Immunomodulatory effects of sucupira (*Pterodon pubescens*) seed infusion on collagen-induced arthritis

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Abstract

Objective

To evaluate the clinical and immunomodulatory efficacy of seed extracts from sucupira branca (Pterodon pubescens *Benth.), a Brazilian anti-inflammatory folk medicine, against collagen II (CII)-induced arthritis (CIA) in mice.*

Methods

Mice were divided into 3 groups: 1) normal control mice received a vehicle (ethanol 15% in water); 2) mice with CIA received the vehicle; and 3) mice with CIA received extract from 1 mg sucupira seeds/day. The daily oral treatments started 21 days after the first collagen immunization, ending 4 weeks later. We analyzed the arthritic index, the histopathology of the joints, the serum anti-CII IgG antibody level, and the absolute counts of the $CD4^+$, $CD8^+$, $CD4^+CD69^+$ and $CD8^+CD69^+$ subsets of inguinal lymph nodes (LN).

Results

Sucupira treatment strongly reduced the severity of arthritis ($p < 0.001$). Vehicle-treated CIA mice exhibited invasive synovial pannus and significant articular leukocyte infiltration, features that were reduced or absent in sucupira-treated mice. Mice with CIA exhibited twice the number of $CD4^+$ and $CD8^+$ LN cells found in control mice. Sucupira-treated mice exhibited these subsets in numbers comparable to the latter. A two-thirds decrease in the level of serum anti-CII IgG antibody and a normalization of the $CD4^+CD69^+$ LN cell number in treated mice hallmark a negative regulatory effect of sucupira on B- and CD4 T-cell activation, respectively. The $CD8^+CD69^+$ cell number remained roughly the same in the 3 groups.

Conclusion

The clinical and immunomodulatory effects of sucupira on CIA provides a further experimental basis for the popular use of sucupira in chronic inflammatory diseases.

Key words

Phytotherapy, sucupira, *Pterodon pubescens*, collagen-induced arthritis, lymphocytes, immunomodulation.

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Introduction

In Brazil, alcoholic infusions (wine, cachaça, etc) using the oily seeds of the sucupira tree (*Pterodon* sp.) have been traditionally prescribed as a folk medicine for the treatment of inflammatory conditions, rheumatic diseases [including rheumatoid arthritis (RA], and pain (1).

Collagen-induced arthritis (CIA) in mice is an experimental model of autoimmunity that in some aspects resembles human RA(2). Cumulative data in the literature have established that both B (3) and T (4) lymphocytes must be present for the full development of CIA. The participation of B cells in the development and severity of CIA can be illustrated by the strong positive correlation between footpad edema indexes and the titers of anti-CII circulating antibodies (5). Among T cells, both the $CD4^+$ and $CD8^+$ subsets were shown to be involved in CIA development. $CD4^+$ lymphocytes represent the major cells responding to CII before CIA onset (6). In their turn $CD8^+$ cells play a co-adjuvant role, as has been demonstrated in CD8-deficient mice, which exhibit a decrease in the incidence and the severity of CIA(7).

We recently completed a study demonstrating that *Pterodon pubescens* Benth. (sucupira branca) seed extracts are very effective in preventing carrageenan-induced paw edema and croton oil-induced ear edema in mice (8), thus supporting their use as an unconventional anti-inflammatory folk medicine.

In the present work we extended our examination of the anti-inflammatory and immunomodulatory properties of sucupira branca seed extracts using the CIAmodel. We evaluated the impact of oral treatment with this sucupira on the clinical evolution of CIA, as well as on the degree of B and T cell activation, as measured by the blood titers of anti-CII antibodies and the number of activated $CD4^+$, $CD8^+$, and $CD4^-/8^-$ lymphocytes in inguinal draining lymph nodes, as measured by the increased expression of the cell surface activation marker CD69 (9).

Materials and methods

Animals

Male DBA/1J mice aged 3 to 5 months were bred in the animal care facilities of the UERJ Biology Institute, and kept under a 13-hour (6:00 AM to 7:00 PM) light regimen with food and water *ad libitum*. Inguinal lymph nodes (LN) were excised after bleeding and killing the mice by cervical dislodgment under deep anesthesia. Experiments were performed with the consent and surveillance of the UERJ ethical committee for the use of animals in research.

