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Immunoenhancing Properties of *Plantago major* Leaf Extract

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***Plantago major* (PM), also known as plantain, is a weed found in temperate zones worldwide. PM leaves have been associated with various biological properties ranging from antiinflammatory, antimicrobial and antitumour to wound healing. However, its mechanism of action associated with boosting of the immune function remains to be elucidated. We found that endotoxin-free methanol extracts from PM leaves, at doses of 50, 100, 250, and 500 μg/mL, were associated with 4.4 ± 1, 6 ± 1, 12 ± 0.4, and 18 ± 0.4-fold increases of nitric oxide (NO) production, and increased TNF-α production (621 ± 31, 721 ± 36, 727 ± 36, and 1056 ± 52 U/mL, respectively) by rat peritoneal macrophages, in the absence of IFN-γ or LPS. NO and TNF-α production by untreated macrophages was negligible. In addition, PM extracts potentiated Con A-induced lymphoproliferation (3- to 12-fold increases) in a dose-dependent fashion, compared with the effect of ConA alone. The regulation of immune parameters induced by plant extracts may be clinically relevant in numerous diseases including chronic viral infections, tuberculosis, AIDS and cancer. Copyright © 2000 John Wiley & Sons, Ltd.**

Keywords: Plantago; leaf extract; macrophage; T cell; immunopotentiation; cell activation.

INTRODUCTION

Plantago major, also known as plantain, waybread, or dooryard plantain, is found on roadsides, fields, lawns and waste places in temperate zones worldwide. *Plantago major* has been used in traditional medicine as an astringent, anaesthetic, antihelmintic, analgesic, analeptic, antiviral, antihistaminic, antiinflammatory, antirheumatic, antitumour, antiulcer, diuretic, hypotensive and expectorant (Matev *et al.*, 1982; Grigorescu *et al.*, 1973; Franca *et al.*, 1996). In addition, plantain is well known to neutralize poisons internally and externally. The fresh leaves are crushed and applied to wounds to prevent or cure infection and hasten healing. Moreover, plantain is a fast pain reliever of stings, bites and poison ivy. The intracellular fluid from *Plantago major* has been also shown to possess prophylactic activity against the development of mammary tumours in mice (Lithander, 1992). However, *Plantago major*'s mechanism(s) of action on immunomodulation remains to be elucidated.

The present study was designed to evaluate the *in vitro* effects of methanol extracts of *Plantago major* leaves on rat peritoneal macrophage and thymic lymphocyte functions. Macrophages play a central role in modulating humoral and cellular immunity against infectious diseases and cancer. Immunomodulatory agents such as inteferon-gamma (IFN-γ) and lipopolysaccharide (LPS) are capable of activating them (Meltzer *et al.*, 1982). Activated macrophages produce mediators of cytotoxicity such as nitric oxide and tumour necrosis factor-alpha (TNF-α) which protect the host against the development of infections and tumours (Nathan and Hibbs, 1991; Hibbs *et al.*, 1988). On the other hand, T lymphocytes respond to antigen challenge by proliferating and expanding the antigen-specific lymphocyte clones thus amplifying immune responses. Functional T cell proliferating activity can be studied by the use of polyclonal mitogens such as concanavalin A (Con A) and phytohaemagglutinin (PHA), which bind to certain sugar residues on T cell surface glycoproteins, including the T cell receptor and CD3 protein, and stimulate T cell proliferative response (Gajewski *et al.*, 1989).

In the present study, LPS was used as a positive control for macrophage activation, and Con A was used to stimulate lymphocyte proliferation. It was found that *Plantago major* leaf extracts activated nitric oxide and TNF-α production by macrophages, and potentiated Con A-mediated lymphocyte proliferation.

MATERIALS AND METHODS

Reagents, culture media, and cell line. Penicillin–

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streptomycin solution, L-glutamine, trypsine-EDTA solution and RPMI 1640 and AIM-V media were obtained from Life Technologies (Grand Island, NY). LPS from *Escherichia coli* serotype 0128:B12, fetal bovine serum (FBS), dimethylsulphoxide (DMSO), Con A, red blood cell lysing buffer, phosphate-buffered saline (PBS), and 3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Actinomycin D was obtained from ICN Pharmaceuticals (Aurora, OH). The murine fibrosarcoma cell line L929 (clone CCL 1) was purchased from the American Type Culture Collection (Rockville, MD), and maintained in RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine, and 0.5% penicillin–streptomycin solution (referred as complete RPMI 1640 medium). [^{3}H]-thymidine was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Chloroform, acetone and methanol were of high performance liquid chromatography (HPLC)-grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI). Extraction columns for solid phase separations were purchased from United Chemical Technologies (Bristol, PA).

