Isolation and Chemical Analysis of a Fatty Acid Fraction of *Kalanchoe pinnata* with a Potent Lymphocyte Suppressive Activity

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Abstract: **Previously we demonstrated that *Kalanchoe pinnata* (KP) leaf extracts inhibited *in vitro* lymphocyte proliferation and showed *in vivo* immunosuppressive activity. Here we attempt to identify the immunosuppressive substances present in KP guided by the lymphoproliferative assays. From the ethanolic extract was purified a fraction (KP12SA) twenty-fold more potent to block murine lymphocyte proliferation than the crude extract. Chemical analysis by ^{1}H- and ^{13}C-NMR, IR and GC-MS of KP12SA (methylated sample) showed 89.3% of palmitic acid (C_{16}), 10.7% of stearic acid (C_{18}) and traces of arachidic (C_{20}) and behenic acids (C_{22}). This study provides evidence that fatty acids present in *Kalanchoe pinnata* may be responsible, at least in part, for its immunosuppressive effect *in vivo*.**

Key words: *Kalanchoe pinnata*, Crassulaceae, ethanolic extract, saturated fatty acid, lymphocyte proliferation.

Introduction

The genus *Kalanchoe* (Crassulaceae) comprises about one hundred species, most of them native from Madagascar (1). *Kalanchoe pinnata* (KP) is a perennial medicinal herb popularly used in Brazil and other parts of the world to treat various inflammatory diseases (2), (3), which contains substances such as bufadienolides (4), terpenoids and flavonoids (5). In a previous work (6), we demonstrated that different extracts from KP leaves blocked human lymphocyte proliferation with no effect on the natural killer activity.

Treatment of mice with KP aqueous extract reduced the proliferative response of spleen cells to the mitogen and antigen; impaired the delayed-type hypersensitive reaction to ovoalbumin (3) and protected mice against progressive infection with *Leishmania amazonensis* (7), (8).

In this study, we describe the chemical composition of a potent fraction of *Kalanchoe pinnata* on lymphocyte proliferation.

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Materials and Methods

General experimental procedures

Reverse phase column chromatographies were carried out on silanized silica gel RP-2 (70 – 230 mesh, Merck) using a gradient of water-methanol. A Sephadex LH-20 gel (25 – 100 mm, Sigma) column was also used. TLC plates (Silica 60 F_{254} Merck; CH_2Cl_2 : MeOH, 98 : 2) were developed with a spray of ceric sulfate solution. ^{1}H- (200 MHz; DMSO-d_6; δ 2.49 as internal reference) and ^{13}C-NMR (50 MHz) spectra were recorded in a Varian Gemini 200.

Plant material

K. pinnata was collected during the autumn season, out of flowering time, at the garden of Universidade Federal do Rio de Janeiro campus (Brazil). A voucher specimen nr 292.697 is deposited at the herbarium of Rio de Janeiro's Botanical Garden.

Ethanolic extraction

Dried leaves (278 g), previously powdered, were macerated in ethanol at room temperature. The ethanolic extract (EE) was concentrated until dryness under vacuum at 65 – 70 °C, yielding 9 g of a dark-green syrupy material.

Purification of EE

The crude EE (9 g) was exhaustively washed with water at room temperature. The water-soluble fraction (EE-1; 4.3 g) was chromatographed on a RP-2 column yielding twelve fractions. The most T-cell suppressive fraction (KP12), eluted with MeOH, was further purified.

KP12 purification

KP12 (739 mg) was washed with CH_2Cl_2, yielding two fractions (KP12S and KP12I). The CH_2Cl_2-soluble fraction (KP12S; 625 mg) was chromatographed on a Sephadex LH-20 column (24 × 1.2 cm) with CH_2Cl_2 : EtOH (1 : 1) to afford only two fractions, KP12SA (532 mg) and KP12SB (14 mg). The most active fraction (KP12SA) was further analysed.

KP12SA: A light brown wax-like material; ^{1}H-NMR (DMSO-d_6, ppm): δ = 0.93 (-C$\underline{H}_3$); 1.25 (intermediary chain C$\underline{H}_2$-); 1.50

(-C<u>H</u>$_2$-CH$_2$-CO$_2$H); 2.20 (-C<u>H</u>$_2$-CO$_2$H); ^{13}C-NMR (DMSO-d_6, ppm): δ = 174.5 (C-1), 33.7 (C-2), 31.3 – 24.5 (5 signals, C-3 to C-14), 22.1 (C-15), 13.9 (C-16). GC/MS data (Table **2**).

