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## Immunostimulatory Effect of *Moringa oleifera* Extracts on Cyclophosphamide-induced Immunosuppressed Mice

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### ABSTRACT

In this study, the effect of *Moringa oleifera* extracts were investigated on an immunosuppressed model of cyclophosphamide-induced Swiss mice. *M. oleifera* seed and root extracts (2.0 g kg<sup>-1</sup> body wt) were administered orally for 7 consecutive days; cyclophosphamide (CP) (200 mg kg<sup>-1</sup> i.p.) was administered by IV on the 4<sup>th</sup> day. Results indicated that the extracts of the *M. oleifera* seeds and roots ameliorated the immunosuppressive effects of CP on delayed type hypersensitivity (DTH) response, leukocyte counts, relative organ weight, IgM concentration, serum TNF- $\alpha$  assay and micro-histological images. This study demonstrated *in vivo* the therapeutic effect of *M. oleifera* on the immune response in clinical application.

### ARTICLE HISTORY

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### KEYWORDS

Bioactivity; immunostimulatory activity; immunosuppression; Swiss mice

## Introduction

There is a growing interest in using natural products to improve the immune response since many natural products have shown immunomodulatory effects and influence on the immune system of the human body.<sup>[1]</sup> Immunosuppression is a state where the body responds inadequately to pathogens and external organism, individuals who are immunocompromised are at higher risk of infection and cancer.<sup>[2]</sup> Immunosuppression can be due to the patient's genetics disposition or due to external source such as radiation, drug or chemotherapy. Most of the synthetic prescription immunostimulants (e.g. Lonquex, Mepact) possess side effects and may contradict with the treatment regime (i.e. Stem cell transplant). Therefore, the study of new immunostimulatory agents have great promises with regard to the prevention and alleviation of the immunosuppressed state.

With the emergence of the recent viral pandemic (SARS-CoV2), the virus main entry point was the angiotensin-converting enzyme 2 receptor, one of

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the main regulator of the inflammatory cascade and fibrosis, which in turn leads to the dysregulation of the patient's immune response. Thus, in severely infected patients, cytokine storm and lung damage were the leading cause of death.<sup>[3]</sup> Immuno-modulating herbs have been suggested in the management of cytokine storm in severe viral infection.<sup>[4,5]</sup> *Moringa oleifera* (Moringaceae) commonly known as 'drumstick tree' or 'horseradish tree' grows in the tropics and subtropics. With its high nutritive values, every part of the tree is suitable for either nutritional, medicinal or commercial purposes. Extracts from the roots and seeds are used as antioxidant, anticancer, anti-inflammatory, anti-diabetic and antimicrobial agent.<sup>[6]</sup> In preclinical research, *M. oleifera* extracts proved to have immunostimulatory effect on both the cell-mediated and humoral immune systems.<sup>[7]</sup> In this study, the immune-stimulatory effect of *M. oleifera* extracts was examined in an immunosuppressed animal model – cyclophosphamide-induced *Swiss* mice.

## Materials and Methods

### Preparation of *M. oleifera* Extracts

The study was conducted at the Faculty of Biotechnology, Department of Biology at Vietnam National University of Agriculture, Hanoi, Vietnam: The fresh plant materials of *M. oleifera* were dried at 50°C until the humidity had reached 5%, ground to a fine powder and extracted with 70% ethanol by percolation in 72 h; the ethanolic extract was vacuum-dried at 40°C then preserved in vacuum packages. The material was then formulated into the form of liquid extract:

100 g *M. oleifera* seeds produced 8 mL of liquid extract (*M. oleifera* seed extract)

100 g *M. oleifera* roots produced 6 mL of liquid extract (*M. oleifera* root extract)

The liquid extract was diluted in distilled water (1 mL 100 g<sup>-1</sup> wt) before oral administration by gastric tube.

