

Original Research Article

Immunomodulatory properties of ethanol extract of *Canarium ovatum* (Burseraceae) pulp

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Abstract

Purpose: To evaluate the immunomodulatory properties of ethanol extract of the pulp of *Canarium ovatum* (COPE).

Methods: The immunomodulatory activity of ethanolic extract of the pulp of *C. ovatum* was investigated in vivo using Balb/C mice. Extract doses of 300 and 600 mg/kg were orally administered to study its effect on delayed type hypersensitivity and humoral antibody response using sheep red blood cells (SRBC). Acute oral toxicity profile and phytochemical analysis were also determined.

Results: Orally-administered COPE did not exhibit any mortality or signs of toxicity at doses 300 - 2000 mg/kg. Phytochemical analysis revealed the presence of biologically-active compounds such as sterols, triterpenes, flavonoids, alkaloids, saponins, glycosides and tannins. Treatment with COPE for 7 days stimulated the early phase of DTH response through significant increase in foot pad thickness (111.87 ± 9.97 % at 300 mg/kg, and 91.27 ± 7.81 % at 600 mg/kg), when compared to distilled water and cyclophosphamide (CP) groups. Similarly, COPE significantly enhanced antibody titer, with highest titer at the dose of 300 mg/kg. Histological observations of the spleen showed follicles with active germinal centers and proliferating lymphocytes, which are consistent with the immunostimulatory effects of COPE.

Conclusion: These results show that COPE has stimulatory effects on cellular and humoral responses in mice, indicating its potential as an immunostimulatory agent.

Keywords: Immunomodulation, *Canarium ovatum*, Delayed-type hypersensitivity reaction, Antibody titer, Pili pulp

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INTRODUCTION

Immunomodulation is a process in which the immune system is either stimulated or suppressed in response to certain conditions [1]. The search for agents with immunomodulatory activities has continued to attract the attention of

researchers. This is due to their potential for alleviating immunological dysfunctions such as acquired immunodeficiency syndrome, and for normalizing the immune system after organ transplantation. Plants are very important sources of compounds that are thought to have significant immunomodulatory activities [2].

Canarium ovatum (Burseraceae) is a native plant in the Philippines. It is considered as one of the most important trees in the Bicol region where its nuts and fruit pulp are used in food and as oil source [3]. Recent investigations on the *C. ovatum* fruit pulp showed that it has good antioxidant activity and anticancer potential [4]. The present study was aimed at assessing the immunomodulatory potential of *C. ovatum* fruit pulp.

EXPERIMENTAL

Plant sample collection and extraction

Canarium ovatum (pili) fruits were obtained from the Pili Research and Technology Development Center (PRTDC) Albay Philippines. In the laboratory, the dried and powdered fruit pulp was macerated in 95 % ethanol (1:3 w/v ratio) for 48 h. Following filtration, the extract was dried under reduced pressure in a rotary evaporator. The resultant dry powder was weighed and dissolved in distilled water to obtain the various concentrations of the extract (COPE) used in subsequent experiments.

Phytochemical screening

Qualitative phytochemical analysis of the crude extract was carried out using standard tests for alkaloids, flavonoids, glycosides, saponins, sterols, tannins and triterpenes [5].

Animals

Male Balb/C mice weighing 25-30 g were used in this study. They were allowed a 7-day acclimatization to laboratory settings before commencement of the study. The mice were maintained on pellet feed and distilled water *ad libitum* in standard environmental conditions of 24 - 27 °C and equal light/dark periods

The animals were handled according to the guidelines of the Animal Care and Use Committee of the University of the Philippines, Manila (Protocol/Approval No.2015-022).

Acute toxicity study

Oral acute toxicity study was carried out in line with the OECD method (guideline no. 423). Three female ICR mice were used per step. After an overnight fast, the extract was administered by oral gavage at a dose of 300 mg/kg, and observations were made at different time intervals from 30 min to 24 h. The animals were observed for weight change, changes in skin, tremor, convulsion, salivation, diarrhea, lethargy,

coma and death, for 14 days. Body weights before and after the treatment were also recorded. Since COPE did not produce any mortality at the dose of 300 mg/kg, the experiment was repeated using a dose of 2000 mg/kg.

Treatment protocol

The results of the acute toxicity study showed no mortality and no sign of toxicity at doses of 300 to 2000 mg/kg of *C. ovatum* pulp extract in mice. Therefore, doses of 300 and 600 mg/kg were chosen for use in subsequent experiments. Four mice groups (5 mice/group) were used. Group I served as vehicle control and was given distilled water, while group II and group III received COPE at doses of 300 mg/kg and 600 mg/kg, respectively. Mice in group IV received cyclophosphamide (20 mg/kg) for induction of immunosuppression.