Preparation of *Plantago major* leaf extract. *Plantago major* (PM) leaves were obtained in a powder form (Lot # 8680) from M. E. Cody Products, Inc. (Westwood, NJ). This leaf powder (400 g) was macerated overnight with 500 mL methanol at room temperature. The extract was filtered, and combined with acetone until an off-white precipitate was formed. This material was then dried under vacuum using a speed vac concentrator (Savant Instruments Inc., Hicksville, N.Y.), dissolved in 15 mL deionized water, and the suspension was centrifuged at 2000 rpm for 10 min. The aqueous fraction was then concentrated to dryness using a speed vac concentrator (Savant Instruments Inc.). The solid was weighed (3.0 g) and labelled PM_1.

Purification of PM_1 fraction. A 2 g capacity copolymeric phase extraction column (CLEAN SCREEN®, United Chemical Technologies, Inc.), composed of $Si(CH_2)_{17}CH_3$ (octadecyl copolymerized on a rigid, purified silica gel support), was conditioned by elution with 5 mL methanol, followed by elution with 5 mL deionized water. The elutions were performed at a fast flow as recommended by the manufacturer. PM_1 (200 mg) was then dissolved in 10 ml deionized water, added to the conditioned column, and eluted as explained above. The eluted fraction was concentrated to dryness under vacuum using a speed vac concentrator (70 mg recovery). The material bound to the column was eluted with 15 mL methanol. The eluted fraction was concentrated as described above and then pre-labelled bound (28- mg recovery). When it was found that the bound fraction retained the biological activity found in PM_1, it was finally labelled PM_2. All fractions were dissolved in PBS, and filtered before use. PM_1 and PM_2 preparations were endotoxin-free at a detection limit of 0.5 ng/mL in the gel clot-based *Limulus* amoebocyte assay (Associates of Cape Cod, Falmouth, MA).

Animals. Sprague-Dawley male rats (200–220 g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). They were given water and food *ad libitum*, and were housed and handled according to guidelines of the Institutional Animal Care and Use Committee (IACUC) (protocol # 659).

Preparation of macrophage cultures. Peritoneal macrophages were harvested by lavaging the peritoneal cavity with cold RPMI 1640 medium immediately after rat death, as described elsewhere (Gomez-Flores *et al.*, 1997b; Calderon *et al.*, 1994). The cell suspension was washed once with this medium, and suspended and adjusted at a concentration of 1.7×10^6 cells/mL in AIM-V medium containing 0.5% penicillin–streptomycin solution (since serum has been reported to potentiate macrophage activation (Chen *et al.*, 1994), the culture medium was changed at this step to the serum-free medium AIM-V which has been observed to support cell culture (Kaldjian *et al.*, 1992). One hundred microlitre-aliquots of this cell suspension were incubated in flat-bottomed 96-well plates (Becton Dickinson, Lincoln Park, NJ) for 2 h at 37 °C in 5% CO_2. Non-adherent cells were removed, and adherent cells (about 70% of the input cells or about 1×10^6 cells/mL) were then incubated 16 h in 100 μL of AIM-V medium in the presence or absence of various concentrations of PM fractions. After incubation, cell monolayers were washed twice in AIM-V. The final cell monolayer consisted of >95% macrophages as judged by Giemsa's stain procedures.

Nitrite determination. Accumulation of nitrite in the supernatants of macrophage cultures was used as an indicator of nitric oxide production by resident or activated cells. Untreated and PM-treated macrophages were incubated in triplicate at 37 °C in 5% CO_2, in a total volume of 200 μL AIM-V medium per well for 3 days. After incubation, supernatants were obtained and nitrite levels were determined with the Griess reagent as reported elsewhere (Gomez-Flores *et al.*, 1997a), using $NaNO_2$ as a standard. Optical densities at 540 nm were then determined in a microplate reader (Molecular Devices Corporation, Palo Alto, CA).

