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Reversibility Capacity of Aqueous Extract of *Moringa oleifera* Leaf on Cyclophosphamide Induced Immunotoxicity in Male Wistar Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author SOO designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors DVD and AAO performed the statistical analysis, managed the analyses of the study. Author SOO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Cyclophosphamide is a chemotherapeutic alkylating drug currently used in combination with other protective agents for the purpose of reducing the adverse toxic effects. Increasingly, plants have become sources of therapeutics that can help to restore host immunity to normal.
Aim: In this study, reversibility capacity of aqueous extract of *Moringa oleifera* leaf on cyclophosphamide-induced toxicity in male wistar rats was investigated.
Methods: Twenty five (25) wistar rats weighing 120 – 200g were used, which were divided into 5 groups of 5 rats each. Group I served as normal control and received water and normal feed. Group II served as standard control and received 30mg/kg cyclophosphamide (a potent immunosuppressant) only. Groups III-V served as test groups and were administered with 125mg/kg

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bw extract +30mg/kg bw CPM, 250mg/kg bw extract +30mg/kg bw CPM and 500mg/kg bw extract +30mg/kg bw CPM respectively *po* once daily for 28 consecutive days. Blood samples were collected through cardiac puncture and transferred into ethylene diaminetetraacetic acid bottles for analyses of hematological parameters such as: Total white blood cell counts and Differential and Platelets count.

Results: The result showed significant ($p<0.05$) reduction in the level of the various immunological parameters assayed. This depicts a possible immunosuppressive potential of *Moringa oleifera* leave extract. However, a dose dependant significant ($p<0.05$) decrease in Total WBC count, Neurophil count, Lymphocyte count, Monocyte count, Total lymphocyte count (TLC) and Platelets count following the co – administration of cyclophosphamide and *Moringa oleifera* leave extract when compared to cyclophosphamide administered groups. However, nonsignificant decrease in eosinophil and Basophil were observed.

Conclusion: Therefore, the results strongly suggest that *Moringa oleifera* leaves may have potential effect as a naturally derived immunostimulant to reverse the immunosuppression induced by cyclophosphamide. Combined administration of *Moringa oleifera* leave extract and cyclophosphamide modulate cellular immunity and could be beneficial in intracellular bacterial and viral infections. Further studies using isolated compounds of the extract are recommended to identify the active agent with its exact mechanism of action.

Keywords: Reversibility; cyclophosphamide; Moringa oleifera; immunosuppression; rats.

1. INTRODUCTION

Immune modulation is the manipulation of immune response to suppress unwanted responses resulting from autoimmunity, allergy, and transplant rejection, and to stimulate protective responses against pathogens that largely elude the immune system. An immune modulator is any substance that affects directly or indirectly the immune response to external agents or therapeutics and prevents or reduces the development of degenerative diseases [1]. They have broad effects on the entire immunity system, but affect primarily cell mediated immunity, while the humoral immunity may be affected indirectly [2]. Immune modulators achieve their effects by boosting specific areas of the immune system, most especially, the innate immunity and the activities of T lymphocytes. They are known to have amplifier and suppressor activities, depending on the immune status of the user [3].

Cyclophosphamide (CPM) is a chemotherapeutic alkylating drug used to treat a variety of cancers, i.e. leukemia, lymphomas, and solid tumors [4]. However, use of CPM is often restricted due to cytotoxic effects that result from formation of reactive metabolites that alkylate DNA and proteins, and also produce cross-links. Among the adverse side-effects that often subsequently result are nausea, mucosal ulceration, vomiting, pulmonary fibrosis, skin pigmentation, hematopoietic suppression, nephrotoxicity, urotoxicity, and hepatotoxicity [5]. To combat this, CPM is currently used in combination with other protective agents with the purpose of reducing the adverse toxic effects. Systemic administration of N-acetylcysteine or sodium-2-mercaptoethane sulfonate (MESNA, a synthetic sulfur agent) protects human/ experimental hosts from CPM-induced hematuria without interfering with CPM efficacy [6]. Several traditionally used medicinal plants and plant products have become potential sources of similar ameliorative agents. Previous studies in our laboratory showed that materials derived from Rhizopora apiculata, Acacia nilotica, or Decalepis hamiltonii exhibited such effects against CPM-induced toxicity [7].

