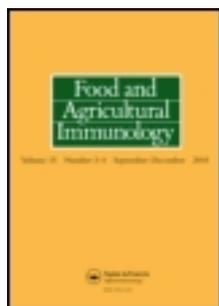


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## Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/cfai20>

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Available online: 09 May 2012

To cite this article: Gabriel Llauradó, Humberto J. Morris, Yamila Lebeque, Adrián Gutiérrez, Roberto Fontaine, Rosa C. Bermúdez & Isabelle Perraud-Gaime (2012): Phytochemical screening and effects on cell-mediated immune response of Pleurotus fruiting bodies powder, Food and Agricultural Immunology, DOI:10.1080/09540105.2012.686988

To link to this article: <http://dx.doi.org/10.1080/09540105.2012.686988>



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## Phytochemical screening and effects on cell-mediated immune response of *Pleurotus* fruiting bodies powder

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(Received 22 August 2011; final version received 13 April 2012)

This study examined the phytochemical profile and the effects of *Pleurotus* fruiting bodies powder on cell-mediated immune response in both *in vivo* and *in vitro* assays. Although carbohydrates (55%, w/w) appear to be the most important immunomodulatory compound, secondary metabolites (terpenoids, phenols and flavonoids) would also enhance immunity. *Pleurotus* powder was administered orally during 7 days to Balb/c mice (1000 mg/kg) and cyclophosphamide (CY; 100 mg/kg) was inoculated intraperitoneally at the beginning of the experiment. The delayed-type hypersensitivity reaction measured at 48 and 72 h after antigen challenge was similar to that of control mice and it was associated with an increase in the mass index of popliteal lymph nodes ( $p < 0.05$ ). An *in vitro* lymphoproliferative-stimulating response was also demonstrated with aqueous and ethanolic extracts obtained from *Pleurotus* powder. These effects suggest that *Pleurotus* supplement could potentiate the cellular immune response and should be promising for further immunotherapy studies.

**Keywords:** cellular; immunomodulating; mice

### 1. Introduction

Mushrooms have been used for many years due to their nutritional and pharmacological properties (Costa, Cunha, & Carvalho, 2006; Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007). The edible mushrooms with medicinal or functional properties include species of *Lentinula*, *Auricularia*, *Hericium*, *Grifola*, *Pleurotus*, *Ganoderma*, *Agaricus* and *Flammulina*. These mushrooms particularly have long been suggested to possess immunomodulatory effects (Smiderle et al., 2008; Smith, Sullivan, & Rowan, 2003; Thekkuttuparambil & Kainoor, 2007).

In the last 5 years, the consumption of mushrooms, either as whole mushroom or extracted supplements, has increased (Wasser, 2010). Most mushroom-derived substances and preparations (extracts, powders and tablets) are usually included in the following categories of products: dietary supplements, functional foods, nutraceuticals, nutriceuticals, phytochemicals and design foods (Chang & Buswell,

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1996, 2003; Wasser, Didukh, & Nevo, 2004) and immunocuticals (Petrova, Wasser, Mahajna, & Denchev, 2005).

The bioactive compounds contained in mushrooms with immunomodulatory and antitumour activities are mainly polysaccharides, polysaccharopeptides and polysaccharide–protein complexes (Hu et al., 2006; Wasser & Weis, 1999). Other substances of therapeutic interest are low-molecular-weight secondary metabolites and mushroom trace elements (Liu et al, 2007; Zaidman, Yassin, Mahajna, & Wasser, 2005) which also play important roles in the immune function.

Immune system is a very complex homeostatic system consisting of a network of interacting cells, tissues and organs. It allows the organism to exist within itself and maintains a surveillance to recognise components considered nonself. The body's immunity has been shown to be suppressed in several diseases, like AIDS and cancer. The chemotherapy and radiotherapy in cancer treatment contribute to further depression of the immune system as a result of the destruction of lymphoid and bone marrow cells (Janeway, Travers, Walpert, & Shlomchik, 2005).