Sucupira extracts

100 g of *Pterodon pubescens* seeds were reduced to a powder and then left to soak in 15% ethanol-water solution in a final volume of 1 L for 2 weeks in a cool dark place. The sucupira infusions were then filtered to remove the insoluble material and the extracts were stored at -20ºC.

Protocol for CIA

Collagen type II (CII), from chicken xiphoid appendix was prepared as previously described (10) and dissolved in 0.05 M acetic acid. For priming, a CII solution was emulsified in complete Freund's adjuvant (CFA; 1:1) and administered intradermally at a dose of 200 µg in 100 µL, into the dorsal tail-base of mice. Twenty-one days later a booster injection of 400 µg CII solution was administered intraperitoneally.

Treatment of CIA

Mice were divided into 3 groups: 1) normal healthy control mice (n = 6) received the vehicle (ethanol 15% in water) alone; 2) mice with CIA (n = 7-15), received the vehicle alone; and 3) mice with CIA (n = 7-15) received sucupira extract. To reproduce the equivalent of the amount taken by humans in wine, the sucupira extract (henceforth referred to as sucupira) was further diluted in vehicle. Fifty µL carrying the active principle extracted from 1 mg of bulk seed were given daily (excepting Sundays) by oral gavage, starting on the day of the CII booster and ending 4 weeks later, one day before the mice were sacrificed. The control groups with and without CIA re-

ceived 50 µL vehicle following the same schedule.

Clinical evaluation of CIA

Paw volume was determined as a measure of arthritis severity using a plethysmometer (Ugo Basile, Varese, Italy) (11). The paw was immersed up to the lateral malleolus in a water-filled cell connected by means of a transducer to a digital display which recorded differences in the water level caused by volume displacement. The volumes measured for the left and right hindpaws on a defined day were compared to the volumes measured for these hindpaws on day 0, thus yielding an edema index (i.e., percentage of swelling or shrinking) for each mouse. The edema indexes were then assembled in order to calculate the mean edema index ± SD for each group for this defined day.

For the histological evaluation, feet were excised, decalcified with 5% HNO_3, embedded in paraffin, cut into 6 µm sections, and stained with hematoxilin and eosin.

Anti-collagen type II IgG detection by ELISA

Volumes of 100 µL/well from a 1 mg CII solution in bicarbonate buffer 0.05 M, pH 9.6, were distributed in 96 flat-bottom wells of polystyrene plates (Falcon 3912; Becton Dickinson Labware, Oxnard, CA) and allowed to stand overnight at ~4°C. The wells were washed 3 times with 200 µL of 10 mM phosphate buffered saline, pH 7.4 (PBS) plus 0.05% Tween 20 (PBS-Tween), and incubated with 200 µL PBS-Tween plus 5 mg/mL BSA (PBS-Tween-BSA) for 1 hour at 4°C.

The next steps were intercalated with 3 washings with PBS-Tween-BSA: 1) addition in triplicate of 50 mLof serum sample diluted 5000-fold in PBS-Tween (PBS-Tween-BSA in control wells), and incubation for 2 hours at 37°C in a humidified chamber; 2) addition of 50 µL/well of rabbit anti-mouse IgG peroxidase-linked conjugate and incubation for 1 hour at 37°C; 3) addition of 50 µL/well of a solution of 0.8 mg/mL o-phenylenediamine plus 0.05% H_2O_2 in 0.3 M sodium citrate-phosphate buffer, pH 6.0, incubation at room temperature for 30 minutes, and stopping of reaction with 50 µL of 4 M H_2SO_4. The absorbances were read at 495 nm in an automated plate reader (Model 550; Bio-Rad Lab., Hercules, CA). Immunoglobulin G serum concentrations were calculated from a standard curve made with the adsorption of different solutions of purified IgG (0.01–1.0 µg/mL) and revelation as described above (steps 2 and 3). Unless stated, all the biochemicals were purchased from Sigma-Aldrich Co., St. Louis, MO.