The use of plants in treatment of various diseases has existed before human written history. Ethnobotany (which means the study of traditional means of using plants by human) has been accepted as helpful approach to find out future medicines. Most pharmaceuticals now accessible to physicians have been noticed to have a long history of use as herbal medicines, including aspirin, digitalis, opium and quinine [8].

Moringa oleifera Lam (syn. *M. ptreygosperma* Gaertn) is a member of *Moringacaea* family, which is a fast growing drought-resistant tree that is native to northern India. It is the most widely spread species of the family, "*Moringacaea"*; containing flavonoids, vitamins A, B, C, E plus Ca^{2+} [9].

M. oleifera is a little beat tall tree, with a height of 10–12 m (32-40 ft) and the trunk can reach a diameter of 45 cm (1.5 ft). The bark has a whitish-grey colour and is surrounded by thick cork. Young shoots have purplish or greenish-white, hairy bark. The tree has an open crown of drooping, fragile branches and the leaves build up feathery foliage of tripinnate leaves [10].

The flowers are fragrant and bisexual, surrounded by five unequal, thinly veined, yellowish-white petals. The flowers are about 1.0-1.5 cm (1/2") long and 2.0 cm (3/4") broad. They grow on slender, hairy stalks in spreading or drooping later flower clusters which have a length of10–25 cm [10]. Flowering begins within the first six months after planting. There are dearth's of literatures to ascertain the immunomodulatory potential of *Moringa oleifera* leaves extract following exposure to an immunosuppressant; hence this study is designed to evaluate the reversibility capacity of aqueous extract of *Moringa oleifera* leaf on cyclophosphamide induced toxicity using wistar rats as models.

2. MATERIALS AND METHODS

2.1 Plant Collection and Identification

Fresh matured leaves of *Moringa oleifera* were collected before sun rise in June at Rumuji area, Emohua L.G.A, Rivers State. The plant was identified by Dr. Chimezie Ekeke, a Herbarium of the Department of Plant Science and Biotechnology, University of Port Harcourt, Choba, Nigeria with the identification Number: UPH-062.

2.2 Preparation of Aqueous Leaf Extract of *Moringa oleifera*

The extraction was done at the Department of Pharmacy and Pharmacognosy, University of Port Harcourt, Rivers State, Nigeria. The fresh leaves were carefully rinsed in distilled water and were air dried for 20 days. The dried leaves were grounded to particle or fine powder, with the aid of domestic electric grinder and 750 g of the leaves was obtained. The dried leaves were suspended in distilled water at room temperature.

The filtrates were pulled together and lyophilized using a freeze dryer. The yield of the aqueous leaf extract of *Moringa oleifera* was 18.22% (w/w). The lyophilized extract was stored in an air tight container and kept in the dark until the time they used.

2.3 Experimental Animals

Twenty five (25) male wistar rats weighing (120-200 g) were used for this study. The animals were purchased from animal farm of University of Nigeria Nsukka, Enugu State, and kept at the animal house, Department of Human Physiology, College of Health Sciences, University of Port Harcourt, Rivers State, Nigeria. The animals were housed in a spacious and well ventilated cage with suitable temperature, relative humidity under 12 hour house light and dark cycle at ambient temperature of 27 ± 1° for acclimatization for two (2) weeks. They were allowed free access to feed diet (Top feeds, Broiler grower-product of Eastern Premier feed mill Ltd) and water *ad libitum* but starved for 12 hours prior to commencing of the experiment.

