L-glutamine, 0.1% RPMI 1640 vitamins solution (Sigma), 1 mM of sodium pyruvate, 10 mM of Hepes, 50 µM of 2-mercaptoethanol, and 50 µg/mL of gentamycin (Sigma). Splenocytes were cultured in 96-well plates at $4x10^5$ cells/well, in 200 µL, in triplicate wells, in the presence of concanavalin A at 2 µg/mL (Con A) alone or various concentrations of plant extracts preparations, as described in Table 1. After 48 h, plates were pulsed with 1 µCi of ^3H-thymidine for 12 h, and proliferation was assessed by measurement of ^3H-thymidine uptake, as described above. The percentage of proliferation inhibition by the extracts was determined in relation to controls stimulated by mitogen in absence of samples.

Mixed lymphocyte reaction (MLR)

BALB/c (H-2^d) mice were weekly immunized with C57Bl/6 (H-2^b) splenocytes intraperitoneally (10^7 cells/mouse). After three weeks of immunization, mice were sacrificed for spleen cell preparation in DMEM medium supplemented as described above. Spleen cells were cultured in 96 plates at $5x10^5$ cells/well in the absence or in the presence of irradiated C57Bl/6 splenocytes at 10^6 cells/well (dose of 3000 rad) and extracts, in triplicates. After 72 h of culture, plates were pulsed with ^3H-thymidine (1 µCi) for 12 h for proliferation assessment, as described above.

Bactericidal assays

Extracts had their antimicrobial activity evaluated at first through disc diffusion in Agar medium method, proposed by Kirby Bauer and described in the Farmacopéia Brasileira (1988). *Escherichia coli* and *Staphylococcus aureus* strains were used. The active extracts were evaluated aiming to identify the minimal inhibitory and bactericidal concentration (MIC and MBC, respectively). The MIC was obtained through dilution in 96-well plates using Mueller Hinton broth (BD, Sparks, MD, USA). The concentrations without grown of bacteria, when observed at microscopy, were used for minimal bactericidal activity determination with culture in plates containing Agar nutrient (BD, Sparks, MD, USA), a solid medium, at 37 °C during 24 h. MBC was considered the one without growing of bacteria observed.

IC50 calculation and statistical analyses

The inhibitory concentrations for 50% of inhibition (IC50) of lymphoproliferation were calculated based in a nonlinear regression (curve fit) and the statistical analyses were made by one-way ANOVA and Newman-Keuls multiple comparison test using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA).

RESULTS

Three species of Rutaceae family had their chloroform and ethyl acetate extracts prepared from different parts of the vegetable, generating eight extracts for investigation of bactericidal and immunomodulatory activities (Table 1). The samples had their cytotoxicity assayed and LC50 values are presented in Table 2. Among the eight samples, EGEL was the one with higher LC50 value. After, PSEA, PSEL and PSCL had the most elevated values of LC50 (Table 2). PSEA was the sample with lowest LC50 value (higher cytotoxicity).

The immunomodulatory activity of the extracts was evaluated using lymphocytes proliferation assays. In the first assay, lymphocytes were activated by the mitogen concanavalin A. The four most active samples were PSEA (31.5 µg/mL), PSCA (31.8 µg/mL), PSCL (37.7 µg/mL), and PSEL (38.7 µg/mL) (Table 1). The second assay for immunomodulatory activity used was the mixed lymphocyte reaction (MLR). Three samples had IC50 values calculated, as shown in Table 1: PSEA (32.3 µg/mL), PSCA (11.4 µg/mL), and PSCL (42.0 µg/mL), being the PSCA the most active extract in this assay. EGCL and GSCL were not active.

The antibacterial activity of extracts was also evaluated. The minimal inhibitory concentration (MIC and MBC) against *S. aureus* for EGEL and GSEL were 1.0 and 5.0 mg/mL, respectively. EGEL, EGCL and GSCL were also active against *S. aureus* in 25%, 30 % and 50% of growth inhibition when compared to the inhibition produced by gentamycin.

