Anti-immunosuppressive Effects of *Chromolaena odorata* (L.f.) King & Robinson (Asteraceae) Leaf Extract in Cyclophosphamide-injected Balb/C Mice

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*Chromolaena odorata* (L.f.) King & Robinson leaf extract was evaluated for its immunomodulating activity in Balb/C mice by employing a number of immune response assays namely macrophage phagocytic activity, splenocyte proliferation, production of reactive oxygen species (ROS), and plasma lysozyme levels. The optimum concentration of *C. odorata* was determined to be 100µg/mL through *in vitro* assays. Subsequently, three (3) groups of 12 mice each were used in *in vivo* experiments, namely: (1) cyclophosphamide-induced (30mg/kg body weight) immunosuppressed mice (Cy- injected or positive control); (2) *C. odorata* extract + Cy-treated mice (C group); and (3) phosphate-buffered saline (PBS)-injected mice as the negative control group. When compared with PBS-treated mice, the Cy-treated mice showed significantly lower phagocytic activity, cellular proliferation, production of reactive oxygen species (ROS), and plasma lysozyme levels. Significant improvement in macrophage phagocytic activity and cellular proliferation was exhibited by the plant extract-treated mice when compared with Cy-injected mice only. The extract from *C. odorata* also improved superoxide production and plasma lysozyme activity compared with the Cy-injected mice. These results demonstrated the immunopotentiating activities of the *C. odorata* leaf extract on the innate immunity of Balb/C mice. Also, the extract could potentially reverse a drug-induced immunosuppression as confirmed through *in vivo* experiments. Indeed, there is a great potential of the plant to be utilized as source of biologically active products and metabolites for drug development.

Key Words: Balb/C mice, *Chromolaena odorata*, cyclophosphamide, immunomodulation, *in vivo*, immunosuppression

INTRODUCTION

Immunomodulation through stimulation or suppression may help in maintaining disease-free state (Ghule et al. 2006). Herbal medicine has become an integral part of standard healthcare, based on a combination of time-honoured traditional usage and ongoing scientific research (Tiwari et al. 2004). Since the ancient systems of medicine, plants have been extensively used as a source of medicine to promote health and to maintain the body’s resistance against infection by potentiating immunity, re-establishing body equilibrium and conditioning of the body tissues. Thus, the development of natural health
products that could potentially modulate the immune system provides an alternative source of bioactive agents with medical significance.

*Chromolaena odorata* (L.f.) King & Robinson (Siam weed) is locally known as 'hagony'. It is considered as an invasive plant species in tropical and subtropical areas of the world. Although regarded as a serious weed, *C. odorata* has potential medical uses (Ling et al. 2007; Taleb-Contini et al. 2006; Owoyele et al. 2005; Phan et al. 2001a, b; Phan et al. 2000; Phan et al. 1998). Traditionally, fresh leaf juice or a decoction of *C. odorata* is used as an astringent, antihemorrhagic, and for treatment of wounds and skin infection. An infusion of the plant is taken to treat malaria, jaundice, and as antipyretic. Recently, a study has demonstrated the antibacterial, antifungal and antiprotozoal effects of the plant species (Vital & Rivera 2009).

The study investigated the immunomodulatory effects of *C. odorata* extract on the enhancement of the innate immune response by employing a number of immune response assays. Specifically, the study assessed the protective effect of the plant extract against a drug-induced immunosuppression in mice.

**MATERIALS AND METHODS**

**Plant material**

Leaves of *Chromolaena odorata* were collected inside the University of the Philippines-Diliman campus. The specimen was sent for authentication and identification at the Jose Vera Santos Memorial Herbarium (PUH) of the Institute of Biology, University of the Philippines, Diliman (Voucher # 9138).

**Preparation of plant extract**

Leaves of *C. odorata* were washed, air-dried until crisp, powdered prior to extraction with 95% ethanol (1:5 w/v) at room temperature for 72 hours. The process was repeated 3 times and the combined ethanol fractions were evaporated under reduced pressure using a rotary evaporator at 55 °C (Ling et al. 2007). The total ethanol extract concentrate yield per gram of dried plant material was determined using the formula: 

\[ \text{yield} = \frac{\text{weight} \times \text{dry-weight}}{\text{weight} \times \text{dry-weight} \times \text{plant material}} \times 100 \]

A stock solution of the plant extract was prepared by dissolving 5mg of the plant extract in phosphate-buffered saline (PBS) solution. The solution was then sterilized using a 0.2μm syringe filter, and stored at -20 °C.