Delayed-type hypersensitivity (DTH) reaction (SRBC) as antigen

Delayed type hypersensitivity reaction was performed using a modified form of the method of Gokhale *et al* [6]. Mice in all the groups were subcutaneously sensitized with 1×10^8 sheep red blood cells SRBC (Fitzgerald Inc., USA). Following the sensitization, group IV mice were intraperitoneally injected with cyclophosphamide 2 h prior to immunization. From day 1 to day 7, mice in groups II and III received COPE at the same doses as earlier indicated, while group I mice were given distilled water. On day 7, the left hind paw thickness was determined in all mice, after which they were subcutaneously challenged with 1×10^8 SRBC which was administered in the same footpad. The immune response was determined by increase in foot pad thickness as measured using a caliper at 0, 2, 24, and 48 h after the SRBC challenge. The values obtained were used to calculate edema index (EI) as in Eq 1.

$$EI (\%) = \{(LT - RT)/RT\}100 \dots\dots\dots (1)$$

where LT and RT are the left and right hind paw thickness, respectively.

Hemagglutination antibody titer

In the test for the *in vivo* antibody production, all mice were sensitized with SRBC (1×10^8), and the plant extract was administered daily through oral gavage from the time of sensitization for 14 days. On day 14, the animals were again challenged with SRBC and blood was collected from each mice through the retro-orbital

puncture. Each blood sample was serially diluted in 20 μ L of normal saline mixed with 20 μ L of SRBC in microtiter plates. The plates were shaken and kept for 1 h to settle at room temperature. They were then examined for hemagglutination. The antibody titer was taken as the highest serial dilution showing visible hemagglutination [6]. The titers were further converted to mean log values for analytical purposes.

Spleen weight index and histology

The spleen of each mouse was weighed to calculate the spleen index. It was then preserved in 10 % formalin and processed for light microscopy at High Precision Laboratory (Quezon City, Philippines). The microscopic section of the spleen from each mouse was examined and scored on a 4-point scale: 0 = normal, 1 = mild, 2 = minimal, 3 = moderate, and 4 = marked [7].

Statistical analysis

Data are presented as mean \pm standard error of the mean (SEM). Groups were compared for statistically significant differences using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test.

RESULTS

Phytochemical screening of ethanol extract of the pulp of *Canarium ovatum* showed that it contained sterols, triterpenes, flavonoids, alkaloids, saponins, glycosides and tannins (Table 1).

Table 1: Phytochemical constituents of *C. ovatum* ethanol pulp extract

Metabolite	Result
Sterols	(+)
Triterpenes	(++)
Flavonoids	(+)
Alkaloids	(+)
Saponins	(+++)
Glycosides	(++)
Tannins	(++)

(+) Traces, (++) moderate, (+++), abundant, (-) absent

The extract did not show any sign of toxicity or mortality when given to the test animals at low (300 mg/kg) or high (2000 mg/kg) dose. In addition, the extract did not produce any significant differences in body weight and spleen weight of the treated mice at the two doses (300 and 600 mg/kg), when compared to the vehicle

control and cyclophosphamide-immunosuppressed group (Table 2).

Table 2: Effects of *Canarium ovatum* extract on body and spleen weights

Group	Treatment (mg/kg)	Body weight (g) ^{ns}	Spleen weight index
I	Distilled water	25.44 \pm 0.99	0.5384 \pm 0.0223
II	300	23.76 \pm 0.73	0.6814 \pm 0.0645
III	600	25.22 \pm 0.69	0.6435 \pm 0.0459
IV	Cyclophosphamide (20)	27.68 \pm 2.39	1.434 \pm .2257*

Values are expressed as mean \pm SEM. * significantly different, compared to control. $p < 0.05$ ($n = 5$)

The administration of the extract for 7 days produced an increase in percentage edema index 2 h after SRBC challenge, and significantly enhanced the titer of circulating antibody, with 300 mg/kg eliciting a higher titer (Table 3). However, cyclophosphamide administration led to decreases in edema index and antibody titer in response to SRBC challenge. After 24 and 48 h of SRBC challenge, the increase in percentage EI was still seen, although the effect was comparable to that seen in group treated with distilled water. The percent edema after 2 h was higher than those seen after 24 and 48 h.

Figure 1 (A to C, inset) shows spleen white pulp with prominent and active germinal centers, and aggregates of darkly-stained lymphocytes within the follicles in mice treated with COPE. A significant increase was observed in the number of proliferating lymphocytes on COPE-treated mice, when compared to the control and cyclophosphamide-immunosuppressed groups (Table 4).