### Drugs and Chemicals

Cyclophosphamide (Endoxan 200 mg, Baxter Oncology GmbH, Germany) was used as the immunosuppressive agent. Levamisole was obtained from Sigma (Aldrich) Chemicals Pvt. Ltd. USA and used as a positive control in this experiment. Sheep red blood cells (SRBC) and OA solution (ovalbumin + Al(OH)<sub>3</sub>) were used as the delayed-type hypersensitivity inducer.

## Animals

Swiss albino mice (*Mus musculus*) of either sex, weighing  $20 \pm 2$  g were purchased from National Institute of Hygiene and Epidemiology. The mice were kept in cages (10 animals per cage) under standard conditions of humidity ( $50 \pm 5\%$ ), temperature ( $25 \pm 2^\circ\text{C}$ ) and light (12 h light/12 h dark cycle) and fed with standard food (National Institute of Hygiene and Epidemiology) and water *ad libitum*. The mice were adapted to their laboratory condition for 7 d before starting the study.

## Animal Study Conduct

Sixty-five mice included in this experiment, were randomly assigned to five groups of 13 mice per cage: control group (Group I), CP-induced group (Group II), positive control group (Group III), M.O seed group (Group IV), M.O root group (group V). On the 4<sup>th</sup> day of the experiment, control group was given an i.p. injection of physiological saline ( $10 \text{ mL kg}^{-1}$ ) and four other groups (Group II–V) were injected CP with a single dose ( $200 \text{ mg kg}^{-1}$ ). Positive control group (Group III): levamisole  $100 \text{ mg kg}^{-1}$ , M.O seed group (Group IV): *M. oleifera* seed extract  $2.0 \text{ g kg}^{-1}$ , and M.O root group (group V): *M. oleifera* root extract  $2.0 \text{ g kg}^{-1}$ , were administered orally levamisole and *M. oleifera* extracts for 7 consecutive days. On the 8<sup>th</sup> day, mice were anesthetized by ketamine ( $100 \text{ mg kg}^{-1}$  i.p.) then euthanized to collect blood samples, spleen and thymus to evaluate immune parameters.

## Delayed Type Hypersensitivity (DTH) Response Estimation

On the 2<sup>th</sup> day of the experiment, animals were given an i.p. injection of sheep red blood cells and injected subcutaneously OVA 0,1 mL into the nape of neck site. DTH reaction was elicited 5 d later by the injection of OVA  $50 \mu\text{L}$  into right hind paw and physiological saline into left one after measuring the thickness of footpad in the center of walking pads using a INSIZE® digital external caliper (Code: 2312–20). After 24 h, the paw volume was measured again to assess the swelling degree through differential percentage between the two footpads.

## Relative Organ Weight

The related weight of the spleen and thymus was calculated as: *Relative organ weight* = *Organ weight (mg)/Body weight (g)*

### **Leukocyte Counts**

On the day of sacrifice, blood samples were collected to determine the number of total WBC, lymphocytes, neutrophils and monocytes by using an HORIBA® ABX MircoES60 blood analyzer.

### **Estimation of IgM, IL-2 and TNF- $\alpha$ Concentration in Serum**

On the 8<sup>th</sup> day of the experiment, blood samples were assayed to measure the concentration of serum IgM, IL-2 and TNF- $\alpha$  by using an ELISA kit (Cloud-Clone Corp., USA) and a BioTek® ELx808 Reader.

### **Histopathological Examination**

A histopathological examination was performed in total of 15 spleens/thymi: 3 each group. After their removal, the organs were weighed using a digital scale (Precisa®, Swiss, Model 321 LX Type 2200 C), then preserved in 10% formalin for fixation. Sections stained with hematoxylin and eosin and examined under a light microscope. The images were photographed digitally at 100x.

### **Statistical Analysis**

Statistical analysis and data plotting were carried out using R version 3.5.3 ( $P < .05$ ).

## **Results**

### **General Result after the Study Period**

#### **Mice survival**

All mice were healthy at the time of sacrifice. There was no adverse effect, no notable change in vital signs, skin, fur and daily behavior were observed.