TNF-α assay. TNF-α production by macrophages was determined by the L929 cell lysing assay. For this bioassay, untreated and PM-treated macrophage monolayers were incubated in triplicate at 37 °C in 5% CO_2, in a total volume of 200 μL of AIM-V medium for 4 h, after which supernatants were collected and kept at −80 °C until use. TNF-α levels in the supernatants were then quantified by the L929 bioassay, as described (Gomez-Flores *et al.*, 1997c). The bioassay was performed in complete RPMI 1640 medium, in the presence of actinomycin D (1 μg/mL final concentration), and using 1/3 serial dilutions of the supernatants. Recombinant murine TNF-α (a gift from NCI Biological Resources Branch, Rockville, MD, lot 88/532) was used as a standard. After 24 h of incubation, cell viability of L929 cells was determined by a colorimetric technique using MTT to a final concentration of 0.5 mg/mL, and incubating the cells for 1.5 h at 37 °C in 5% CO_2. After the incubation period, supernatants were discarded, and formazan crystals were dissolved in DMSO. Optical densities were then determined in a microplate reader (Molecular Devices Corporation) at 540 nm. TNF-α levels (U/mL) represented the inverse of the dilution causing 50% cytotoxicity.

Effect of LPS on macrophage activation. We utilized

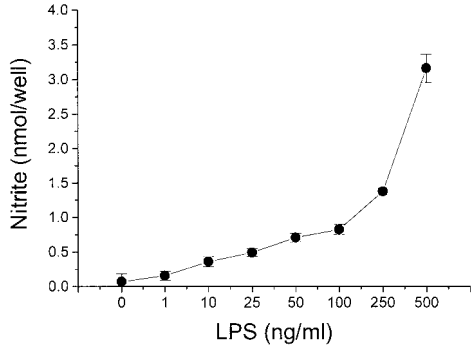

Figure 1. Effect of LPS on nitric oxide production by rat peritoneal macrophages. Peritoneal macrophages were treated with LPS (1–500 ng/mL) for 16 h, washed, and incubated for an additional 3 days. Nitric oxide production was then determined as explained in the text. Data represent mean ± SD of nitrite levels of triplicate determinations.

LPS as a positive control for macrophage activation. Resident peritoneal macrophage cultures prepared as described above, were incubated for 16 h with LPS (1–500 ng/mL), after which macrophage monolayers were washed twice, and incubated for an additional 3 days. After incubation, supernatants were obtained, and nitrite levels were determined as explained above.

T cell culture preparation and proliferation assay. Thymus was immediately removed after rat death, and was mechanically dissociated into a single-cell suspension in RPMI 1640 medium, as reported elsewhere (Riley *et al.*, 1998). The resulting cell suspension was washed three times in this medium, and suspended in AIM-V medium. The culture medium was changed at this step to the serum-free medium AIM-V which has been observed to support cell culture (Kaldjian *et al.*, 1992). T cell proliferation was determined by [^{3}H]-thymidine uptake as previously reported (Gomez-Flores and Weber, 1999). Immediately after rat death, single-cell thymus suspensions were prepared as described above, and adjusted to 1×10^7 cells/mL. Cell suspensions (100 μL) were added to round-bottomed 96-well plates (Becton Dickinson) containing triplicate cultures (100 μL) of AIM-V medium (unstimulated control) or the mitogen Con A at submaximal and maximal concentrations of 0.625, 1.25, and 2.5 μg/mL. After incubation for 44 h at 37 °C with 5% CO_2. [^{3}H]-methylthymidine (6.7 Ci/mmol, ICN Pharmaceuticals Inc., Costa Mesa, CA) was added (1 μCi/10 μL/well), and cultures were incubated for an additional 4 h. Cell cultures were then harvested with a semiautomatic cell harvester (Tomtec, Orange, CT), and cell-incorporated radioactivity was determined by liquid scintillation spectrophotometry using a Microbeta Plus liquid scintillation counter (model 1450, Wallac OY, Turku, Finland) with a counting efficiency for tritium of 35%. Proliferative responses of thymic lymphocyte to maximal and submaximal concentrations of Con A were used for data analysis.