Use of immunomodulating therapeutic agents can solve these problems largely and efforts to find new immunomodulators are ongoing. *Pleurotus* species, like many edible and medicinal mushrooms, are a good source of immunomodulators and substances considered as 'host defence potentiators' (HDPs) as judged by their immunostimulating properties (Gregori, Švigel, & Pohleven, 2007; Sedaghat & Ghazanfari, 2011; Wasonga, Okoth, Mukuria, & Omwandho, 2008).

In previous papers, we reported the immunomodulating effects of a hot-water extract prepared from the mycelium of *P. ostreatus* on the immunosuppression caused by cyclophosphamide in mice (Morris et al., 2003) as well as the *in vitro* effects of five water-soluble fractions on macrophage activation (Morris, Lebeque, Fontaine, Bermúdez, Llauradó, & Marcos, 2007). Taking into account that *Pleurotus* fruiting bodies obtained under good manufacture practices can be used in the formulation of biologically active nutritional supplements such as, powders, capsules and tablets, the present study examined the phytochemical profile and the effects of *Pleurotus* fruiting bodies powder on cell-mediated immune response in both *in vivo* and *in vitro* assays.

## 2. Materials and methods

### 2.1 Mushroom material

*Pleurotus* sp. strain CCEBI-3024 (Pleurotaceae) is deposited at the Culture Collection of the Centre of Studies for Industrial Biotechnology (CEBI). For maintenance, slants with solid medium of agar–dextrose–potato (PDA) incubated at 37 °C for 7 days were used.

### 2.2 Preparation of *Pleurotus* fruiting bodies powder

*Pleurotus* sp. cultivation was performed by solid-state fermentation of mushroom spawn on pasteurised coffee pulp used as substrate in plastic bags of 2 kg (30 × 40 cm; Bermúdez, García, Gross, & Serrano, 2001). The fruiting bodies were harvested, sliced into small pieces and dried at 45 °C for 24 h. The dried material was milled,

and the resulting powder was preserved from light and humidity in plastic bags for further use.

### **2.3 Phytochemical profile of *Pleurotus* fruiting bodies powder**

The sugar and protein contents in the powder were determined by the method of Dubois, Gilles, Hamilton, Rebers, & Smith (1956) and by Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951) using glucose and bovine serum albumin (BSA) as standards, respectively. Total lipids were measured according to Association of Official Analytical Chemists (AOAC, 1995) approved method.

The powder was extracted with hot water and ethanol to obtain both aqueous and ethanolic extracts for assessing the phytochemical profile and the *in vitro* lymphoproliferative activity.

The phytochemicals contained in both aqueous and ethanolic extracts were estimated qualitatively according to Harbourne (1984). Dragendorff's and Wagner reagents were used for alkaloid detection. Fehling and Benedict reagents were used for reducing sugars detection. The flavonoid content was determined according to the Rosemheim and concentrated sulphuric acid methods. Lieberman–Burchard and Solkowski assays were used for terpenoids identification. The presence of phenols and tannins was assessed by the reaction with  $\text{FeCl}_3$ . Amino acids detection was performed by the ninhydrine assay. All reagents for the phytochemical tests were freshly prepared following standard procedures.

### **2.4 Animals and treatments**

Pathogen-free male Balb/c mice were purchased from the National Centre for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). The 20- to 25-g mice were fed a standard diet and acidified water *ad libitum*. Fifteen mice were divided into three groups ( $n=5$ ). The two experimental groups were treated intraperitoneally (i.p) with cyclophosphamide (CY) USP 23 for injection obtained from JSLYP (China) at 100 mg/kg on day 0. The *Pleurotus* sp. fruiting bodies powder was administered by oral route (1000 mg/kg) for 7 days to the 'CY-*Pleurotus*' group, whereas physiological saline solution was administered to the 'CY-Saline' group in a similar schedule. Non-treated mice were used as controls in the experiment.

The research was approved by the institutional Ethical Committee (University of Oriente) and has been performed in accordance with Cuban legislation and the National Research Council Guidelines for the Care and Use of Laboratory Animals.