Methylation: Methylated samples from KP12SA (30 mg) and palmitic acid (Merck Lot 69370) were prepared as described by Schmitz and Pelka (9).

GC-MS: A HP6890 gas chromatograph using a HP5-MS capillary column (30 m × 0.25 mm I.D) was used for KP12SA analysis using palmitic acid as standard with injections of 1 μl for both samples (helium as carrier gas). Injector and detector temperatures were held at 270 °C. Column temperature was held at 70 °C for 1 min, and then programmed at a rate of 10 °C/min to 290 °C. Fatty acid methyl esters were identified by comparison with library standards data.

Methylated palmitic acid: GC/MS; *Tetradecanoic acid methyl ester* (C_{15}) t_{R1} 12.55 – 12.79 min (traces), *m/z* = 242 ($M^{+\cdot}$), 199, 143, 87, 74, 55; *12-Methyltetradecanoic acid methyl ester* (C_{16}) t_{R2} 13.56 – 13.70 min (traces), *m/z* = 256 ($M^{+\cdot}$), 213, 143, 87, 74, 55; *Palmitic acid methyl ester* (C_{17}) t_{R3} 14.78 min (97.6%), *m/z* = 270 ($M^{+\cdot}$), 227, 143, 87, 74, 55; *Heptadecanoic acid methyl ester* (C_{18}) t_{R4} 15.21 min (traces), *m/z* = 284 ($M^{+\cdot}$), 241, 185, 143, 87, 74, 55; *Stearic acid methyl ester* (C_{19}) t_{R5} 16.50 min (2.4%), *m/z* = 298 ($M^{+\cdot}$), 255, 143, 87, 74, 55.

Cells

BALB/c mice (20 g) lymphocytes from inguinal lymph nodes and human peripheral blood lymphocytes (PBMN) were prepared as described elsewhere (6), (7).

T Cell proliferation

Murine or human lymphocytes were cultivated as described elsewhere (6), (7). Briefly: murine cells (4 × 10^6 cells/ml) were cultivated with 2.5 μg/ml ConA (10) (concanavalin A, Sigma Chem. Co) 48 h at 37 °C in 5% CO_2, in the absence or presence of different concentrations of crude extract or fractions. In control experiments, lymphocytes were cultivated in the presence of palmitic acid (Merck), cyclosporin A (Sandoz Pharmaceuticals, USA) or azathioprine (Microbiologica Química e Farmacêutica LTDA, Brazil). Proliferation was measured by incorporation of 0.5 μCi [^3H]-thymidine/well added 6 h before the end of the culture period. The cells were collected onto glass fibre filters using a dot-blot apparatus (11) and the incorporated radioactivity measured by liquid scintillation spectrometry. PBMN were cultured in the same way, except that the stimulus used was the human T cell mitogen phytohemagglutinin (PHA) at 5 μg/ml.

Results and Discussion

In this study we used the ethanolic extract (EE) obtained from leaves collected during the autumn season, in a region at sea level, where the plant grew under direct sunlight and with a low supply of water.

The fractionation of EE was guided by the murine and human T cell proliferation *in vitro*. Unprimed T cells are normally induced to proliferate by the lectins Con A and PHA (10). These mitogens activate T cells by indirectly crosslinking their antigen receptors (TCR), and therefore serve well for monitoring T cell activation in a mixed cell population (10), (12). Table **1** shows that EE was more potent to inhibit human lymphocyte proliferation (IC_{50} = 17.5 μg/ml) than murine proliferation (IC_{50} = 500 μg/ml). This different effect of EE may be due to the different stages of activation of these two kinds of cells.

The chromatography of the water-soluble fraction (EE-1), which corresponds to 47.8% of EE, on a reverse-phase column resulted in twelve fractions. The most apolar of them (KP12) showed a very high inhibition of murine T cell proliferation (Table **1**). KP12, corresponding to 8.2% of EE, was washed with CH_2Cl_2 yielding the soluble fraction KP12S which was much more active than the insoluble KP12I fraction on both murine and human cells. Further fractionation of KP12S on Sephadex LH-20 produced two subfractions. KP12SA which maintained its strong inhibitory activity and KP12SB, which was much less active, with an IC_{50} higher than 50 μg/ml for both murine and human cells. The observation that fractionation of KP12S was not accompanied by further increase in its inhibitory activity may be due to the elimination of substances which acted in synergy with the most active substances concentrated in KP12SA.