Statistical analysis. The results were expressed as mean ± SD of triplicate determinations per treatment from a representative experiment. All experiments were repeated at least three times with similar results. Statistical analysis were performed by the Student's *t*-test and one-way analysis of variance.

RESULTS

Effect of LPS on macrophage activation

As shown in Fig. 1, a dose-dependent significant ($p < 0.001$) increase in nitric oxide production was observed in LPS-treated cells. LPS was used as a positive control for macrophage activation.

Induction of nitric oxide and TNF-α by *Plantago major* fractions

Acetone-mediated precipitation of a methanolic extract of PM produced an off-white, water-soluble fraction named PM_1 (0.7% recovery). PM_1 activated macrophages to produce nitric oxide (Fig. 2a) and TNF-α (Fig. 2b) in a dose-dependent fashion. PM_1, at doses of 50, 100, 250 and 500 μg/mL, caused 4.4 ± 1, 6 ± 1, 12 ± 0.4 and 18 ± 0.4-fold significant ($p < 0.001$) increases of nitric oxide production by macrophages, compared with untreated control (Fig. 2a). PM_1 also stimulated the production of significant ($p < 0.001$) TNF-α levels (621 ± 31, 721 ± 36, 727 ± 36 and 1056 ± 52 U/mL, at PM_1 doses of 50, 100, 250 and 500 μg/mL, respectively) by

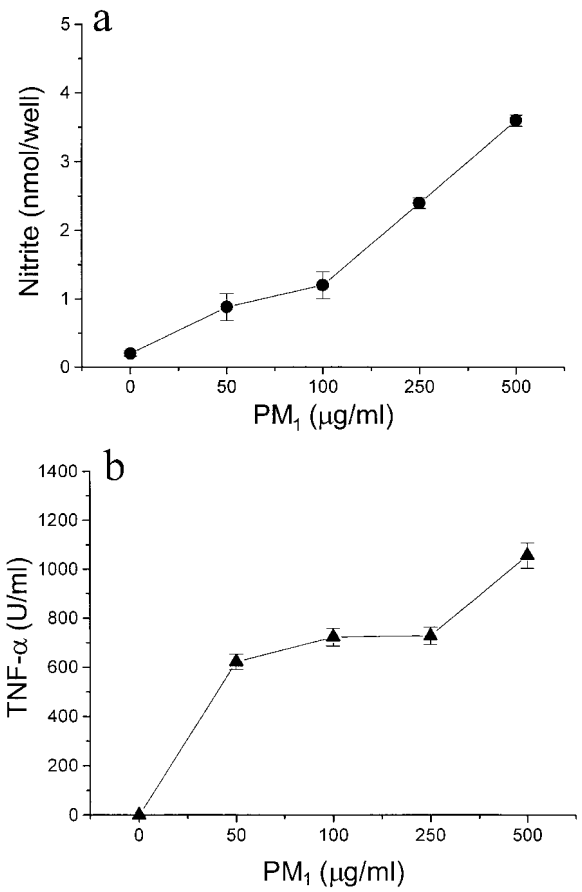

Figure 2. Effect of PM_1 fraction on the production of nitric oxide and TNF-α by macrophages. Peritoneal macrophages were treated with PM_1 (50–500 μg/mL) for 16 h, washed, and incubated for an additional 4 h and 3 days to determine TNF-α and nitrite levels, respectively. Nitrite (a) and TNF-α (b) production was then determined as explained in the text. Data represent mean ± SD of nitrite and TNF-α levels of triplicate determinations.

these cells (TNF-α production by untreated macrophages was negligible) (Fig. 2b). PM_1 fraction was further purified by phase extraction (United Chemical Technologies, Inc.). Upon percolation elution (the unbound fraction showed negligible activity to macrophages and lymphocytes, data not shown), the column was eluted with methanol. The eluted material, named PM_2, was more potent than PM_1 in activating macrophages in a dose-dependent manner. PM_2, at doses of 50, 100, 250 and 500 μg/mL, caused significant ($p < 0.001$) 11 ± 0.66, 14 ± 0.84, 36 ± 2.16, and 46 ± 2.76-fold increases of nitric oxide production by macrophages, compared with the untreated control (Fig. 3a). PM_2 was also associated with significant ($p < 0.001$) increases in TNF-α production (1000 ± 60, 1150 ± 69, 1200 ± 72 and 1550 ± 93U/mL, at PM_2 doses of 50, 100, 250 and 500 μg/mL respectively) by these cells (TNF-α production by untreated macrophages was negligible) (Fig. 3b).