Labeling of cell surface antigens for flow cytometry

Inguinal LN were minced and pressed through a 200 mesh stainless steel grid with the aid of a rubber policeman in Eagle's minimum essential medium, Earle's base, pH 7.2 (Sigma-Aldrich) diluted 1:2 with 0.9 g% NaCl (MEM/3) plus 5% fetal bovine serum (FCS, Fazenda Pigue, Nova Friburgo, RJ). Surface staining was carried out in Eppendorf tubes on melting ice in the dark using 1.5 x 10^6 cells of pools of equal cell numbers from 6-15 mice. Cells were first incubated with 1 µg of anti-CD16/32 specific monoclonal antibody (mAb; clone 2.4G2; Pharmingen, San Diego, CA) for 15 minutes to block Fc receptor antibody binding. Fluorescein (FITC)-conjugated anti-CD8α (clone 53.6.7), R-phycoerythrin (PE)-conjugated anti-CD4 (clone GK1.5, Becton Dickinson Biosciences, Palo Alto, CA), and biotin-conjugated anti-CD69 (clone H1.2F3, Pharmingen) mAbs were then added to the cells in a 200 µL final volume of MEM/3-FCS. Suspensions were incubated for 45 minutes and cells were washed 3 times with 1 mLMEM1/3-FCS (x 1000 g, 1.5 minutes, 8°C). Labeling with anti-CD69 was revealed by a further incubation of cells with 1 µg streptavidin-Tricolor (Caltag Laboratories, San Francisco, CA) in 200 µL. Propidium iodide (PI; Sigma-Aldrich) was added in a final concentration of 2 µg/mL moments before acquisition in order to exclude dead cells from the analysis.

Flow cytometry was carried out in a FACSCAN II apparatus (Becton Dickinson Biosciences) equipped with an air-cooled 488 nm argon ion laser of 15 mW, using 530 nm (for FITC) and 585 nm (for PE and PI) band pass filters, and a 650 nm (for Tricolor and PI) long pass filter. Fluorescence overlap compensation was obtained from single antibody labelings. Events were harvested by the usual criteria of size (FSC), granularity (SSC), and PI exclusion. Collection and analysis were done through the CELLQUEST program (Becton Dickinson).

Statistics

Comparisons between groups were made using the Student's t test.

Results

Oral administration of sucupira reduced CII-induced joint injury. As shown in Figure 1, daily sucupira treatment starting on the day of the CII booster and ending one day before animal sacrifice decreased markedly the development of arthritis. Histological examination of the foot joints of mice with established CIArevealed their disseminated invasion by a hypertrophic fibroblastoid-like synovial pannus (Fig. 2B; p) and intense leukocyte infiltration (Fig. 2B; li). However, under sucupira treatment these features were strongly reduced or more frequently absent, animals exhibiting normal synovial membranes (Fig. 2C; sm), little or no articular cartilage (c) and bone (b) erosion, and a remarkable reduction in leukocyte infiltrates, thus resembling the joints of vehicle-treated control mice (Fig. 2A).

Sucupira treatment also markedly decreased the circulating titers of anti-CII antibodies. Mice with CIA exhibited a strong positive correlation between circulating levels of IgG anti-type II collagen and the severity of arthritis (5). A decrease of two-thirds in blood anti-CII IgG levels in sucupira-treated mice (Fig. 3) indicated that this medicine was able to reduce the level of CII-specific B-cell activation.

Sucupira treatment avoided the LN enlargement observed in CII-primed mice. The inguinal LN of mice with CIA exhibited roughly twice the total number of $CD4^+$, $CD8^+$, and $CD4^-/8^-$

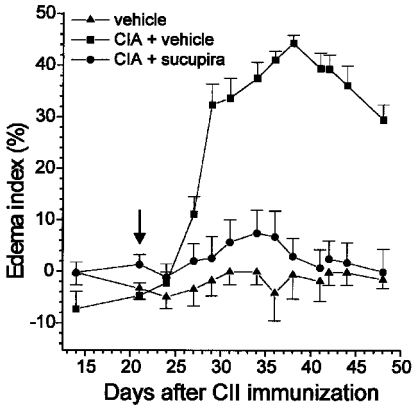

Fig. 1. Therapeutic effect of sucupira in CIA. Male DBA/1 mice were divided into three groups. One group (n = 6) represented control mice treated with the vehicle alone (▲). The other two groups (n = 15) were primed with 200 µg CII in CFA intradermally and boosted 21 days later with 400 µg CII intraperitoneally. These groups were treated either with vehicle (■) or sucupira extract (●). Vehicle or sucuira (50 µL) were administered daily and orally, the treatment starting on the day of the CII-booster (day 21; ➔) and being maintained up to one day before the sacrifice of the mice (day 47 in this representative experiment). To measure disease severity, the volumes of the left and right hindpaws were measured with a plethysmometer on a defined day and compared to the volumes measured for these hindpaws on day 0. The differences were expressed as an edema index (percentages of swelling or shrinking) for each animal. Individual edema indexes were then assembled in order to calculate the mean edema index ± SD for each group on this defined day. Differences between CIA untreated and CIA sucupira treated animals were all significant above day 29 ($P < 0.001$).

