

ANAPSOS (*POLYPODIUM LEUCOTOMOS*) MODULATES LYMPHOID CELLS AND THE EXPRESSION OF ADHESION MOLECULES

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Anapsos is a medical prescription registered in the Health Ministry of Spain, that is obtained from the rhizomes of the fern *Polypodium leucotomos*. An immunomodulating effect of Anapsos on certain lymphocyte subsets and cytokines has already been described in the literature. The current study extends and supports part of the aforementioned results of the product on the immune system, showing the ability of Anapsos to stimulate proliferation and activation of T and natural killer lymphocytes, as well as an important down-regulating effect on CD11, CD18 and CD62-L adhesion molecules, both on peripheral blood mononuclear cells and on U-937 and HL-60 cell lines.

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INTRODUCTION

The fern *Polypodium leucotomos*, grows in the rainforest of Central and South America. It has been the subject of research in the last years, in order to analyse its possible immunomodulating effect [1, 2] and its application in some autoimmune diseases [3–5]. *In vitro* and *in vivo* studies performed with a phytoextract called Anapsos, obtained from the rhizomes of this fern, have already shown changes in certain immune cell subsets and cytokines [6–9].

Anapsos is a medical prescription registered since 1975 in the Spanish Health Department as Regender[®] (Health National Registration Number: 55958) and Armaya Fuerte[®] (Health National Registration Number: 55756), being Psoriasis and Atopic Dermatitis its two authorised indications. Most of its clinical effects on the aforementioned diseases have already been confirmed and documented in open and double blind placebo-controlled clinical trials [1, 10–13]. It is administered orally (360–720 mg day⁻¹) and only minor side effects such as abdominal pain have been reported, pointing out these data to its good tolerance and safety like a medicine. The product has also been described to have anti-tumoural activity [14].

In this study, we show the ability of Anapsos to stimulate proliferation and activation of T and natural killer (NK) lymphocytes as well as its important down-regulating effect on CD11, CD18 and CD62-L adhesion molecules, both on peripheral blood mononuclear cells (PBMNC) and U-937 and HL-60 cell lines.

MATERIALS AND METHODS

Preparation of Anapsos

Standardisation of Anapsos is carried out all along the manufacturing process, from botanical identification, till obtaining the extract, including planting crop and harvesting of the rhizomes. Briefly, the rhizomes of the *P. leucotomos* are harvested at a height of 2000 m in the Ecological Recovery and Experimental Plantations in Guatemala (property of ASAC Pharmaceutical International A.I.E.). Once the rhizomes had been identified by botanical experts, they are dehydrated under controlled conditions. The dry rhizomes suitably ground, are extracted with different polar and non-polar solvents at a temperature no higher than 60 °C. The extract thus obtained (Anapsos) is concentrated under controlled conditions of vacuum and temperature and includes water-soluble and lipo-soluble compounds, always containing stable rates for its different phytochemicals [fumaric acid, quinic acid, malic acid, fumarates, quinate, malate sugars and terpenes (wallichiene)]. Standardisation of Anapsos through these

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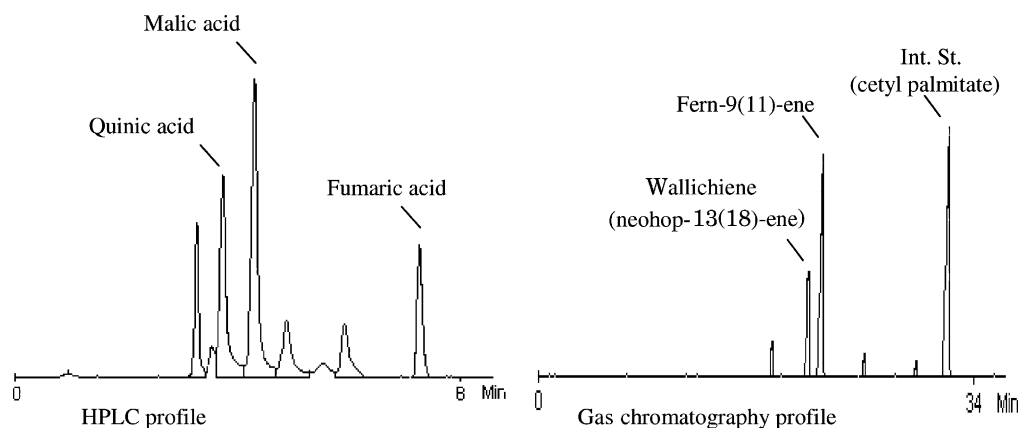


Fig. 1. Quantitative and qualitative HPLC and gas-chromatography finger prints for usual water- and lipo-soluble components of Anapsos.

well known aforementioned markers always ensures the reproducibility among those extracts belonging to different batches. Thus, and prior to be accepted for use, all the extracts will have to show identical quantitative and qualitative HPLC and gas-chromatography finger prints for their usual water and lipo-soluble components (Fig. 1).