**Experimental animals**

Balb/C mice obtained from the Marine Science Institute (MSI), University of the Philippines, Diliman and the National Institutes of Health (NIH), University of the Philippines, Manila were used in assessing immune response activities in vivo. The animals were initially acclimated to laboratory conditions for one week prior to experiment proper. The animals were housed in appropriate cages and were fed Purina Feeds and sterile drinking water ad libitum. The experimental set-up and procedures were approved by the College of Science Animal Care and Use Committee (CSACUC), UP Diliman.

**Experimental set-up**

Thirty-six (36) mice were divided into 3 groups of 12 mice each. *C. odorata* extract (5 mg/kg body weight) was intraperitoneally (i.p.) injected in one group for a period of ten days. Another group of mice was i.p. injected with sterile PBS at a volume dose of 50 mL/kg body weight (negative control). The last group was injected with 0.2 mL of 30 mg/kg body weight of cyclophosphamide (Endoxan®, i.p.), a known immunosuppressant, at days 1, 4, 7, and 10 of the treatment period. Cyclophosphamide was also administered to groups of mice treated with *C. odorata* on the same period, 1 hour after administration of the plant extract. Mice were sacrificed on the 11th day and immune response assays were performed.

**Immune response assays**

**Splenic macrophage phagocytic activity**

In assessing the effect of the plant extracts on the phagocytic activity of splenic macrophages, the protocol of Zelikoff (1997) was used with some modifications. Mice were sacrificed and each of their spleens was collected aseptically. Cell suspension was prepared by placing the spleen on sterile wire mesh and mechanically dispersed using a syringe plunger in RPMI 1640 medium (Gibco® cat. no. 31800-022) supplemented with 10% heat-inactivated FCS (Gibco® cat. no. 16000-044), 50 μM 2-mercaptoethanol (Riedel-de Haén 62736), 100U/100 μg penicillin-streptomycin (Gibco® cat. no. 15140-122) and 0.25 μg/mL amphotericin B (Gibco® cat. no. 041-95780). The viability of splenocytes was determined by trypan-blue dye exclusion technique. The cell number was adjusted to 5x10⁶ cells/mL. During the viability counts, splenic macrophages were morphologically distinguished from lymphocytes based on their larger size.

To one hundred microliters (100 μL) of 5x10⁶ splenic macrophages, 0.05 mL of opsonized yeast cells (5x10⁶ cells/mL) was added. The mixture was incubated for
60 minutes. Then, 20 µL aliquot was placed on glass slide for smearing. Cell smears were air-dried at room temperature, fixed with 95% ethanol and stained with Giemsa counterstained with eosin. A total of 100 cells were counted per slide at 1000x magnification. Phagocytic activity was calculated using the formula: (Number of cells with engulfed yeast cell particles / 100 cells) x 100.

**Lipopolysaccharide (LPS)-sensitive cell proliferation assay**

Another set of mice were sacrificed and each of their spleens was collected aseptically as described in supplemented RPMI 1640 phenol red-free medium (RPMI 1640 PR- Sigma® cat. no. R8755). The viability of the cells was determined by trypan-blue dye exclusion technique. The cell number was adjusted to 1x10⁶ cells/mL.

