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Short Communication

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Cytotoxicity of Dehydrocrotonin (a Nor-Clerodane from *Croton cajucara)* on Human Lymphocytes

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SUMMARY. **Trans-Dehydrocrotonin, a 19-nor-clerodane, is the major norditerpene obtained from *Croton cajucara,* a Brazilian medicinal plant which presents important biological effects, such as antineoplastic and antiulcerogenic activities. In this work, we analyzed the effect of this sesquiterpene lactone on normal human lymphocytes. The cell viability was verified after treatment for 24 and 72 h with trans-dehydrocrotonin, in the presence and absence of phytohemagglutin (specific mitogen for this cell), through three endpoints to assess cytotoxicity *in vitro:* MTT reduction (mitochondrial function), protein quantification (cell number) and phosphatase activity (cell metabolism). When the cells were treated with dehydrocrotonin in the presence of mitogen, no toxic effect was observed. Nevertheless, in the absence of mitogen, the IC50 was 450 μM for MTT reduction and phosphatase activity. Moreover, in this condition, trans-dehydrocrotonin caused stimulation of protein content from 100 μM. Our results suggest that phytohemagglutin protects human lymphocytes against the trans-dehydrocrotonin toxic effect.**

INTRODUCTION

A significant number of cytotoxic sesquiterpene lactones has been isolated along the course of a continuous search for antitumoral agents from plant sources. Beyond their potential as antineoplastic agents, these compounds also present other interesting biological effects, including anti-ulcerogenic, anti-inflammatory, neurocytotoxic and cardiotonic activities [1]. *Croton cajucara* Benth (Euphorbiaceae), commonly known as "sacaca", is widely used as a tea, extracted mainly from its bark and leaves, by Amazonian folk medicine for the treatment of a wide range of gastrointestinal symptoms [2]. The major secondary metabolite, trans-dehydrocrotonin (DCTN), a 19-nor-clerodane, is the principal *nor*diterpene obtained from this plant, which presents important biological activities as antineoplastic and antiulcer [3-5]. Primary lymphocytes culture is important for analysis of celular and molecular events ocurring during immune responses.

The phosphatases are hydrolases that catalyze the hydrolysis of monoester phosphates. Previous studies in our laboratory showed that phosphatases level is an useful tool for studing cell viability [6,7]. These enzymes are divided into three groups: acid phosphatases, alkaline phosphatases and protein phosphatases (PPs). The PPs have been classified by structure and substrate specificity [8].

Protein phosphorylation by kinases may be reverted by protein phosphatases, which are divided into serine/threonine phosphatases and tyrosine phosphatases, based on their substrate specificity [9,10]. In lymphocytes membranes there is an important protein tyrosine phosphatase (CD45) which plays an important role in these cells proliferation [11]. Numerous assays have been recently developed as alternatives for eval-

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uating the toxicity *in vitro* of several compounds. Thus, the therapeutic and toxicological effects of a compound are important parameters in the verification of its applicability in pharmacology [12]. The aim of this work was to examine the cytotoxicity of DHC on human lymphocytes, assessing its effect by MTT reduction [(3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], protein content and protein phosphatase activity.

MATERIALS AND METHODS

DHC obtention

DHC was obtained from *C. cajucara* (Sacaca) barks, as described by Souza Brito et al. 13. The extraction of the powdered bark of *Croton cajucara* was carried out with hexane by a standard method and characterized by spectroscopic methods as IR, UV, MS and 1H-and 13C-NMR, as recently described . The purity was over 99%, analyzed by NMR technique [14].

Lymphocytes isolation and culture conditions

The blood was collected from healthy donators and all experiments were performed according to institutional guidelines approved by Ethics Committee of the Faculty of medicine of State University of Campinas (Proc. N° 311/2000). Peripheral blood was diluited 1:1 with non-suplemented RPMI medium and centrifuged in the presence of hystopaque for 30'(1,500 rpm). Afterwards, cells were ressuspended in RPMI 1640 suplemented with 10% fetal bovine serum, 100 IU/ml penicilin and 100 µg/ml streptomycin and plated at density of $1x10^6$/ml in 24-well plate. The lymphocytes were cultured in a humidified incubator with 5% CO_2 in air, at 37 °C for 48 h, period after which the medium was removed and replaced by the medium containing DHC at concentration ranging from 10 to 100 nM. Endpoints evaluated were: phosphatase (405 nm), protein content (660 nm) and MTT reduction (570 nm).

MTT assay

The cells were washed once with phosphate buffered saline (PBS) before adding 0.1 ml serum-free medium containing MTT (1 mg/mL) to each well. After incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1 ml ethanol with stirring for 15 min on a microtitre plate shaker and the absorbance was read at 570 nm [15].

Protein determination

The protein concentration was determined by a modification of Lowry's method [16].