Therefore, Anapsos can be included among the emerging concept of 'phytodrugs', a category applicable to natural compounds of constant and stable chemical characteristics [15].

For the *in vitro* research study, Anapsos was filtered by 0.22 μm to ensure sterility, freeze-dried and granted by ASAC at 20 mg of Anapsos per vial. For the *in vivo* studies, we used the authorised registered medicine containing capsules of 120 mg of Anapsos each.

Test sample

In vivo. Anapsos was administered (720 mg day⁻¹, oral capsules) to a group of 10 healthy people of both sexes, aged 21–35, for 11 days, and PBMNC were obtained on the previous day of treatment and on days 1, 4 and 11 after treatment. This dose is the one currently used for the treatment of psoriasis and atopic dermatitis.

In vitro. PBMNC were simultaneously obtained from a second group of 10 healthy people of both sexes, also aged 21–35.

Two different myeloid tumoural lines, HL-60 and U-937, were also used for evaluation. The HL-60 line was established from a acute promyelocytic leukaemia, lacks specific markers for lymphoid cells and expresses receptors for Fc and complement. The U-937 line, established from a diffuse histiocytic lymphoma, is one of the few cell lines displaying many monocytic characteristics and has thus served as a model for monocyte/macrophage differentiation *in vitro*.

Cell culture. PBMNC were obtained by gradient density centrifugation (Ficoll–Hypaque). A lymphocyte purity

over 90% was reached in all the samples. PBMNC from the non-treated people, as well as myeloid tumoural cells, were adjusted at $1 \times 10^6 \text{ ml}^{-1}$ with RPMI 1640 culture medium, supplemented with 10% foetal calf serum, 1% antibiotic and 1% glutamine 2 mM. PBMNC were placed onto 12-well plates per triplicate at a volume of 1 ml well⁻¹ and incubated at 37 °C and 5% CO₂ for 0, 6, 18, 24, or 48 h, without any stimulus or stimulated with 10 $\mu\text{g ml}^{-1}$ of phytohemagglutinin (PHA), 150 $\mu\text{g ml}^{-1}$ of Anapsos as has been shown in previous studies [9] or PHA + Anapsos. Myeloid tumoural cells were cultured for 0, 6 or 24 h, without any stimulus or stimulated with 150 $\mu\text{g ml}^{-1}$ of Anapsos, 20 $\mu\text{g ml}^{-1}$ of lipopolysaccharide (LPS) and LPS + Anapsos. PBMNC obtained from the treated group, were directly analysed.

Phenotypical analysis

The different lymphocyte subsets were subsequently analysed and quantified using phenotypical analysis by flow cytometry with double or triple labelling, using the most appropriate combination of monoclonal antibodies in each case: CD2, CD3, CD4, CD16, CD56, CD69, CD11a, CD11b, CD18 and CD62-L (L-selectin) (Caltag). Flow cytometry was carried out with the FACS Vantage flow cytometer (Becton & Dickinson), equipped with an INNOVA 621 II Enterprise Ion Laser. Ten thousand events per lymphocyte population were acquired and analysed. Data samples were analysed using the Cell Quest Software (Becton & Dickinson).

Statistics

All statistical calculations were performed using the SPSS Windows Release 10.0 software package. Data are presented as arithmetic mean values \pm SEM. Parametric (Student's *t*-test for paired and unpaired data) and non-parametric Mann–Whitney *U*-test (two-tailed) were used to compare between groups. Probability values <0.05 were considered significant.