The effect of the plant extract was evaluated according to a rapid colorimetric assay for cellular growth and survival developed by Mosman (1983) with some modifications. Reaction tubes containing supplemented RPMI 1640 PR- and cell suspension were prepared with and without lipopolysaccharide (LPS, Sigma® cat. no. L3129; stock concentration = 2 mg/mL; final concentration 10 µg/mL). Lipopolysaccharide was used to stimulate the proliferation of B lymphocytes in culture. Reaction tubes containing cell suspension in supplemented culture media and PBS served as control. Two hundred microliters of the mixture (0.20 mL) was placed in 96-well plate, incubated at 37 °C in a humid atmosphere with 5% CO₂ (Anaeropack CO₂, Mitsubishi Gas Chemical Co., Inc.). After 48 hours of incubation, the growth culture medium from each well was removed by aspiration. Forty microliters of 1x RPMI 1640 PR- (supplemented with 100U/100µg penicillin/streptomycin and 0.25 µg/mL Amphotericin B) and 20 µL of salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma® cat. no. M2128; stock concentration = 5 mg/mL in PBS) was then added to all wells, and incubated for 3 hours at 37 °C in a humid atmosphere. The reaction was stopped by adding 100 µL per well of acid isopropanol (0.04N HCl in isopropanol). After a few minutes at room temperature, the optical density (OD) was measured on a Multiskan EX Thermo Electron Corporation Elisa Reader at 595 nm. Cell proliferation was calculated using the formula: % Proliferation = [(OD sample – OD control)/OD control] x 100.

**Splenic macrophage extracellular superoxide anion production**

Assay for the assessment of superoxide anion produced outside the mitochondria was performed according to the protocol of Zelikoff et al. (1996). Splenic macrophages obtained from the previous preparation were adjusted to a cell concentration of 4x10⁶ cells/mL. Cells (125 µL of 10⁶ cells/mL) was added to each of four previously labeled (1 to 4) microcentrifuge tubes containing 250 µL ferricytochrome C (Sigma® cat. no. C7752; stock concentration = 4 mg/mL in Locke’s mammalian saline solution; final concentration = 2 mg/mL). 62.5 µL of bovine superoxide dismutase (SOD, Sigma® cat. no. S2515; stock concentration = 300 µg/mL; final concentration = 37.5 µg/mL) was added to the second and fourth tubes. Ten microliters (10 µL) of phorbol 12-myristate 13-acetate (Sigma® cat. no. P8139; stock concentration = 1 mg/mL DMSO; working concentration = 100 µg/mL Hank’s balanced salt solution) was added to the third and fourth tubes at a final concentration of 2 µg/mL. Mammalian physiological saline solution (Locke’s) was added to each tube to bring to the final volume up to 0.5 mL. An additional tube (labeled as “B”) containing all the reagents, but without cells, served as the reaction blank. Each tube was vortexed for approximately 30 seconds and then, 200 µL aliquots (2x10⁶ cells/well) was placed into the individual wells of a 96-well microtiter plate and the absorbance measured at 492 nm for up to 1 hour. Time points suggested for measurement include: 0, 15, 30, 45, and 60 minutes; plates were incubated at 37°C in a humidified environment between readings. The rate of superoxide anion radical production can be determined from measurements taken over time, while OD readings at a single time point (time of peak superoxide anion radical production = 60 minutes) can also be used to make comparisons between different exposure groups. Change in absorbance was calculated by subtracting the mean of the “blank” wells and the wells containing SOD from the absorbance measured in the non-SOD-containing wells. By multiplying the change in absorbance by 15.87, the nmol concentration of SOD-inhibitable superoxide anion radical can be computed. Data were expressed as nmol O₂⁻/2x10⁵ cells/unit time.

**Plasma lysozyme assay**

Blood samples were extracted from the external jugular vein of the mice from the three experimental groups and centrifuged at 1,000 rpm for 10 minutes. After centrifugation, plasma was collected and stored at -20°C until the assay was performed.

Lysozyme activity was determined by a microtitre plate method which measures the lysis of a suspension of Micrococcus lysodeikticus (75 mg 100 mL⁻¹ of 0.1M phosphate/citrate buffer with 0.09% NaCl, pH 5.6). One hundred and seventy-five microliters (175 µL) of the bacterial suspension was added at 25 µL of each plasma sample, and to hen egg white lysozyme standard (Sigma®, cat. No. L-6876; 0 to 50 µg mL⁻¹ of 0.1M phosphate/citrate buffer with 0.09% NaCl, pH 5.8) in flat bottomed, 96-well plates, in duplicate wells per sample. The plates were incubated for 30 minutes. The rate of lysis was...
determined against *M. lysodeikticus* (Sigma®, cat. no. M-3770) blank at 450 nm on a Statfax 2100 multiscan plate reader. Lysozyme activity was calculated from the standard curve, and expressed as μg lysozyme/mg protein (Secombes 1998). Protein concentration was determined using the Bio-Rad protein assay kit by following the manufacturer’s instructions.