### **Effect of *M. oleifera* Extracts on Delayed Type Hypersensitivity (DTH) Response**

Administration of CP (200 mg kg<sup>-1</sup>, i.p) showed a decrease in the DTH response (Table 1). There was an increase in the paw volume at groups treated with levamisole and the *M. oleifera* root extract, but not with the seed extract (Table 1).

**Table 1.** Effect of *Moringa oleifera* extracts on swelling degree of footpads and relative organ weight of the spleen and thymus.

Group	Treatment	Swelling of footpads (%)	Relative weight of spleen (mg g <sup>-1</sup> )	Relative weight of thymus (mg g <sup>-1</sup> )
I	Control	59.42 ± 13.79	6.43 ± 1.03	3.22 ± 0.64
II	Cyclophosphamide (CP)	43.75 ± 10.08	2.99 ± 0.59	0.82 ± 0.18
III	CP + Levamisole	68.01 ± 14.22	3.32 ± 0.71	0.67 ± 0.22
IV	CP + <i>M. Oleifera</i> seeds extract	44.73 ± 12.86	4.41 ± 1.38	0.89 ± 0.29
V	CP + <i>M. Oleifera</i> roots extract	71.87 ± 23.13	2.81 ± 0.63	0.68 ± 0.17

### Effect of *M. oleifera* Extracts on Relative Organ Weight

The relative thymus weights of the animals were not affected by the *M. oleifera* seed and root extracts. However, there was an increase in relative spleen weight at mice treated *M. oleifera* seed extract (Table 1).

### Effect of *M. oleifera* Extracts on Leukocyte Counts

In mice treated with CP the leukocyte counts were reduced (Table 2). Levamisole increased the white blood cells (WBC), lymphocytes (LYM) and the neutrophil (NEU), but had no effect on the monocytes (MONO). There was no difference in the leukocyte count in groups treated with *M. oleifera* seeds and roots extracts.

### Effect of *M. oleifera* Extracts on Serum IgM, IL-2 and TNF- $\alpha$ Concentrations

Levamisole and *M. oleifera* seed extracts increased IgM concentration (Fig. 1). Levamisole and *M. oleifera* root extracts improved TNF- $\alpha$  concentration. There was no notable difference observed between groups treated with *M. oleifera* extracts and CP-induced group in terms of IL-2 concentration.

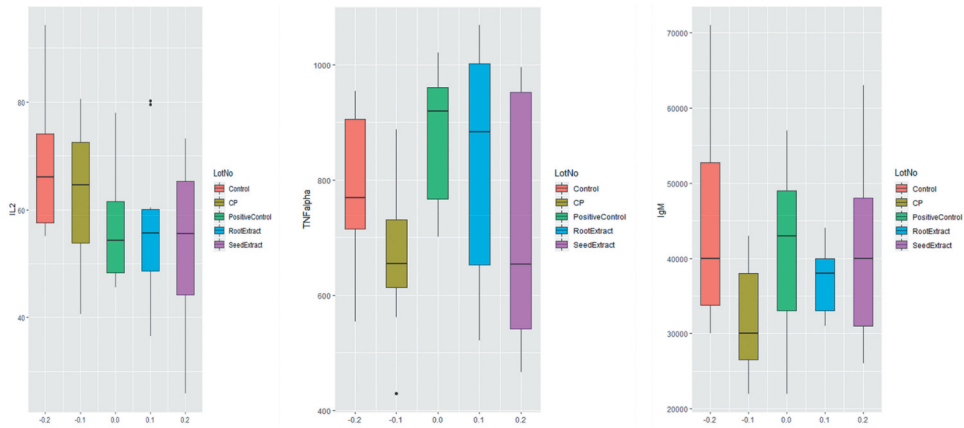
### Histopathological Study of Spleen and Thymus

Histological imaging demonstrated that in the spleen and thymus of CP-induced animals, the number of lymphocytes decreased (Figs. 2 and Figs. 3).