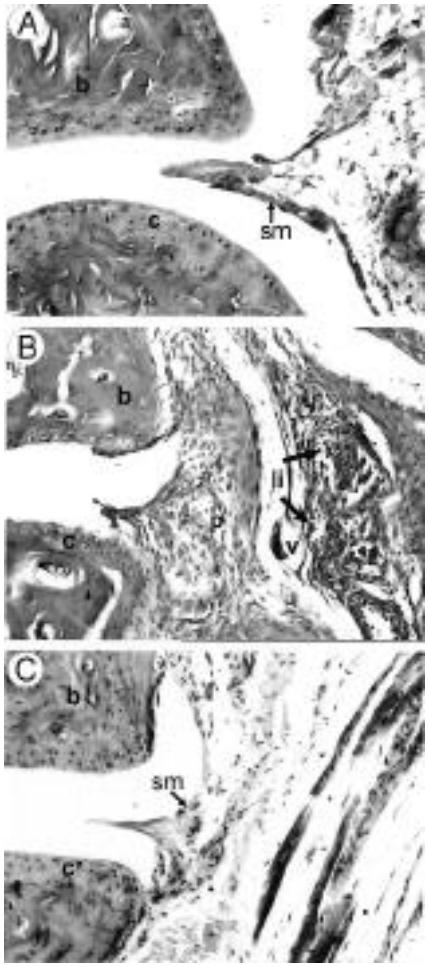

Fig. 2. Histological examination of sucupira-treated mice. Six µm sections of fixed/decalcified feet articulations of vehicle-treated control mice (**A**), vehicle-treated CIA mice (**B**), and sucupira-treated CIAmice (**C**) were stained with hematoxilin/eosin and photographed under light microscopy. The slides show: 1) hypertrophy of the synovial membrane (**sm**) forming a pannus (**p**) in the vehicle-treated CIA mice and the thin pattern of this membrane in control as well as sucupira-treated mice and; 2) an enlarged vessel (**v**) and the leukocyte infiltrate (**li**, arrows) in vehicle-treated CIAmice, uncommon images in the joints of sucupira-treated mice. **b**, bone; **c**, cartilage. Magnification, 180 x.

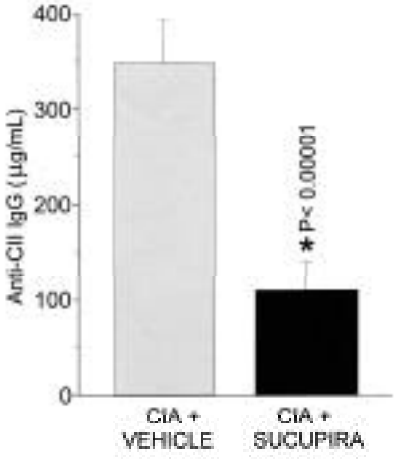

Fig. 3. Anti-CII IgG circulating titers in sucupira-treated mice. Vehicle-treated CIA mice (n = 12) and sucupira-treated CIAmice (n = 15) were sacrificed at day 49 and their sera were tested individually for anti-CII IgG titers by ELISA. Vehicle-treated normal mice exhibited undetectable sera levels of anti-CII IgG. Bars represent the mean µg/mLtiters ± SD for each group.