**Statistical analysis**

Results were expressed as mean ± SE. Data obtained were subjected for normality (Kolmogrov-Smirnov’s test) and homogeneity (Levene’s test) of variance tests prior to any appropriate statistical analysis. ANOVA (parametric) and Kruskal-Wallis test (non-parametric) were appropriately used to analyze results obtained. *P* value of less than 0.05 was considered statistically significant. Least Significant Difference (LSD) was used as a posteriori test to compare the means.

**Waste Disposal**

Balb/C mice carcasses were buried in a designated plot. Waste laboratory chemicals and materials used in the study were disposed in compliance with the biological and chemical safety guidelines of the Research and Analytical Services Laboratory, Natural Sciences Research Institute, University of the Philippines, Diliman.

**RESULTS**

**Description of plant extract**

Out of 275g of *C. odorata* dried leaves, a total of 16.2g of air-dried crude extract was obtained (5.89% yield). The dried crude extract is dark green in color and has a sticky or paste-like consistency. The crude extract was placed in sterile Petri dishes sealed with parafilm, then stored in resealable plastic bags at 4 °C.

**Phagocytic activity of murine splenic macrophages**

A significant decrease in the ability of macrophages to engulf opsonized yeast cells was observed in immunosuppressed mice compared with the negative control (PBS; *p*=0.010) and extract-treated mice (C; *p*=0.003) as shown in Figure 1. The plant extract significantly enhanced the phagocytosis activity of macrophages. *C. odorata* significantly increased the mean percentage phagocytic activity to 21.42% from that of the Cy-treated group (4.33%).

**Proliferation of Lipopolysaccharide (LPS)-sensitive spleen cell**

Figure 2 shows that Cy-treatment (positive control) significantly decreased the mean percentage of cell proliferation.
proliferation to almost 10% from that of PBS-treated group (negative control) with (58.46%; p=0.001). Without LPS, cyclophosphamide inhibited spleen cell proliferation by 63.92% compared with the negative control group. The plant crude extract significantly enhanced cellular proliferation, which reversed the inhibitory effects of cyclophosphamide treatment. Specifically, the extract from *C. odorata* significantly induced the proliferation of LPS-sensitive cells (70.43%; p=0.001). In the absence of a mitogen, a 69.27% mean cell proliferation activation was detected when compared with that of immunosuppressed group (p=0.028).

**Splenic macrophage production of extracellular superoxide anion**

As shown in Figure 3, cyclophosphamide decreased the production of superoxide anion compared with PBS-treated mice. The extract of *C. odorata* enhanced the activity compared when compared with the cyclophosphamide treated mice. However, the observed effects of the crude extract were not statistically significant compared with those of the Cy-treated mice.

**Plasma lysozyme assay**

Results (Figure 4) showed that significantly (p=0.025) higher levels of plasma lysozyme were detected in PBS-treated mice (negative control) compared with Cy-treated mice (positive control). However, the observed increase in the lysozyme level induced by *C. odorata* treatment was found to be insignificant compared with that of the positive control group.

**DISCUSSION**

In recent years, focus on the use of non-traditional approaches to prevent and treat diseases has been revived all over the world. The evidences collected from previous research studies on natural products showed immense potential of medicinal plants used in traditional systems (Ajaya-Kumar et al. 2004) either as an alternative or complimentary medicine to the conventional treatment procedures. A large number of plants used in traditional medicines have been shown to possess nonspecifically immunomodulating activities (Choi et al. 2004) and are being extensively explored for their potential in the treatment and prevention of chronic diseases.