DISCUSSION

Rutaceae species are widely known by their ethnobotanical uses in many countries in the world (Lorenzi, 2002; Moshi, 2002). Diverse biological activities of species from this family have been demonstrated, such as anti-ulcer (Li, 2005), giardicidal (Amaral, 2006), acetylcholinesterase inhibition (Barbosa-Filho, 2006), antiplasmodial (Dolabela, 2008), and trypanocidal activity (Mafezoli, 2000). Limonoids and flavonoids are among the active chemical components of Rutaceae species already reported (Nakagawa, 2006).

In this work the bactericidal and immunomodulatory activities of three Rutaceae species from Brazilian semi-arid region were analyzed. Leaves or aerial parts of the vegetable were used to prepare extracts with different solvents, allowing the extraction of compounds with different polarities from different organs of the plant.

The cytotoxicity of extracts from the three Rutaceae species studied (*Esenbeckia grandiflora, Pilocarpus spicatus* and *Galipea simplicifolia*) was first investigated to determine the LC50 values. PSCA, EGCL, and GSEL had lower LC50 values. The first two were

Table 1. Extracts prepared from Rutaceae family species.

Sample	Specie	Localization of collect (Rio de Contas/Bahia State/Brazil)	Solv ent used	Vegetable part	Voucher specimen number
EGEL	*Esenbeckia grandiflora* Mart.	13°35'S/41°39'W	ethyl acetate	leaves	HUEFS 59.790
EGCL	*Esenbeckia grandiflora* Mart.	13°35'S/41°39'W	chloroform	leaves	HUEFS 59.790
PSEA	*Pilocarpus spicatus* A.St.-Hil.	13°36'15"S/41°45'34"W	ethyl acetate	aerial parts*	HUEFS 59.786
PSCA	*Pilocarpus spicatus* A.St.-Hil.	13°36'15"S/41°45'34"W	chloroform	aerial parts*	HUEFS 59.786
PSCL	*Pilocarpus spicatus* A.St.-Hil.	13°36'15"S/41°45'34"W	chloroform	leaves	HUEFS 59.786
PSEL	*Pilocarpus spicatus* A.St.-Hil.	13°36'15"S/41°45'34"W	ethyl acetate	leaves	HUEFS 59.786
GSEL	*Galipea simplicifolia* Schult.	13°35'S/41°39'W	ethyl acetate	leaves	HUEFS 59.792
GSCL	*Galipea simplicifolia* Schult.	13°35'S/41°39'W	chloroform	leaves	HUEFS 59.792

*Branches and leaves

prepared with chloroform and the last one with ethyl acetate. The ethyl acetate extract from leaves of *Galipea simplicifolia* (GSEL) had the lowest LC50 (6.92 µg/mL), indicating a high toxicity.

Four samples inhibited lymphocyte proliferation induced by concanavalin A and three of them inhibited the MLR. Although there are data from the literature reporting biological activity of species from the Rutaceae family, our results did not confirm this at least for these three studied species studied regarding immunomodulatory potential. This affirmative is based on values of IC50 related to the LC50 ones. If observed, the samples inhibited the lymphocyte proliferation in both models but the cytotoxicity values, expressed as LC50, were very similar to the IC50 values. This means that the inhibition of lymphoproliferation observed may be due to the cytotoxic effects and not by a specific mechanism. This highlights the need of toxicological tests for the use of these plant species based in folk medicine, since biological activity is popularly considered but not its toxicity.

For the samples assayed, two of them presented antibacterial activity, EGEL and GSEL. According to the consulted literature, none of these genera had bactericidal activity reported so far. Thus, by the first time is reported the bactericidal activity for these taxa.

In summary, our results show the pharmacological potential for the three species from Rutaceae family, especially regarding bactericidal activity. Further studies are needed in order to identify the active compounds present in these extracts aiming to the identification of those with low toxicity.

Table 2. IC50 and LC50 values (µg/mL) of samples.

	Cytotoxicity	Lymphoproliferation	Myxed Lymphocyte Reaction
EGEL	47.75	NC	NC
EGCL	NC	NC	NC
PSEA	24.3	31.5	32.3
PSCA	12.2	31.8	11.4
PSCL	33.7	37.7	42.0
PSEL	22.8	38.7	NC
GSEL	6.92	NC	NC
GSCL	NC	NC	NC

NC= non-calculated; IC50 = inhibitory concentration for 50 % and LC50= lethal concentration for 50 %.

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