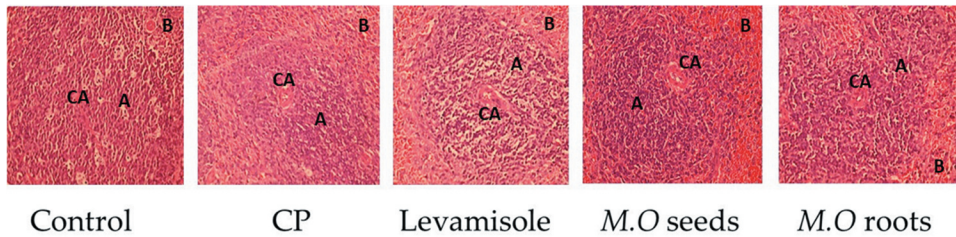
**Table 2.** Effect of *Moringa oleifera* extracts on the total leukocyte counts (TLC).

Group	Treatments	TLC Count (Means $\pm$ SD)			
		WBC (G/L)	LYM (G/L)	NEU (G/L)	MONO (G/L)
I	Control	4.58 $\pm$ 0.94	3.08 $\pm$ 0.94	1.08 $\pm$ 0.34	0.42 $\pm$ 0.12
II	Cyclophosphamide (CP)	1.75 $\pm$ 0.47	1.03 $\pm$ 0.32	0.38 $\pm$ 0.12	0.19 $\pm$ 0.06
III	CP + Levamisole	2.17 $\pm$ 0.45	1.41 $\pm$ 0.55	0.55 $\pm$ 0.18	0.14 $\pm$ 0.05
IV	CP + <i>M. Oleifera</i> seeds extract	1.47 $\pm$ 0.29	0.98 $\pm$ 0.28	0.31 $\pm$ 0.08	0.15 $\pm$ 0.05
V	CP + <i>M. Oleifera</i> roots extract	1.49 $\pm$ 0.34	0.91 $\pm$ 0.29	0.34 $\pm$ 0.10	0.20 $\pm$ 0.06

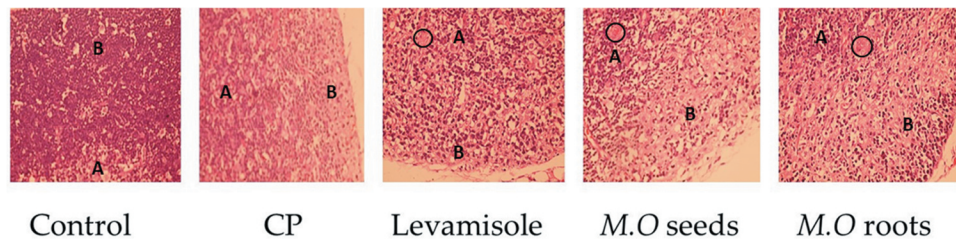
White blood cells (WBC), lymphocytes (LYM), neutrophil (NEU), monocytes (MONO).



**Figure 1.** Effect of *Moringa oleifera* extracts on serum IgM, IL-2 and TNF- $\alpha$  concentration.



**Figure 2.** Micro-histopathological images of mouse spleen (HE x 100); (a). White pulp, (b). Red pulp, CA. Central Artery. The number of lymphocytes decreased in the Cyclophosphamide (CP) treated group; Levamisole and the *Moringa oleifera* seeds/roots extracts improved the lymphocyte count.



**Figure 3.** Micro-histopathological images of mouse thymus (HE x 100); (a). Medulla, (b). Cortex, the circle indicate a Hassall's corpuscle. The number of lymphocytes decreased in the Cyclophosphamide (CP) treated group; Levamisole and the *Moringa oleifera* seeds/roots extracts improved the lymphocyte count.

Levamisole and the seed and root extracts of *M. oleifera* restored the lymphocyte counts in the spleen and thymus.