### **2.5 In vivo evaluation of *Pleurotus* powder effects on cell immunity**

The effect of *Pleurotus* powder on cell-mediated immunity was determined by the delayed-type hypersensitivity (DTH) reaction (Kim et al., 1998). Mice were immunised by an intradermal (i.d) injection of 50  $\mu\text{l}$  of 5 mg/ml BSA emulsified in Complete Freund Adjuvant (CFA; Sigma, St. Louis, MO, USA) at two sites on the abdomen. Eight days after immunisation, the mice were rechallenged by injection of 20  $\mu\text{l}$  of 5 mg/ml BSA into one rear foot pad, while the other received a comparable volume of phosphate buffered saline (PBS). Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge by use of a micrometer (Mitutoyo, Tokyo,

Japan). The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS-injected foot pads. A similar immunisation protocol was applied to control animals.

The popliteal lymph nodes (right and left) of the antigen sensitised and rechallenged animals of DTH experiment were removed and washed with PBS pH 7.4. Excess humidity was discarded with a filter paper, and the lymph nodes were immediately weighed separately in an electronic analytical balance (Sartorius). The mass index was expressed as the relation between the weight of the popliteal node belonging to BSA-injected foot pad with respect to that of PBS-injected pad (Descotes, 2006).

### 2.6 *In vitro* evaluation of nutritional supplement on cell immunity

The *in vitro* lymphoproliferative test was carried out according to a modification of the method described by Soto-Velazco, López, Vázquez, Valls, and Álvarez (2002) using murine spleen lymphocytes instead of human lymphocytes. The suspension of splenocytes was obtained by the gentle teasing of spleens in RPMI-1640 (Sigma, St. Louis, MO, USA) containing 8% fetal calf serum and supplemented with antibiotics. Viable cells estimated by the Trypan Blue exclusion method were counted with a Neubauer chamber, and the cell concentration was adjusted to  $2 \times 10^6$  cells/ml.

Briefly, phytohaemagglutinin (PHA; 200  $\mu$ l) was added to conical tubes of 15 ml containing 5 ml of supplemented RPMI-1640 to a final concentration of 5  $\mu$ g/tube, followed by the addition of  $2 \times 10^6$  cells and 100  $\mu$ l of powder extracts (aqueous or ethanolic). The tubes were incubated at 37 °C for 27 h and then 500  $\mu$ l of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT; 5 mg/ml) was added. After the incubation of the resulting mixture at 37 °C for 4 h, the tubes were centrifuged 10 min at 1500 rpm. The supernatants were discarded and 1 ml of isopropanol was added to each tube. The absorbance of the mixture was measured at 570 nm in a Genesys 10 UV/VIS spectrophotometer. The stimulation index was calculated considering the absorbance of control cultures without PHA as the unit.

### 2.7 *Statistical analysis*

The results were expressed as the arithmetic means  $\pm$  standard deviation (SD). The Kruskal–Wallis rank test followed by the Student–Newman–Keuls test was applied to determine the significance of differences between treatments in DTH assay. The Mann–Whitney test was used to compare the two means in the experiment of popliteal lymph nodes mass index. Differences at  $p < 0.05$  were accepted as significant. The software Statgraphics Plus version 5.1 (Statistical Graphics Corporation, 1994–2001) was used in all the analysis.

## 3. Results and discussion

A wide variety of bioactive substances isolated from many species of mushrooms has been identified. Most of them are high-molecular-weight polysaccharides and proteoglycans or low-molecular-weight secondary metabolites such as terpenoids, steroids and phenols (Zaidman et al., 2005).

Dried *Pleurotus* mushroom would become an attractive alternative for the development of drugs and immunocuticals preparations. The powder evaluated in this work contained 55% (w/w) carbohydrate, 20% (w/w) protein and 4% (w/w) lipids. Although, polysaccharides appear to be the most important bioactive component with respect to immunomodulation, the presence of different secondary metabolites in *Pleurotus* sp. fruiting bodies powder could lead to a synergy in the immune-enhancing activity.

Phytochemical analysis is commonly used to detect qualitatively secondary metabolites present in plants. The result of the preliminary phytochemical test shows that both aqueous and ethanolic extracts contain alkaloids, phenolic compounds like flavonoids and tannins, reducing sugars and amino acids (Table 1). These phytoconstituents were present in varying amounts. Remarkably, the ethanolic extract from *Pleurotus* powder exhibited a very strong terpenoid reaction. Fats and oil were generally absent in the extracts due to the polarity of the solvents used in their preparation.