RESULTS

In vitro results

Unlike PHA, stimulation with Anapsos alone showed a trend to increase the percentage of CD4+ cells along the culture, that reached maximum levels at 18 h, these values being significant versus unstimulated cells (59 ± 3 vs 46 ± 2 , $P = 0.007$). Similar results were found when PHA + Anapsos stimulation was used (62 ± 5 vs 46 ± 2 , $P = 0.008$) [Fig. 2(A)]. In the first 6 h of culture, stimulation with Anapsos (vs unstimulated cells) was able to increase the NK cells (CD3-CD16+CD56+) (20 ± 2 vs 14 ± 3 , $P = 0.03$) in a very similar way to that found with PHA (23 ± 2 vs 14 ± 3 , $P = 0.01$). However, costimulation with PHA and Anapsos showed an important increase in the percentage of NK cells during the first 18–24 h of culture, that yielded its most significant values at 6 h, when compared with PHA (39 ± 4 vs 23 ± 2 , $P < 0.001$) or Anapsos alone (39 ± 4 vs 20 ± 3 , $P = 0.002$) [Fig. 2(B)]. *In vitro* stimulation with PHA alone (vs unstimulated cells) tended to cause the expected decrease in the percentage of cells expressing CD2 and CD3 surface antigens; similar results were obtained with PHA + Anapsos stimulation (data not shown).

Increase in activation markers such as CD69 was also observed on NK cells after *in vitro* stimulation with

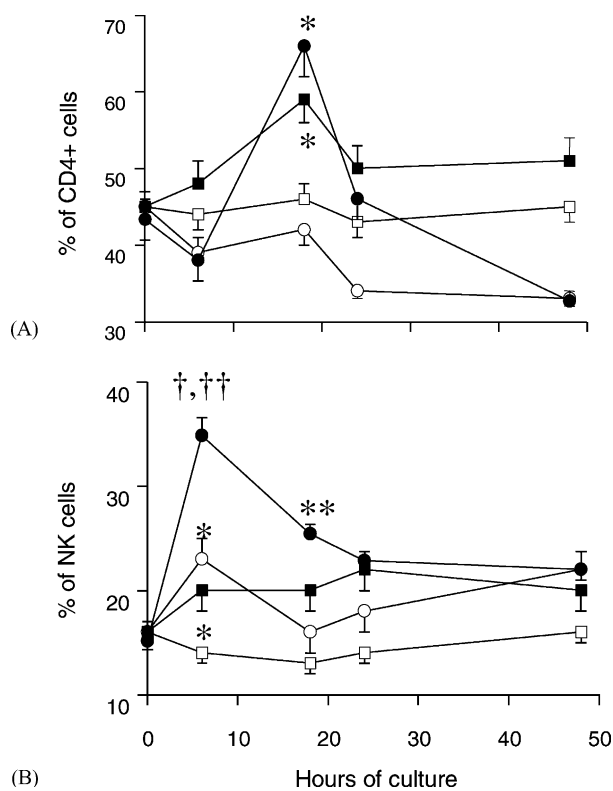


Fig. 2. *In vitro* expression of: (A) CD4+ cells and (B) NK (CD3-CD16+CD56+) cells on non-stimulated (\square), or stimulated PBMNC with PHA (\circ), ANP (\blacksquare) or PHA + ANP (\bullet). Bars express the mean \pm SEM of 10 individuals assayed in triplicate cultures. (* $P < 0.05$ vs control cells; ** $P < 0.01$ vs control cells; † $P < 0.001$ vs PHA stimulated cells; †† $P < 0.005$ vs ANP stimulated cells).