## Discussion

*M. oleifera* is a fast-growing, drought-resistant tree found in tropical and subtropical regions of South Asia, including Vietnam.<sup>[8]</sup> In recent years, *M. oleifera* has been widely cultivated not only for human consumption as vegetables but also for traditional herbal medicine. Extracts from *M. oleifera* components contain various polyphenols, which are under preclinical research to determine their potential effects on improving health or treatment of diseases, especially on immunodeficiency disorders.<sup>[9]</sup> In the current study, the main objective was to evaluate the immunostimulatory effects of *M. oleifera* root and seed extracts and before sacrificing the experimental animals, the mice were also observed for changes in vital signs and daily behavior. No adverse effects were noted in mice administered with *M. oleifera* in this study, indicating the safety of *M. oleifera* on mice.

*M. oleifera* extracts have been tested on animal model to demonstrate its effect on the immune system. Leaf extract of *M. oleifera* at 500 mg kg<sup>-1</sup> and 1000 mg kg<sup>-1</sup> showed notable immunostimulatory effect on both the cell-mediated and humoral immune systems in the *Wistar albino* rat.<sup>[10]</sup> Methanolic *M. oleifera* increased the levels of serum immunoglobulins, the circulating antibody titer, adhesion of neutrophils, attenuation of cyclophosphamide-induced neutropenia and phagocytic index. *M. oleifera* leaves at 125 mg kg<sup>-1</sup> was reported as having potential as an immuno-stimulant to reverse the immunosuppression induced by cyclophosphamide in *Wistar* rat.<sup>[11]</sup> The ethanolic extract of seeds of *M. oleifera* stimulated immune-mediated inflammation in *Wistar* rats.<sup>[12]</sup>

In DTH test, the DTH response, which directly correlates with the cell mediated immunity was found to be considerable in mice treated with levamisole and *M. oleifera* extracts. This may be explained that after challenged by antigen, the sensitized T-lymphocytes were converted to lymphoblast, secreting a variety of molecules including pro-inflammatory lymphocytes, attracting phagocytes to the site of reaction.<sup>[13]</sup> An increase in footpad thickness indicated the stimulatory effect of *M. oleifera* extracts on the lymphocytes and accessory cell types required for the expression of this reaction. Cytokines secreted by a board of cells including lymphocytes have an important role in inflammation response of the body, especially IL-2 and TNF- $\alpha$ . IL-2 has a wide spectrum of immune promoting activities, such as inducing the differentiation of lymphocytes, promoting the function of NK cells, and releasing interferons.<sup>[14]</sup> In this study, there was no change due to *M. oleifera* extracts in IL-2 concentration. However, TNF- $\alpha$  concentration increased in groups treated with *M. oleifera* seeds and root extracts. Antibody production induced by thymus-dependent antigen (sheep red blood cells) requires the cooperation of T- and B-lymphocytes and macrophages. The high value of the IgM titer in mice treated with *M. oleifera* extracts expressed the immuno-

stimulatory effects of the extracts of *M. oleifera* seeds and roots through the humoral immunity.

The increase in thymus and spleen weight was accompanied by an increase in its cell counts, improving the stimulatory effect of this extract on the lymphocytes and bone marrow hematopoietic cells. This result was consistent with the histopathological change in thymus and spleen in experimental groups (Figs. 1 and Figs. 2). Bone marrow is a site of continued proliferation and turnover of blood cells, and is a source of cells involved in immune reactivity. T-lymphocytes and other key cells of immune system are known to activate the production of antibody polymorphic-nuclear granulocyte to destroy the antigen. Loss of stem cells and inability of bone marrow to regenerate new blood cells will result in leukopenia. In this study, *M. oleifera* extracts improved leucocyte counts suggesting an important role of *M. oleifera* extracts on the bone marrow activity suppression induced by CP.

The results indicated that *M. oleifera* extracts stimulate the humoral as well as the cellular immunity. Possibly the use of *M. oleifera* extracts may improve patient's prognosis with immunodeficiency disorders, either due to aging or as side effect of treatment regime. This study suggests the immune-stimulatory effect of *M. oleifera* extract and warrant further study for its safety and efficacy in clinical practice.

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