Among the mentioned bioactive components identified in aqueous and ethanolic extracts, phenolic compounds have been studied for their antioxidant properties, and they also play an important role in cancer prevention (Dubost, Ou, & Beelman, 2007). An isoflavone (Genestein) isolated from *Flammulina velutipes* produced a growth inhibition of the human ovarian carcinoma SKOV-3 in a dose-dependent manner (Li & Mi, 2003).

Reducing sugars are structural constituents of beta-D-glucans, components of mycetes' cell walls with a well-documented immunity-stimulating effect (Rop, Mlcek, & Jurikova, 2009). On the other hand, amino acids are the structural units of proteins that may be associated to polysaccharides to form immunomodulating complexes, like PSP isolated from the mycelium of *Trametes versicolor* (Hobbs, 2004). Moreover, fungal immunomodulatory proteins, purified from medicinal mushrooms comprise a group of novel proteins, possess immunomodulatory properties and have a strong potential of being applied to food or pharmaceutical products for commercial development (Ou, Hsiao, Wang, Ko, & Lin, 2009).

Terpenoids have been found in ethanolic extract and their ability to stimulate the immune system is known. For instance, the triterpene lanostenoide obtained from

Table 1. Phytochemical profile of *Pleurotus* sp. fruiting bodies powder.

Metabolites	Assays	Aqueous extract	Ethanolic extract
Alkaloids	Dragendorff	+++	+++
	Wagner	++	++
Terpenoids	Solkowski	–	+
	Lieberman–Burchard	–	+++
Reducing sugar	Fehling	+	+
	Benedict	+	+
Phenols and tannins	FeCl <sub>3</sub>	+	+
Amino acids	Ninhydrine	+	+++
Flavonoids	Concentrated H <sub>2</sub> SO <sub>4</sub>	+	+
	Rosemheim	+	+

Note: The phytochemicals contained in both aqueous and ethanolic extracts were estimated qualitatively according to Harbourne (1984). Three replicates were used for each assay. Legend: (–) none, (+) present, (++) mild, (+++) marked.

*Ganoderma applanatus*, inhibited the carcinogenesis induced by the Epstein Barr virus (Gao, Zhou, Huang, & Xu, 2003).

Recent studies in *Pleurotus* species have evidenced several therapeutic effects related with the immune system, such as antitumour, antimicrobial and antiviral activities. Most of these investigations, both *in vivo* and *in vitro*, have been carried out with extracts and isolated compounds (polysaccharides, proteins and DNA; Gregori et al., 2007). Therefore, the study of a dried pulverised preparation of *Pleurotus* fruiting bodies would be of interest in the development of new pharmaceutical formulations.

The immunomodulating properties of *Pleurotus* powder administered therapeutically to cyclophosphamide treated mice on cell-mediated immune response were assessed by the assay of induction of DTH response.

Mice supplemented with *Pleurotus* powder showed a higher DTH response as judged by the increase of foot pad swelling compared to saline control group, particularly at 48 and 72 h after antigen rechallenge ( $p < 0,05$ ; Table 2). The DTH response mounted at these times by CY-*Pleurotus* group was similar to that of control mice. The reconstitution of DTH response reflected the induction of CD4<sup>+</sup> Th1 cells and the activation of macrophages by cytokines: tumour necrosis factor alpha (TNF- $\alpha$ ) and gamma interferon (IFN- $\gamma$ ; Janeway et al., 2005; Kim et al., 1998).

Macrophages have been suggested to play important roles in immunological surveillance. The ability to activate murine peritoneal macrophages *in vitro* by five water-soluble fractions (F-I to F-V) extracted from mycelium of *Pleurotus* sp. was demonstrated in a previous work, as judged by the increase found in glucose consumption and in lysosomal enzyme (acid phosphatase) activity (Morris et al., 2007).

The effects of two glucans obtained from *Pleurotus ostreatus* and yeast were assessed in different immunological functions of mice (Paulik, Svrcek, Mojzisova, Durove, Benisek, & Huska, 1996). Both glucans augmented the DTH response compared to control mice, but the induction was higher for *Pleurotus* glucan. A significant increase in the number of T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>) was found in

Table 2. Effect of therapeutic administration of *Pleurotus* powder on the delayed-type hypersensitivity response (foot pad thickness) of cyclophosphamide treated Balb/c mice.

