PHYTOCHEMICAL CHARACTERIZATION AND ANALYSIS OF IMMUNOMODULATORY POTENTIAL IN LEAVES OF *Celtis fluminensis* CARAUTA

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Introduction

The genus *Celtis* is the most populous in the Cannabaceae family, with around 70 known species. Several species of this genus exhibit biological activities, presenting substances with potential to be new candidates for drugs in the pharmaceutical industry. Anti-inflammatory, antioxidant, and antimicrobial activities are generally described for the genus, often related to the presence of phenolic metabolites, fatty acids, and terpenes[1].

Despite numerous studies on the genus and its species, there is a lack of phytochemical studies focusing on the therapeutic activities of *Celtis fluminensis*. This species is endemic to Brazil, native to the Atlantic Forest, primarily distributed across the Southeast states and in Bahia. It is a shrub-like species, commonly known as "esporão-de-galo," "juá," and "juá-mirim." It has a smooth, brown stem, curved stipular thorns, and lanceolate oval foliage, among other characteristics[2].

Studying the phytochemical composition of this species is important and can contribute to identifying prototypes for the development of new drugs, assist in the development of physiological and pharmacological theories, and enhance ethnopharmacological knowledge in communities[3]. The aim of this study was to utilize leaf samples of *C. fluminensis* to characterize its chemical constituents and evaluate the immunomodulatory potential and toxicity of the extract, fractions, and substances from the species.

Material and Methods

The solvents used for the phytochemical processes were: hexane, dichloromethane, chloroform, ethyl acetate, ethanol and methanol, acetonitrile, 96% formic acid, distilled water, and ultrapure water. Microcrystalline cellulose was used in the fractionation process. For instrumentation, the following were utilized: an ultrasonic washer, rotary evaporator, ultra-efficient liquid chromatography, gas chromatograph, diode array detector (DAD). A high-resolution mass spectrometer was used as the analyzer.

For biological analysis, the murine macrophage cell line RAW 264.7 was cultured in DMEM-F12 medium supplemented with fetal bovine serum (FBS). The quantification of nitric oxide (NO) production was performed using the Griess assay (1% p-aminobenzenesulfonamide + 0.1% naphthalenediammine dichloride in 5% phosphoric acid), measuring absorbance with a plate spectrophotometer. The cytotoxicity test was conducted using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT).

The *C. fluminensis* sample was collected in 2022 in São Francisco de Itabapoana, in the State of Rio de Janeiro. The leaves of the species, after drying and grinding, were subjected to ethanol extraction using ultrasonic equipment. The crude ethanol extract was fractionated using cellulose as the stationary phase, successively eluted with solvents of increasing polarity, resulting in the hexane (FH), chloroform (FC), ethyl acetate (FA), and methanol (FM) fractions. The FH and FC fractions were analyzed by Gas Chromatography (GC), while FM underwent analysis by Ultra-Efficient Liquid Chromatography (UELC), both coupled to the mass spectrometer[4].

The extract and the organic fractions were also sent for biological analyses. RAW 264.7 macrophages were seeded in 96-well culture plates with DMEM-F12 medium supplemented with 2% FBS.

After 24 hours, samples containing LPS (cellular stimulus) were added to the cell monolayer in serial dilutions (0.8, 4, 20, and 100 µg/mL) in triplicate. The culture of macrophages stimulated only with LPS was used as a positive control, while the culture of macrophages without stimulation or treatment was used as a negative control. The culture plate was maintained in an incubator at 37ºC with 5% CO_2, and after 24 hours, the supernatant was collected to assess NO inhibition, while the viability of the cell monolayer was evaluated using the MTT method. The quantification of nitric oxide (NO) production was performed by Griess assay, and the absorbance was measured in a plate spectrophotometer at 570 nm. For the cytotoxicity test, 10 µL/well of MTT solution (5 mg/mL) was added to the RAW 264.7 cell culture. After 2 hours in the incubator, the supernatant was discarded, and the generated formazan crystals were solubilized in isopropanol with added hydrochloric acid. The minimum concentration corresponding to 50% cytotoxicity (CC_{50}) were calculated, along with the selectivity index (CC_{50}/IC_{50})[5].

Results and Discussion

The hexane fraction presented, among other substances, tetradecanoic acid, vitamin E, β-sitosterol, β-amyrin, and friedelan-3-one. Vitamin E was also identified in the chloroform fraction, along with hydrocarbons. In the FM fraction, the identified compounds were dihexoside kaempferol, kaempferol-O-sambubioside, apigenin-7-O-dihexoside, tectorigenin-glucosyl-glucoside, apiin, and diosmetin-apiosyl-glucoside.

The FC and FA fractions exhibited a greater potential to inhibit NO production by stimulated macrophages, with IC_{50} values of 20.7 ± 1.8 and 21.1 ± 1.7, respectively. The FH fraction also showed activity in this regard, while the FM fraction showed no potential. The extract exhibited 26.4 µg/mL; however, the ability to modulate NO production was evident given its low CC_{50} value (39.2 ± 1.0), indicating cytotoxicity and therefore non-selectivity. The FC and FA fractions showed low or no toxicity at concentrations where the inhibitory activity of NO production was present in a selective manner, both exhibiting $CC_{50} \geq 70$ µg/mL. The selectivity indices (CC_{50}/IC_{50}) for these fractions varied from 3.5 to 3.7. In contrast, the FH and FM fractions displayed low or no toxicity, with $CC_{50} \geq 100$ µg/mL.

Conclusion

The results obtained showed similarities between the identified substances and those described in the literature for leaves of the genus *Celtis*, such as β-sitosterol, vitamin E, and kaempferol. The FC and FA fractions exhibited greater immunomodulatory potential and low cytotoxicity. Due to the higher quantitative yield of the FC fraction in the fractionation process, studies will continue to emphasize this fraction. Obtaining viable and safe results can contribute to research for future drug candidates. Additionally, it may aid in ethnopharmacological studies in communities that may use *C. fluminensis* as a therapeutic input.

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