The ^{1}H- and ^{13}C-NMR analysis of KP12SA showed an aliphatic saturated chain. The triplet signal at 0.93 ppm (-C<u>H</u>$_3$) corresponds to the terminal methyl group (-<u>C</u>H$_3$; 13.6 ppm). The long intermediary proton methylene chain was assigned at 1.25 pm. The corresponding carbon signals were listed between 33.7 and 22.1 ppm.

The signal at 174.5 ppm was attributed to the carbonyl group of an acidic function. Methylene protons in the α and β positions to -COOH were listed at 2.20 (triplet) and 1.50 ppm (multiplet), respectively. No signals for olefinic carbons were observed, in agreement with the ^{1}H-NMR spectrum. The approached proton test (APT) ^{13}C-NMR spectrum did not show any -CH signal, only confirming the -CH$_3$ group.

Table 1 Inhibition of the proliferative response of murine and human T cells by *K. pinnata* EE, its fractions and positive control drugs.

Sample	Murine T cells ((IC_{50}) μg/ml)	Human T cells ((IC_{50}) μg/ml)
EE	500.0	17.5
KP12	17.0	ND
KP12S	22.5	12.5
KP12I	>50.0	>50.0
KP12SA	24.0	12.0
KP12SB	>50.0	>50.0
palmitic acid	33.5	6.0
cyclosporin A	1.8	ND
azathioprine	ND	0.2

Murine and human T cells were stimulated with 2.5 μg/ml Con A/48 h or 5 μg/ml PHA/96 h, respectively, in the presence of various concentrations of KP ethanolic extract (EE), its fractions (KP12, KP12S, KP12I, KP12SA or KP12SB) and positive control drugs (cyclosporin A, azathioprine or palmitic acid) in triplicate samples for each concentration point. The lymphoproliferative responses were measured by incorporation of [^3H]-thymidine and the results obtained from three independent experiments were expressed as ((IC_{50}) μg/ml). Controls cultured with the mitogens alone induced a proliferation of 35,300 ± 660 cpm (murine cells) and 42,550 ± 1300 cpm (human cells). ND = not determined.

Table 2 Composition of a methylated KP12SA sample determined by GC-MS.

Substance	t_R (min)	Percentage	Fragments (*m/z*)
Palmitic acid methyl ester (C_{17})	14.63	89.3	270, 227, 143, 87, 74, 55
Stearic acid methyl ester (C_{19})	16.52	10.7	298, 255, 143, 87, 74, 55
Arachidic acid methyl ester (C_{21})	18.19 – 18.34	Traces	326, 283, 87, 74, 55
Behenic acid methyl ester (C_{23})	19.87	Traces	354, 87, 74, 55

A HP6890 gas chromatograph using a HP5-MS capillary column with injections of 1 μl for both samples. Equipment coupled to a HP5972 mass spectrometer. Fatty acid methyl esters were identified by comparison with data of a library standards.

The fatty acid composition was also confirmed by the IR analysis. We observed a large band at 3400 cm^{-1} attributed to the axial deformation of O-H. Bands between 2960 – 2870 cm^{-1} correspond characteristically to saturated chains. The absorption at 1715 cm^{-1} was attributed to the carbonyl of carboxyl group.

These experiments revealed that KP12SA could be a single or a mixture of two or more saturated fatty acids. In order to identify its composition, a sample was methylated and submitted to GC-MS. Two major signals were listed (Table **2**), one at t_R 14.63 min and other at t_R 16.52 min. The methyl esters corresponding to these peaks were identified by comparison of their retention time and mass spectrum fragmentation pattern with library standards data.

The major substance corresponds to 89.3 % of KP12SA (t_R 14.63 min) and was identified as palmitic acid (C_{16}). Stearic acid (C_{18}) was present at 10.7 % and corresponds to the signal at 16.52 min. Traces of two other saturated fatty acids were identified as arachidic (C_{20}) and behenic (C_{22}) acids.

Our data indicate that KP12SA, which is mainly constituted of palmitic acid, is a better inhibitor of lymphocyte proliferation than the crude extract, specially in regard to murine cells.

Many studies have investigated the level and composition of fat consumed in human and rat diet and its effects on the immune system (13), (14). Studies showed that inhibitory effect of unsaturated fatty acids on human and murine lymphocytes (15 – 17) is more pronounced than that found with saturated fatty acids, such as palmitic and stearic acids. Our study provides, for the first time, evidence that an enriched saturated fatty acid fraction from *Kalanchoe pinnata* plays an important role on lymphocyte proliferation, which may explain its immunosuppressive effect *in vivo*.

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