Table 4: Spleen histology

Group	Treatment (mg/kg)	Number of proliferating lymphocytes	
I	Distilled water	3.50 \pm 0.18 ^b	2.52 \pm 0.07 ^c
II	300	3.54 \pm 0.12 ^b	3.48 \pm 0.06 ^a
III	600	3.73 \pm 0.08 ^b	3.50 \pm 0.06 ^a
IV	Cyclophosphamide (20)	1.13 \pm 0.48 ^a	1.45 \pm 0.13 ^b

Values are presented as mean \pm standard error. Different letters indicate significant differences between groups ($p < 0.05$).

DISCUSSION

DTH which is usually used to determine reaction to the antigen SRBC, indicates potentiating effect on T-lymphocytes and accessory cell types [6]. The early phase of DTH reaction involves clonal expansion of lymphocytes, increased vascular permeability, induction of local inflammation, and influx of neutrophils [8]. The results of the

Table 3: Effect of *Canarium ovatum* extract on circulating antibody titer and on footpad thickness

Group	Treatment (mg/kg)	Antibody (HA) titer	% EI after 2 h	% EI after 24 h	% EI after 48 h
I	Distilled water	3.33±0.26 ^a	79.31 ± 19.92 ^b	32.23 ± 13.58 ^a	25.54 ± 12.83 ^a
II	COPE (300)	6.80±0.14 ^b	111.87±9.97 ^a	31.14 ± 2.69 ^a	24.28 ± 6.00 ^a
III	COPE (600)	6.51±0.27 ^b	91.27 ± 7.81 ^a	27.01 ± 6.21 ^a	24.31 ± 7.80 ^a
IV	Cyclophosphamide (20)	2.77±0.00 ^a	15.84 ± 4.82 ^c	5.34 ± 4.31 ^b	3.12 ± 4.38 ^b

Values are presented as mean ± DSEM (n = 5). Different letters indicate significant differences between groups ($p < 0.05$)

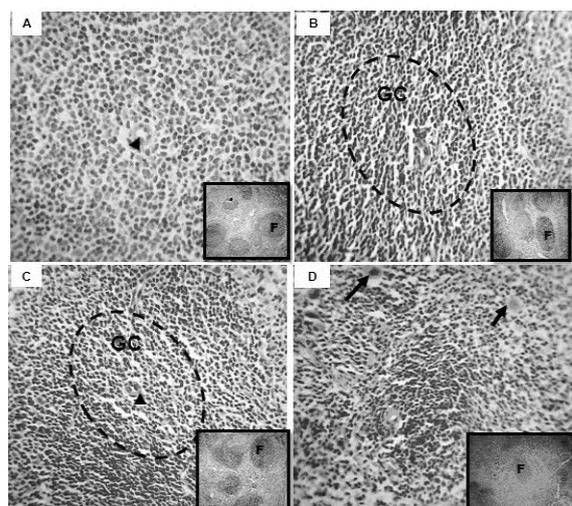


Figure 1: Histological features of lymphocytes of spleen tissues treated with distilled water (A), and COPE at 300 mg/kg (B), 600 mg/kg (C) or cyclophosphamide (D). Arrows indicate myeloid cells, arrowheads for central artery and GC for germinal centers. H & E stain, x400. Inset: Follicles (F). H & E, x50

present study showed that COPE exerted pronounced effects on early phase of DTH reaction as shown by increases in edema at early stage after SRBC challenge, thereby confirming its stimulatory activity on T-lymphocytes.

Humoral immunity or response involves the interaction of antigens with B-cells which then differentiate into plasma cells [9]. The plasma cells then produce antibodies which mediate the humoral response. In this study, administration of COPE produced an increase in the antibody titer in mice. This enhancement of antibody responsiveness to SRBC indicates increases in the population of cells such as macrophages, and T and B lymphocytes which are involved in antibody production [8]. Thus, COPE exerts a potentiating effect on the humoral response.

The spleen plays a vital role in immune response. It filters the blood and it is also the site of antibody synthesis, which makes it an important organ for evaluation of changes in the

immune system [7,10]. Microscopic observations of the spleen showed that treatment with COPE led to pronounced germinal centers and significant increases in the number of lymphocytes. These results suggest that the humoral immune response was enhanced by COPE. It has been suggested that prominent germinal centers are indicative of increases in the population of proliferating B-lymphocytes and stimulation of humoral response [11,12].

The results from phytochemical screening of COPE revealed the presence of sterols, flavonoids, triterpenes, alkaloids, saponins, glycosides and tannins. These compounds are known to modulate the immune system [12]. Thus, their presence further validates the immune-modulatory potential of COPE.

CONCLUSION

The results obtained in the present study indicate that ethanol extract of the pulp of *Canarium ovatum* possesses stimulatory effects on cell-mediated and humoral immune functions in mice.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims related to the content of this article will be borne by them. Salvador-Membreve, Cajuday and Serrano conceived and designed the study. Baldo helped in collecting

and analyzing the data. All authors participated in writing the manuscript and approved its publication.

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