Lymphoproliferation mediated by PM_2

PM_2 induced 1.8 to 2-fold significant ($p < 0.001$) increases in the proliferation of thymic lymphocytes (1200 to 1400 cpm, at doses of 62.5 to 250 μg/mL

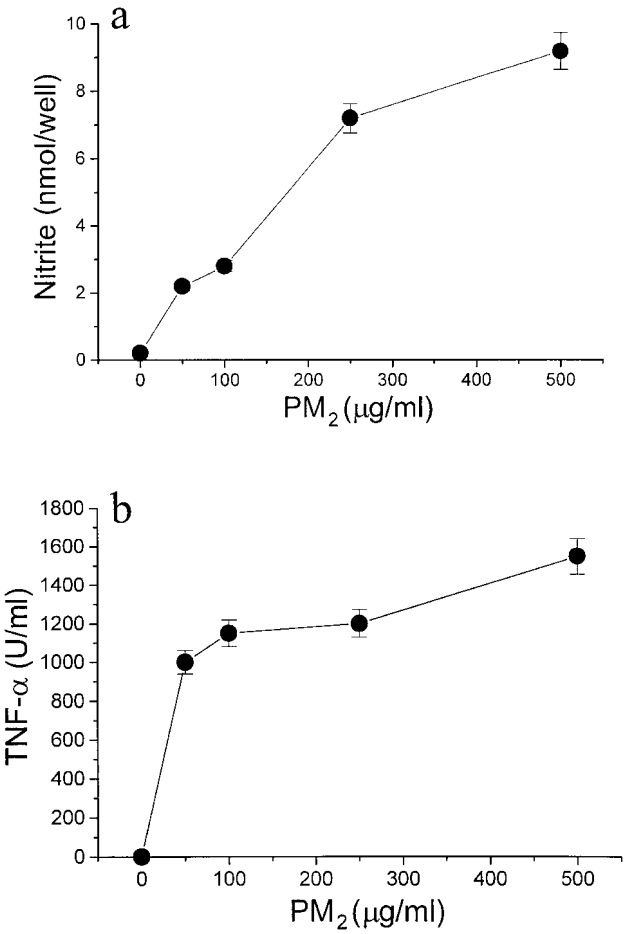

Figure 3. Effect of PM_2 fraction on the production of nitric oxide and TNF-α by macrophages. Peritoneal macrophages were treated with PM_2 (50–500 μg/mL) for 16 h, washed, and incubated for an additional 4 h and 3 days to determine TNF-α and nitrite levels, respectively. Nitrite (a) and TNF-α (b) production was then determined as explained in the text. Data represent mean ± SD of nitrite and TNF-α levels of triplicate determinations.