Trypan blue exclusion

The number of cells was quantified using the tripan blue, which is excluded by viable cells [17].

Phosphatase assay

The enzyme was obtained after lysis of the cells with acetate buffer 0.1 mM, pH 5.0. The reaction mixture (final volume 0.5 mL) contained 100 mM acetate buffer pH 5.0, 5 mM pNPP and cell extract. After a 40 min incubation at 37 °C, the reaction was stopped by adding 0.5 mL of 1 M NaOH. The amount of pNP released was determined by measuring the absorbance at 405 nm [6].

Purification of protein tyrosine phosphatase from membrane of human lymphocytes

Protein tyrosine phosphatase was purified [18], and the homogeneity of the enzyme was evaluated by SDS-PAGE.

Statistical evaluation

All experiments were performed in 24-well tissue culture plates, in triplicate, and the results shown in graphics represent the mean ± the standard deviation.

RESULTS AND DISCUSSION

In this work, we studied the effect on human lymphocytes of trans-dehydrocrotonin (Fig. 1), a type of sesquiterpene, isolated from stem barks of *Croton cajucara*.

The structures of the cytotoxic sesquiterpene lactones are diverse in two aspects: they show considerable variation in carbon skeletons and contain a variety of combinations of functional groups. The first problem was the determination

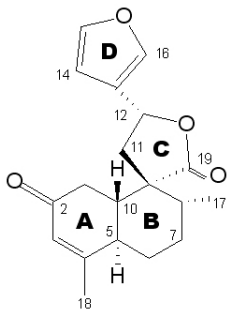

Figure 1. Chemical structure of trans-dehydrocrotonin.

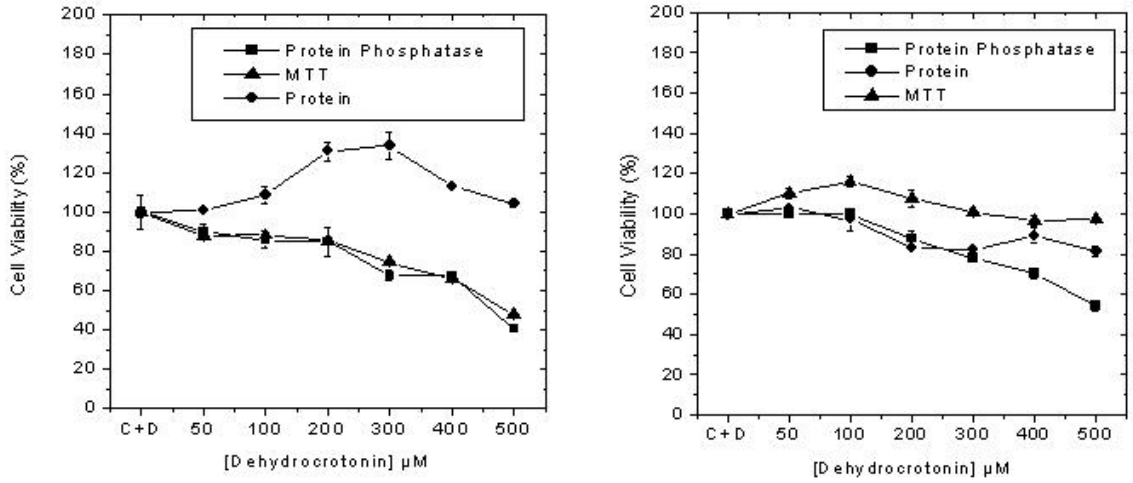

Figure 2. Effect of DHC on cell viability in the absence (**A**) and presence (**B**) of phytohemaglutunin. The curves show the effects of the DHC on human lymphocytes assessed by MTT reduction (A_{570}), protein content (A_{660}) and protein phosphatase. The viability was expressed relatively to the control (100%) and each point represents the mean ± sd of three experiments carried out in eight replicates.

of which functional groups contributed to cytotoxicity. Preliminary evidence suggested that α-methylene-γ-lactone was one of greatest importance. It has been possible to demonstrate that cytotoxicity is critically dependent upon the presence of this funcional group. The α-methylene-γ-lactone reacts rapidly with cysteine to form stable adducts, while endocyclic α,β-unsaturated-γ-lactones, react slowly with cysteine, to form unstable adducts [4] .