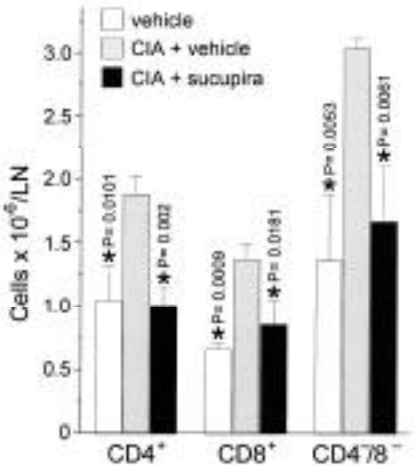

Fig. 4. Total cell numbers of CD4/CD8 defined subsets in draining LN of sucupira-treated mice. Pooled inguinal LN cells from vehicle-treated control mice (n = 6), vehicle-treated CIAmice (n = 7-10), and sucupira-treated CIA mice (n = 7-10) were stained with anti-CD4 and anti-CD8 mAbs and analyzed by flow cytometry. Total cell numbers were obtained by multiplying the mean number of cells per LN by the percentages of CD4+, CD8+, or CD4-/8- cells among them. Results show the mean ± SD of 3 independent experiments. Pvalues show the statistical significance between vehicle-treated control groups or sucupira-treated CIAgroups and vehicle-treated CIAgroups.

cells found in sucupira-treated CIA mice and control animals (Fig. 4), although the relative numbers (i.e., percentages) of these cells did not differ among these groups (n = 3, not shown). These results indicate that sucupira treatment counteracted the expansion of these subsets as observed in CIA.

Sucupira treatment reduced the overall increase in activated $CD4^+$ cells but not the increase in activated $CD8^+$ cells. The up-regulation of CD69 surface antigen is a characteristic of recently antigen-activated T, B, and NK lymphocytes and neutrophils (9). We therefore used the expression of this antigen to assess the ratios and absolute numbers of activated cells among $CD4^+$, $CD8^+$, and $CD4^-/8^-$ subpopulations in the LN of mice with CIAwhich were or were not treated with sucupira. Sucupira-treated mice exhibited relative increases of $CD69^+$ cells in all three of these subpopulations when compared to untreated CIA animals. The increase was more marked among $CD8^+$ LN cells, which exhibited nearly twice the percentages of $CD69^+$ cells found in CIAmice (not shown). However, when assessed as total counts, the sucupira treatment shifted to a normalization of the number of $CD4^+CD69^+$ and $CD4^-/8^-$ $CD69^+$ cells in the LN of mice (significant only for the former in the analysis of three experiments) (Fig. 5). The total numbers of $CD8^+CD69^+$ cells in the LN of sucupira-treated mice exhibited instead a tendency to increase when compared to the LN of CIAor control mice,

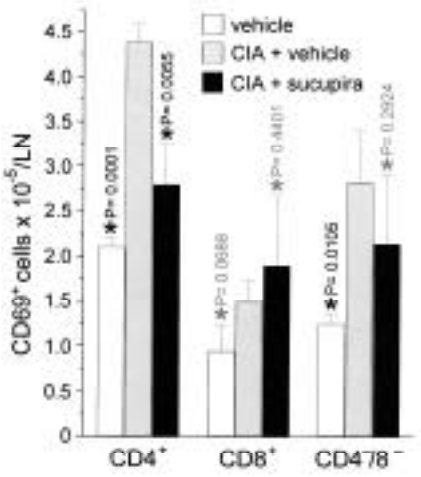

Fig. 5. Total CD69+ activated cell numbers among CD4/CD8 defined subsets in the draining LN of sucupira-treated mice. Pooled inguinal LN cells from vehicle-treated control mice (n = 6), vehicle-treated CIAmice (n = 7-10), and sucupira-treated CIAmice (n = 7-10) were stained with anti-CD4, anti-CD8, and anti-CD69 mAbs and analyzed by flow cytometry. Total CD69+ cell numbers per subset were obtained by multiplying the mean number of cells per LN versus the percentages of CD4+, CD8+, or CD4-/8- cells versus the percentage of CD69+ cells in these subsets. Results show the mean ± SD of 3 independent experiments. P values show the statistical significance between vehicle-treated control groups or sucupira-treated CIAgroups and vehicle-treated CIAgroups.

a consequence of the relative increase of these cells in the former.

Discussion

Sucupira treatment was able to reduce the level of CII-specific B-cell activation *in vivo*, a condition required to reduce the severity of the disease (5). Sucupira treatment also avoided the increases in the total numbers of $CD4^+$, $CD8^+$, and $CD4^-/8^-$ (i.e., a subset rich in B cells) lymphocyte subpopulations, which were observed in draining LN of untreated CIA mice. Additionally, this treatment avoided the increase in the total number of $CD4^+CD69^+$ activated cells observed in the LN of the latter. Together, these observations are indicative that sucupira impairs the activation and expansion of the lymphocytes involved in CIAdevelopment.