	Foot pad thickness (mm)		
	24 h	48 h	72 h
<b>Control</b>	0.48 $\pm$ 0.07 <sup>a</sup>	0.46 $\pm$ 0.04 <sup>a</sup>	0.38 $\pm$ 0.07 <sup>a</sup>
CY- <i>Pleurotus</i>	0.14 $\pm$ 0.05 <sup>b</sup>	0.43 $\pm$ 0.02 <sup>a</sup>	0.29 $\pm$ 0.05 <sup>a</sup>
CY-Saline	0.11 $\pm$ 0.06 <sup>b</sup>	0.39 $\pm$ 0.01 <sup>b</sup>	0.12 $\pm$ 0.04 <sup>b</sup>

Note: Mice were immunised by an intradermal (i.d.) injection of 50  $\mu$ l of 5 mg/ml bovine serum albumin (BSA) emulsified in Complete Freund Adjuvant (CFA) at two sites on the abdomen. Eight days after immunisation, the mice were rechallenged by injection of 20  $\mu$ l of 5 mg/ml BSA into one rear foot pad, while the other received a comparable volume of PBS. Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge. The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS-injected foot pads. All values are expressed as the arithmetic mean  $\pm$  SD of five mice. Different letters indicate significant differences among groups (Kruskal-Wallis, Student-Newman-Keuls,  $p < 0.05$ ).

mice administered with a water-soluble polysaccharide extracted from the fermentation broth of *Pleurotus citrinopileatus* (Wang, Hu, Liang, & Yeh, 2005).

These findings suggest that oral administration of edible mushroom-derived products with potential immunomodulating activities would stimulate the immune system after their absorption in the gastrointestinal tract and the activation of gut-associated lymphoid tissues, thus integrating different elements of the immune function.

However, the results of the administration of an aqueous extract of *P. florida* to female Balb/c mice showed that DTH responses were not affected by various doses of the product in different routes (i.p. or oral). With respect to viability of splenic cells and histological changes in secondary lymphoid tissues, this study indicated that the effects of *P. florida* on cellular responses depend on doses and routes of administration (Sedaghat & Ghazanfari, 2011).

In our study, the DTH reconstitution was associated with the increase observed in the mass index of popliteal lymph nodes of the *Pleurotus* supplemented animals ( $p < 0.05$ ; Figure 1). Antigens are concentrated in the secondary lymphoid organs, including lymph nodes, where they are presented by mature dendritic cells, the most efficient type of antigen-presenting cell for initiating responses of naive T cells (Janeway et al., 2005).

Considering the possible sex-based differences on cellular immune response, the use of both male and female animals is recommended in future preclinical evaluations of *Pleurotus* nutritional supplements.

Cellular immune response was also evaluated by the *in vitro* lymphoproliferative response of murine splenocytes. The incubation of splenocytes with aqueous and ethanolic extracts derived from *Pleurotus* powder for 72 h led to stimulation indexes of 1.90 and 1.28, respectively (Figure 2). The higher index obtained for aqueous