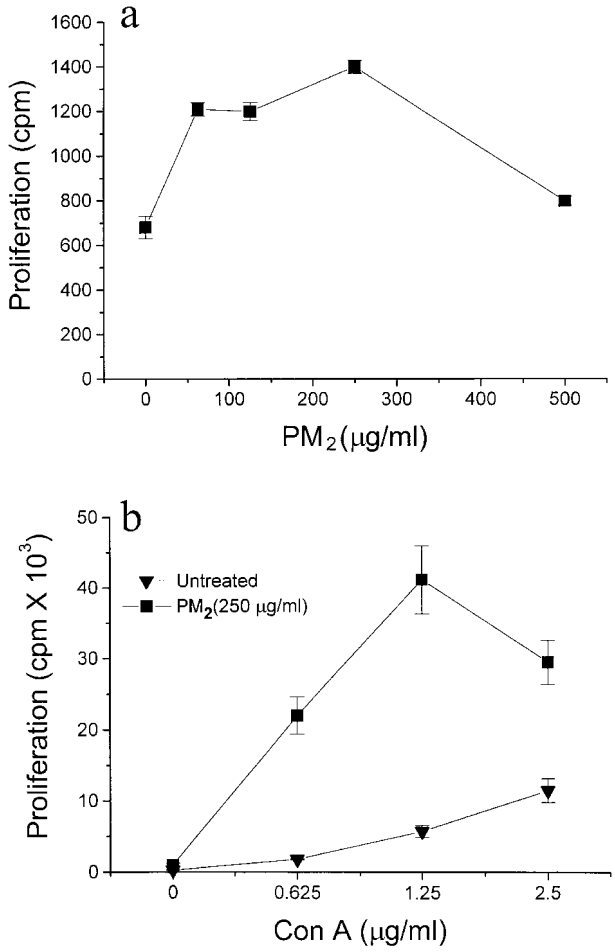

Figure 4. Effect of PM_2 fraction on T lymphocyte proliferation. Thymic cells were treated with PM_2 (100–500 μg/mL) (a), and with PM_2 (250 μg/mL) plus Con A (0.625–2.5 μg/mL) (b) for 48 h. After this, [^{3}H]thymidine incorporation was determined as explained in the text. Data represent mean ± SD of triplicate determinations.

respectively, compared with the untreated control (680 cpm)) (Fig. 4a), and significantly ($p < 0.001$) potentiated Con A-mediated thymic lymphocyte proliferation (3- to 12-fold increases) compared with the effect of Con A alone (Fig. 4b).

DISCUSSION

Mankind has a great history of the use of higher plant extracts for the therapy of diverse maladies. Medicinal plants play a major role in the life of many people worldwide, and their usage has increased significantly. Epidemiological studies have associated a reduced risk of infectious diseases and cancer with a diet high in fruits and vegetables, and have determined that molecules such as β-carotene, tocopherols, vitamin C and flavonoids, confer some of this protective benefit. Finding additional agents for human or agricultural use based upon higher plant extracts, may contribute to increasing the number of plant compounds of potentially beneficial application.

Plantago major has been broadly used to treat diverse medical conditions ranging from pain to infectious diseases and cancer (Matev *et al.*, 1982; Grigorescu *et*

al., 1973; Franca *et al.*, 1996; Lithander, 1992). However, its mechanism(s) of action associated with boosting the immune response remains to be elucidated. In this study, we found that a methanolic extract from *Plantago major* leaves, enhanced nitric oxide and TNF-α production by macrophages, and stimulated lymphocyte proliferation. Nitric oxide is the most studied reactive nitrogen intermediate. It plays a relevant role against intramacrophage infections caused by *Cryptococcus*, *Schistosoma*, *Leishmania*, *Francisella*, *Listeria* and *Mycobacteria* (Nathan and Hibbs, 1991). The major effect of nitric oxide is to inhibit ATP and DNA synthesis. Nitric oxide production by macrophages depends on activation of the inducible enzyme nitric oxide synthase which can be mediated by interferons, TNF-α or LPS (Moncada *et al.*, 1991). In addition, monokines such as interleukin-1, interleukin-6 and TNF-α are essential mediators of host inflammatory responses in natural immunity (Bonta and Ben-Efraim, 1993). On the other hand, in response to antigen challenge, T cells secrete lymphokines whose function is to promote the proliferation and differentiation of T and B lymphocytes, and macrophages (Mackay, 1993). The presence of immunopotentiating agents in *Plantago major* might explain its prophylactic action against the development of mammary tumors in mice (Lithander, 1992). Further investigation is underway to characterize the active constituents present in *Plantago major* leaf extract.

By stimulating non-specific cellular immune responses, most pathogens can be eliminated before they can cause the ill feeling associated with disease. Plant extracts are potentially curative. Some of these extracts can boost humoral (Kroes *et al.*, 1993; Rehman *et al.*, 1999) and cell-mediated immunity (Upadhyay *et al.*, 1992; Kim *et al.*, 1999; Coeugniet and Elek, 1987) against viruses (Tan *et al.*, 1998; Calixto *et al.*, 1998; Houghton, 1996), bacteria (Boyanova and Neshev, 1999; Caceres *et al.*, 1993), fungi (Ali *et al.*, 1999; Matos *et al.*, 1999), protozoa (Phillipson and Wright, 1991; Sharma and Sharma, 1998) and cancer (Wong *et al.*, 1994; Chung, 1999).

The regulation of immune parameters induced by plant extracts, may be clinically relevant in numerous disease processes including chronic viral infections, tuberculosis, AIDS and cancer.

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