Leaf extract of *C. odorata* was prepared by employing an alcohol extraction procedure. Only crude extracts were prepared to evaluate the immunomodulatory activities of the plant species instead of purifying its active components. Though it may be rational to use single plant
Anti-immunosuppressive Effects of Chromolaena odorata in mice

Figure 3. Effects of 100µg/mL of *Chromolaena odorata* (Lf.) King & Robinson leaf extract on O$_2^*$ production of murine macrophages at 30 min incubation. Each value is expressed as nmol O$_2$/2x10$^5$ cells/30min. *C. odorata* induced respiratory oxidative burst among the treatments. *Black bars - stimulated; white bars - unstimulated.

Figure 4. Effects of 100µg/mL of *Chromolaena odorata* (Lf.) King & Robinson leaf extract on plasma lysozyme activity. Each value is expressed as µg lysozyme per mg protein. Each bar represents the mean±SEM. * = significantly different from Cy-treated mice.
or its single constituent, it has been a general experience that the total plant extract shows more efficacies versus single constituent (Bin-Hafeez et al., 2001). Moreover, there are substances other than the pure compound that may contribute for the immunomodulatory activity or the synergistic interaction among the different components of the extract (Ajaya-Kumar et al. 2004).

*C. odorata* extract significantly increased immunoactivity of macrophages and lymphocytes in an immunosuppressed animal model. Immunosuppression, particularly of humoral immunity, is a common consequence of long-term cyclophosphamide (Cy) chemotherapy in cancer patients (Bin-Hafeez et al. 2001). Cyclophosphamide is known to cause leucopenia as it is cytotoxic not only to cancer cells but to leukocytes as well. In effect, a single dose of Cy inhibits the proliferative response of B cells and has a particularly intense effect on short-lived lymphocytes known to include a great proportion of B-cells (Bafna & Mishra 2006).

In this study, cyclophosphamide treatment impaired macrophage activation and proliferation as well as macrophage phagocytic activity consistent with the previously reported findings of Zhu et al. (2007). Likewise, the drug reduced the release of plasma lysozyme as compared to the PBS-treated mice. However, *in vivo* treatments with low dose of *C. odorata* (5mg/kg body weight) ameliorated the inhibitory effects induced by the drug through significant enhancement of phagocytosis and cellular proliferation, in the absence or presence of LPS. Phagocytosis represents an important innate defense mechanism and this phylogenetically conserved process is critical for innate immunity (Greenberg & Grinstein 2002). The ability of the plant extract to induce the proliferation of LPS-sensitive splenic lymphocyte population possibly suggests that the extract might contain active components that may be involved in B cell proliferation stimulation, which could be associated to humoral-mediated immunity that may be involved in B cell proliferation stimulation, and, with the aid of other cytotoxic mechanisms, helps the host to clear the infection. In non-physiological terms, the NADPH oxidase system can also be activated by some compounds (such as phorbol myristate acetate and synthetic chemotactic peptides), which are also able to trigger respiratory burst (Lunardi et al. 2006).

Previous report have assessed the immunomodulatory effect of two species of *Chromolaena* namely, *C. hirsuta* and *C. squalida*, on the inhibition of oxygen free radicals. Results have shown that five methoxylated flavonoids isolated from the two species inhibited reactive oxygen species generation by opsonized-zymosan polymorphonuclear leukocytes (Taleb-Contini et al., 2006). Similar to the present study, there was some degree of inhibition in superoxide anion production in extract-treated mice compared with PBS-treated mice, but this was not statistically significant. Nevertheless, previous findings on the efficacy of *C. odorata* as an antimicrobial, antiprotozoal and antifungal (Taleb-Contini et al., 2003; Suksamrarn et al. 2004; Ngon-Ngane et al. 2006; Vital 2008; Vital and Rivera 2009) suggest the capacity of the plant extract to induce degradation of pathogens possibly through the release of lytic enzymes from macrophages. The results in this study could also suggest that there could be another antimicrobial mechanism, aside from ROS production, that is induced by *C. odorata* extracts. The decrease in the production of reactive oxygen species could also indicate the possible antioxidant property of *C. odorata* plant extract.

This study assessed the immunomodulatory effects of *C. odorata in vivo*. The results showed that the plant crude extract could potentially reverse the inhibitory effects of cyclophosphamide in the various immune parameters measured in this study. These could provide baseline information in considering the efficacy of the plant extract as immuno-nutritional supplement.

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