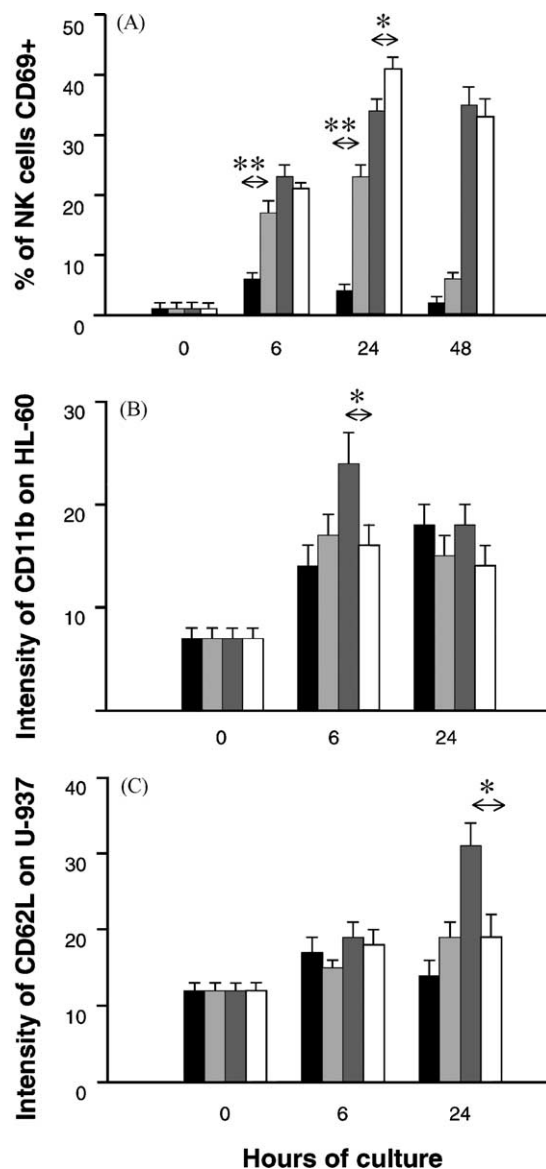


Fig. 3. (A) Simultaneous expression of NK (CD16+ and/or CD56+) and CD69 antigens on cultured PBMNC. (B) CD11b intensity expression on HL-60 cell line. (C) CD62-L intensity expression on U-937 cell line. Bars express the mean \pm SEM of 10 individuals assayed in triplicate cultures. (* $P < 0.01$; ** $P < 0.001$). (\blacksquare) Non-stimulated cells. (\square) Stimulated cells with ANP. (\blacksquare) Stimulated cells with PHA (A) or LPS (B, C). (\square) Stimulated cells with PHA + ANP (A) or LPS + ANP (B, C).

Anapsos and/or PHA. Anapsos induces expression of CD69 during the early 24 h of culture ($P < 0.001$). The costimulation with PHA and Anapsos increases the expression induced by PHA alone ($P < 0.01$). After 24 h of culture no effect of Anapsos was observed [Fig. 3(A)]. In contrast to the aforementioned effects observed on CD69, Anapsos was able to suppress significantly the LPS-induced CD11b and CD62-L fluorescence increase on tumour cell lines ($P < 0.01$) [Fig. 3(B, C)] and to reduce PHA-induced lymphocyte aggregation (Fig. 4). The same effect was observed with CD11a and CD11b on lymphocytes and monocytes, respectively (data not shown).