The cytotoxic activity of DHC on HL60 cells was measured by MTT reduction (mitochondrial function), protein quantification (cell number) and phosphatase activity (cellular metabolism).Trans-dehydrocrotonin was used at concentrations up to 500 µM. As shown in the Figure 2A, the IC_{50} value was 450 µM when MTT redictions and phosphatase activity were used as parameters and, for protein content, a stimulation was observed when dehydrocrotonin was utilized in concentrations higher than 100 µM, in the absence of phytohemagglutinin. Conversely, in the presence of lectin, this lactone was less toxic (Fig. 2B). These results were similar to those obtained for promyelocytic leukemia cell line, HL60 (IC_{50} = 360 µM; data not shown). Figure 3 shows the inhibitory effect of trans-dehydrocrotonin (50%) on the protein tyrosine phosphatase (CD45), which revealed that the cytotoxic effect of this compound on human lymphocytes might be related to CD45 inhibition. This result confirms the mechanism of sesquiterpene lactones involved in adduct formation with enzymes that present –SH

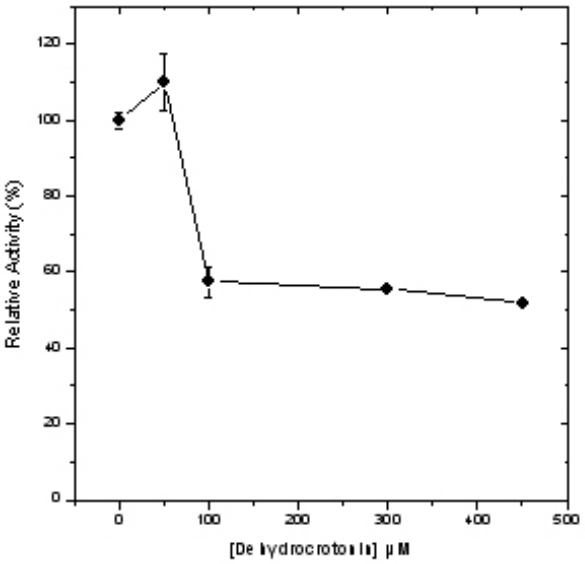

Figure 3. Effect of dehydrocrotonin on human lymphocytes protein tyrosine phosphatase (CD45) activivy. The enzyme was purified and the phosphatase assay was performed in the presence of 100 mM acetate buffer (pH 5.0) and 5 mM pNPP. After a 40 min incubation at 37 °C, the reaction was stopped with NaOH. The amount of pNP released was determined according to described in Materials and Methods.

groups (Michael Reaction). Therefore, the inhibition of CD45 probably was due to this mechanism.

Trans-dehydrocrotonin prevented the mitogenic effect of okadaic acid (OKA) on human lymphocytes (Fig. 4). This compound is a specific inhibitor of protein serine/threonine phosphatases (PP1 and PP2A) and its effect is tran-

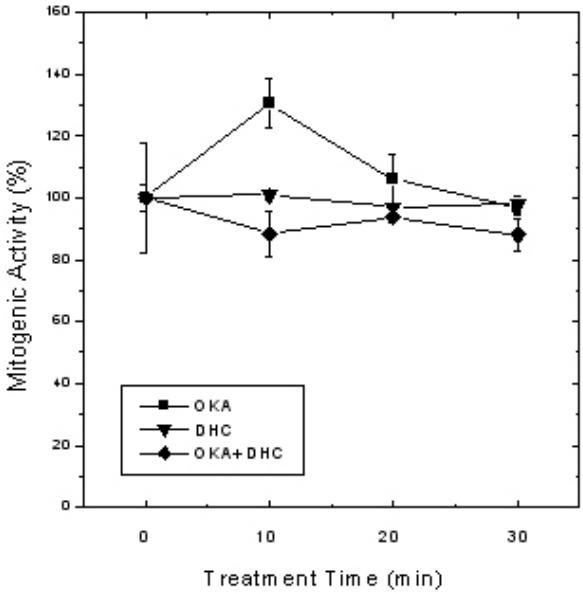

Figure 4. Effect of trans-dehydrocrotonin on mitogenic activity of okadaic acid on lymphocytes culture. The DHC (100 µM) was addictioned on the culture medium with okadaic acid (10 nM) and after 10 min the cell number was determinated in the presence of tripan blue.

sient because OKA presents activity as tumor promoter through the activation of protein kinase C. We have shown previously that DHC inhibited the human promyelocytic leukemia cells growth. Our results suggest that this sesquiterpene lactone could acts on indifferentiated cells (blasts).

Sesquiterpene lactones present functional groups that are responsible by their biological effects. In the specific case of DHC, analyzing its structure, it can be found three funcional groups highly reactives: O=C-C=C (ring A), the lactone (ring C) and ciclopentenone (ring D). As the structure of DHC is not planar, the group O=C-C=C is suggested to be the most available, since it may function as Michael acceptor, characterized by nucleophilic attack involving -SH groups of some proteins, GSH and nitrogen bases, mainly guanine [19,20].

Finally, when taken together, our results indicate that DHC might present specific action on tumour cells, since this lactone presented less toxic effect on normal lymphocytes, and the cytotoxic effect of DHC on human lymphocytes could be related to the inhibition of protein tyrosine phopshatase.

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