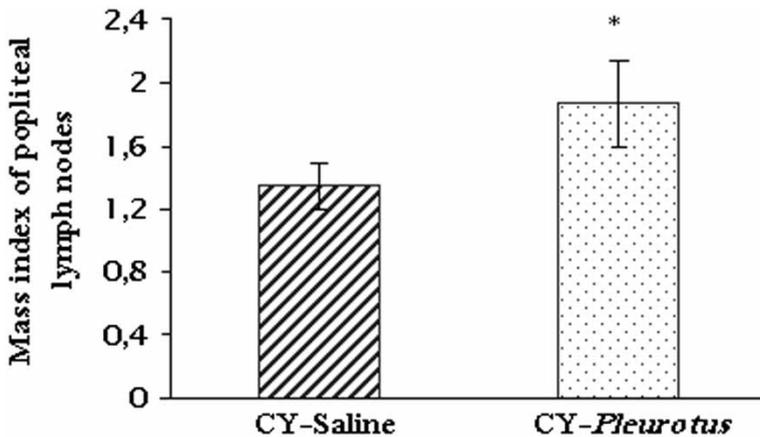


Figure 1. Mass index of popliteal lymph nodes of cyclophosphamide treated Balb/c mice administered or not with *Pleurotus* powder. The popliteal lymph nodes (right and left) of the antigen sensitised and rechallenged animals of DTH experiment were removed and weighed separately. The mass index was expressed as the relation between the weight of the popliteal node belonging to BSA-injected foot pad with respect to that of PBS-injected pad. The (\*) reflects significant differences among the groups in the Mann-Whitney test ( $p < 0.05$ ).

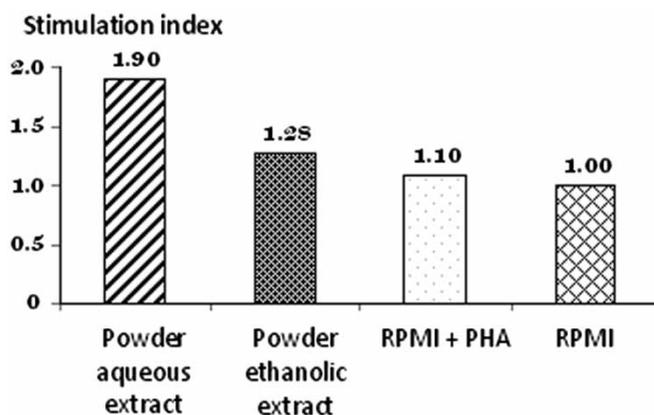


Figure 2. *In vitro* lymphoproliferative-stimulating response of aqueous and ethanolic extracts obtained from *Pleurotus* powder on murine spleen cells. Phytohaemagglutinin (PHA; 200  $\mu$ l) was added to tubes containing 5 ml of supplemented RPMI-1640 to a final concentration of 5  $\mu$ g/tube, followed by the addition of  $2 \times 10^6$  cells and 100  $\mu$ l of powder extracts (aqueous or ethanolic). The tubes were incubated at 37 °C for 72 h and then 500  $\mu$ l of MTT (5 mg/ml) was added. After the incubation of the resulting mixture at 37 °C for 4 h, the tubes were centrifuged, the supernatants were discarded and 1 ml of isopropanol was added. The absorbance was measured at 570 nm. The experiments were done in triplicate. The stimulation index was calculated considering the absorbance of control cultures without PHA as the unit.

extract could be related with the presence in this fraction of immunomodulating glucans.

It has been reported that the incubation of human mononuclear cells with different extracts from *Ganoderma lucidum* led to the stimulation of lymphoproliferative response (Soto-Velazco et al., 2002). On the other hand, glucans isolated from *P. florida* fruiting bodies significantly induced the proliferative response as well as phagocytic activity of fish leukocytes (*Catla catla*) *in vitro* (Kamilya, Ghosh, Bandyopadhyay, Mal, & Maiti, 2006).

In sum, the nutritional supplement of *Pleurotus* sp. provides immunological benefits in terms of the stimulation of cell-mediated immune responses. *Pleurotus* powder could potentiate the host defence mechanisms, and further studies are needed to address effective phytochemicals and their mechanisms.

#### 4. Conclusion

This study shows that *Pleurotus* fruiting bodies powder exhibited immunomodulating properties, as judged by the *in vivo* stimulation of cell immune response when administered orally to cyclophosphamide-treated Balb/c mice, and the *in vitro* lymphoproliferative-stimulating response. Although most of the phytoconstituents identified in *Pleurotus* powder have been implicated as immunomodulatory agents, we are unable, at present, to link the observed activity to any of the constituents. Moreover, phytochemical(s) can act alone or in combination to effect physiological changes. These effects on cell immunity are especially valuable in the prophylaxis of tumours, immunodeficiencies and as co-adjuvant in chemotherapy.

## Acknowledgements

We would like to thank the Cuban Ministry of Science, Technology and Environment (Territorial Project 9072 of the Program for Development of Health Products and Services) and the University of Oriente's authorities that enabled us to carry out this research.

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