Conversely to $CD4^+$ lymphocytes, CD8 lymphocytes are the main cells responsive to CII after CIA development. At this time, the latter have a suppressor function, revealed in adoptive transferred mice (12) and in CD8 deficient mice, which are more susceptible to a second induction of CIA after remission of the initial disease (7). The fact that activated $CD8^+CD69^+$ cells were distributed in much higher percentages in the LN of sucupira-treated mice might suggest also their involvement in the control of CIA development.

The target cell(s) on which sucupira exerts its primary immunomodulatory effect in CIA is an open question. *In vitro* sucupira is able to decrease significantly IL-6 production by human mononuclear cells and nitric oxide production by a murine macrophage cell line (unpublished results). These observations suggest that sucupira could reduce inflammatory processes, thus impairing the accessory cell functions necessary for the activation of lymphocyte subsets and the full development of CIA. In support of this view are results showing that sucupira impairs CIA development when given in a prophylactic regimen (i.e., daily treatments starting 3 weeks before CII priming). In a similar way, using a delayed type hypersensitivity model, a 7-day treatment with daily oral sucupira doses similar to those used for CIA before sheep red blood cell (SRBC) injection, followed by 5-day oral doses, reduced significantly ($P < 0.05$) the foot edema evoked by a sub-plantar challenge of mice with SRBC (unpublished results).

A previous study on other *Pterodon* species (*P. emarginatus* and *P. polygalaeflorus*), ascribed the anti-inflammatory capacity of the seeds of these trees to vouacapan derivatives (tetracyclic furane-diterpenes) (13). However, the seed extracts of *Pterodon pubescens* contain a very complex mixture of long-chain alcohols, non-cyclic sesquiterpenes, monocyclic sesquiterpenes, acyclic diterpenes, and tri- and tetracyclic diterpenes, most of which are still uncharacterized.

From the bulk extracts of sucupira seeds we have recently identified, by GC/MS or NMR, some major components such as derivatives of vouacapan and the non-cyclic sesquiterpenes farnesol and geranylgeraniol. Importantly, complex sub-fractions of these extracts rich in vouacapan derivatives or farnesol were very active in preventing carrageenan-induced paw edema (single dose on the order of 0.1 μg kg^{-1} b.w.) and croton oil-induced ear edema (single dose in the order of 0.1 μg/ear) in mice ($P < 0.05$ to $P < 0.001$). A third fraction composed only of geranylgeraniol was very active (on the order of 0.1 ng/ear) only in the latter assay ($P < 0.001$) (8). Additionally, preliminary experiments using the mouse carrageenan-induced pleurisy model revealed a remarkable inhibition ($P < 0.05$) of pleural leukocyte infiltration following single treatments with the vouacapans-enriched fraction or the geranylgeraniol fraction, both at a best dose of 0.1 μg kg^{-1} b.w. (unpublished results).

Therefore, although there are other substances present in two of these fractions, the above-mentioned compounds are the first candidates for the anti-edematogenic and anti-inflammatory activity exhibited in these models, as well as in CIA. In this context, we must recall that farnesylation or geranylgeranylation are post-translational modifications required for the localization of proteins in cell membranes, thus allowing their role in signaling processes. For instance statins, drugs that inhibit these prenylations, have been shown to increase cyclooxygenase-2 expression and prostacyclin formation by human aortic smooth muscle cells, the effect being reversed with the addition of geranylgeranyl-pyrophosphate (14). Therefore, we can not rule out the possibility that the continuous supply of putative prenylation precursors (i.e., farnesol and geranylgeraniol) provided in sucupira could be negatively affecting the cellular signaling events involved in CIA development, such as the production of prostaglandins. Curiously, sucupira extracts are able to inhibit the platelet aggregation induced by arachidonic acid in rabbit plasma (unpublished results).

In summary, sucupira exerts potent clinical benefits on CIA development, impairing both the B and T cell responses involved in disease development. The results demonstrate that sucupira seed extracts contain immunomodulatory substances, which could account

for its beneficial effect on chronic inflammatory conditions as in rheumatic diseases. The results also launch the perspective of the use of this folk medicine in other situations of autoimmune disease or in allograft rejection.

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