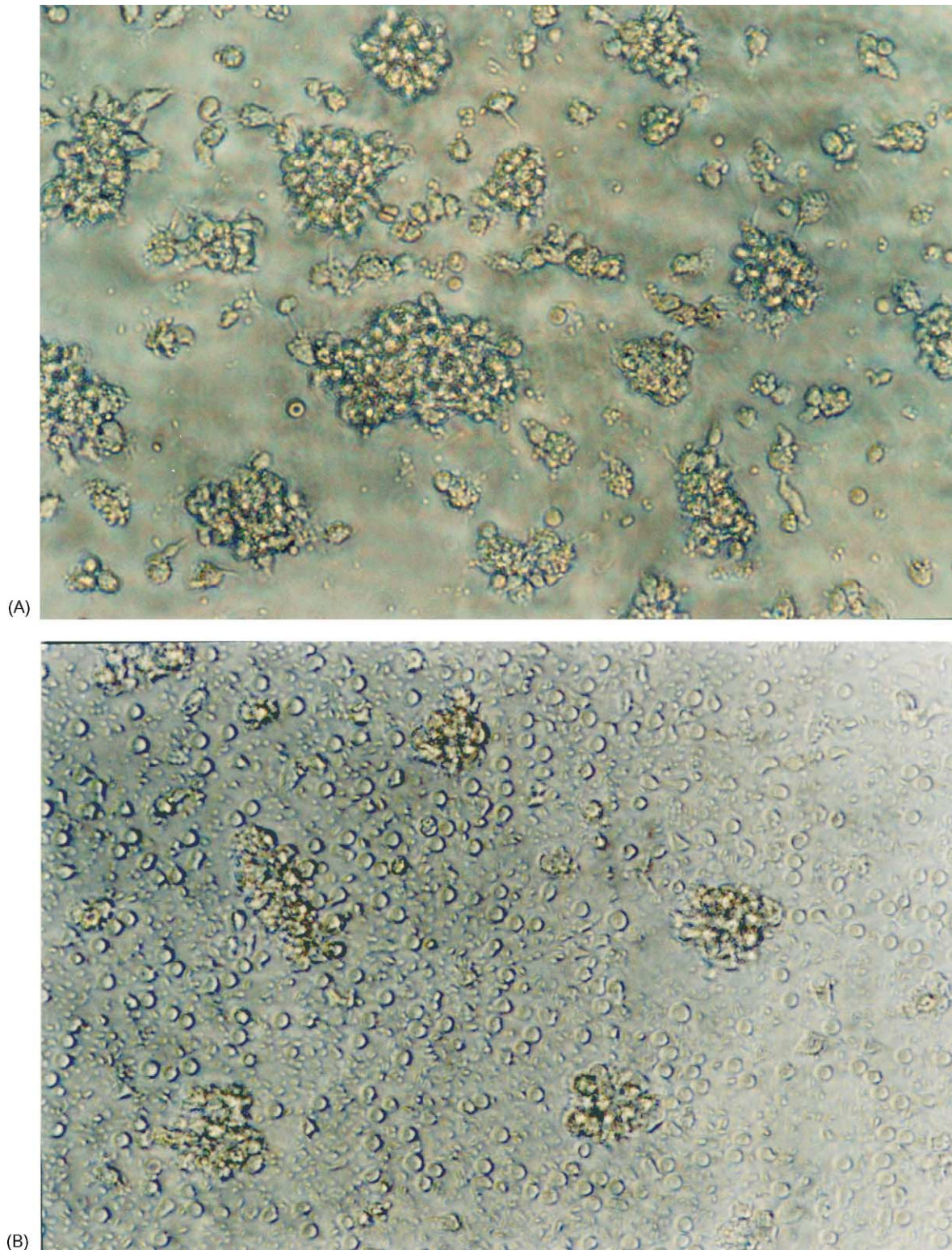


Fig. 4. *In vitro* lymphocyte aggregation under stimulation with: (A) PHA or (B) PHA + ANP.

In vivo results

A trend to increase the percentage of CD4+ and NK cells was also observed *in vivo* after the Anapsos oral intake, but without reaching significant levels. However, *in vivo* effects of Anapsos on the expression of the adhesion molecules were much more marked than those found *in*

vitro. Thus, Anapsos was able to down-regulate CD11b expression on monocytes (70 ± 4 vs 97 ± 3 , $P < 0.001$) and CD11a (46 ± 2 vs 62 ± 4 , $P = 0.007$), CD11b (4 ± 1 vs 14 ± 2 , $P = 0.02$) and CD18 (52 ± 3 vs 73 ± 3 , $P = 0.002$) expression on lymphocytes, to a higher extent than that observed *in vitro* [Fig. 5(A, B)]. Although the above

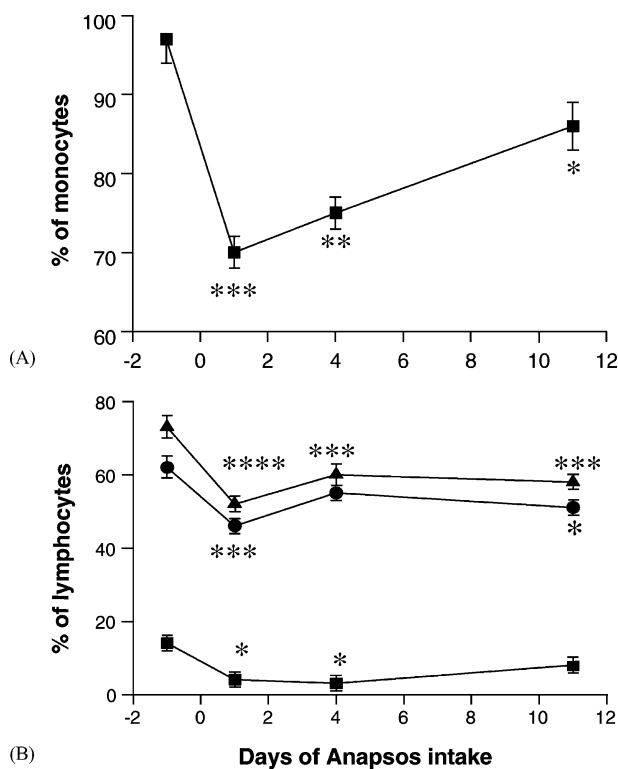


Fig. 5. *In vivo* expression of: (A) CD11b on monocytes and (B) CD11a (●), CD11b (■) or CD18 (▲) on lymphocytes during Anapsos intake. Bars express the mean \pm SEM of 10 individuals. (* $P < 0.05$ vs day - 1; ** $P < 0.01$ vs day - 1; *** $P < 0.005$ vs day - 1; **** $P < 0.001$ vs day - 1).

mentioned significant values are referred to the first 24 h after the Anapsos intake (day + 1, maximum reached effect), the decrease in these values was maintained all along the culture.

DISCUSSION

Anapsos is a purified and standardised phytodrug obtained from the dried rhizomes of the fern *P. leucotomos*, which grows in Central America. Some of its compounds have showed anti-degenerative, neurotrophic and immunomodulating activities [9, 15, 16].

Flow cytometry is a very sensitive method to identify and characterise cell populations. One of its most common applications in the clinical setting include immunophenotyping of the cell surface differentiation antigens with fluorescently conjugated monoclonal antibodies [17]. Analysis of different immune subsets carried out on several *in vivo* and *in vitro* studies performed with Anapsos, both in animals and in patients, have already shown a pleiotropic activity. This modulating effect has been related with its efficacy to treat certain autoimmune diseases and neurodegenerative disorders [3–6, 9, 12, 15, 16].

The present study describes an ability of Anapsos to increase the percentage of T lymphocytes in their helper population (CD4+). Most antibody-dependent cellular cytotoxicity in peripheral blood is mediated by NK cells

and also by a small subpopulation of T lymphocytes which expresses CD16 [18]. The current results also indicate an ability of Anapsos to up-regulate the percentage of the aforementioned cytotoxic cells. The significant increase observed in the co-expression of NK cells with membrane antigens like CD69 means that Anapsos is equally capable of activating these cells. This activation is observed during the early 24 h of culture. After that, this effect is not observed anymore due to either the depletion of the product in the medium or to the normal down-regulation of that marker. The activation of these cells allows to explain the above-mentioned anti-tumoural activity of the product [14] and supports the results of previous studies on cytokines, where a main effect of the product on cellular immunity was shown [9, 19, 20].

Leukocytes possess specific receptors for antigen adhesion molecules to interact with other cells and carry out their function. In the inflammatory response, the adhesion molecules are also implicated. However, although inflammation is usually a physiological phenomenon it can also become pathological when reaches extremes, deriving in different diseases that usually course with an abnormal increase on the expression of adhesion molecules [21, 22]. Thus, medicines capable of reducing the overexpression of the adhesion molecules in these processes could be useful in their treatment.

In this study we show an ability of Anapsos to down-regulate both *in vitro* and *in vivo*, the expression of certain adhesion molecules belonging to the β 2-integrin family (CD11a, CD11b, CD18) on leukocytes, being this effect responsible for the observed *in vitro* decrease of the PHA-induced lymphocyte aggregation. Anapsos also avoids the LPS-induced increased expression of different adhesion molecules (CD11b, CD62-L) spontaneously expressed on myeloid tumoural cell lines. The mechanism exerted by Anapsos on the adhesion molecules is still not known, but it could be related with its pleiotropic ability to down-regulate the secretion of proinflammatory cytokines such as IL-1 β and to up-regulate anti-inflammatory cytokines such as IL-10 [8, 9]. The down-regulating effect that Anapsos exerts on the aforementioned adhesion molecules lets us justify why the product has shown to be efficient on several autoimmune diseases [3, 4, 23] and makes it interesting for being evaluated in other autoimmune pathologies.

Different components of Anapsos justify its pleiotropic activity. Thus, the Anapsos stimulating effects observed on lymphocyte subsets, as well as the synergistic effects shown with PHA, can be related with some of the aforementioned compounds present in the extract. Likewise, the fumarates and fumaric acid of the extract can be responsible for the ability of the product to down-regulate the expression of adhesion molecules and proinflammatory cytokines [24, 25].

Further clinical studies will be necessary to elucidate the relationship between the phenotypic changes observed with Anapsos and their function, as well as its inhibiting activity on the adhesion molecule expression.

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