All animals received human care according to the criteria outlined in the guide for the care and the use of Laboratory animals prepared by the National Academy of Science and published by the National Institute of Health. The ethical regulations are accordance with National and Institutional guidelines for the protection of animals' welfare were strictly adhered to during the experiments [11].

2.4 Experimental Design

Twenty five (25) experimental male rats were randomly grouped into five groups (I-V) of five rats (n= 5) in each group after the period of acclimatization.

Group I: Served as normal control, and received water and normal feed only
Group II: Served as Standard control, and received 30mg/kg cyclophosphamide for the last 4 days (24 – 28th days).
Group III: Received 125mg/kg aqueous extract of *Moringa oleifera* + 30mg/kg of cyclophosphamide
Group IV: Received 250mg/kg aqueous extract of *Moringa oleifera* + 30mg/kg of cyclophosphamide
Group V: Received 500mg/kg aqueous extract of *Moringa oleifera* + 30mg/kg of cyclophosphamide

The different animal groups received 1ml of the respective doses of the extract using an oral gavage once daily (9am-10am), for a period of 28 days. The doses of the extract chosen were

based on the results of acute toxicity (LD_{50}) study [12], which was obtained for aqueous leaf extract of *Moringa oleifera* as 1 g/kg.

Five (5) animals from each group except the lethality group were sacrificed on the 29th day and 5 ml of blood was collected by cardiac puncture into EDTA bottles for hematological evaluation viz: Total white blood cell counts (WBCs), Differential WBCs, Platelets etc, using an automated machine Beacon formulated by Mindray Haematological Analyzer, Bc 2300, USA.

2.5 Statistical Analysis

Data were expressed in mean ± SEM. The data were analyzed using One way Analysis of Variance (ANOVA) and Dunnett's Post hoc (multiple comparison), which was used to decide the level of significance between control and test groups. All statistical analysis was done using Statistical Package for Social Science (SPSS), version 20.0 software. The difference was considered significant at ($P<0.05$).

3. RESULTS

Animals' body weight before and after administration of *Moringa oleifera* leaf extract and cyclophosphamide is as shown in Table 1. There was a significant ($p<0.05$) reduction in body weight of animals administered with cyclophosphamide when compared with control group. A significant ($p<0.05$) increase in the animals' body weight was recorded with the animals administered with 500mg/kg extract + CPM when compared with the cyclophosphamide administered group.

Table 2 depicts mean changes in total WBC, RBC and platelets following administration of *Moringa oleifera* leaf extract at different concentrations and Cyclophosphamide (Immunosuppressant).

It is reported that cyclophosphamide induces a decrease in white blood cells and lymphocyte in mice [13]. In this albino rat model there was also significant ($p<0.05$) reduction in total white blood cell count, red blood cell count and platelet count was observed following cyclophosphamide administration when compared to the control. However, co – administration of graded doses of *Moringa oleifera leaves* and cyclophosphamide (125mg/kg extract + CPM, 250mg/kg bw extract + CPM and 500mg/kg bw extract + CPM) revealed a dose dependent significant ($p<0.05$) increase in the total white blood cell count, and platelet count compared with the cyclophosphamide administered group.

The lower dose of the extract and simultaneous administration of cyclophosphamide (CPM) showed a significant ($p<0.05$) increase in red blood cell count compared with cyclophosphamide administered group.

The percentage (%) changes in white blood cell differentials following administration of *Moringa oleifera* leaf extract at different concentration and cyclophosphamide are as shown in Table 3. A significant ($p<0.05$) reduction in lymphocyte count, monocyte count and total lymphocyte count was seen following cyclophosphamide induction when compared with control. A dose dependent significant ($p<0.05$) increase was observed in the total lymphocyte count following the administration of 125mg/kg bw extract + CPM, 250 mg/kg bw extract + CPM and 500mg/kg bw extract + CPM when compared with the cyclophosphamide administered group. A significant ($p<0.05$) increase in the lymphocyte count was observed following administration of 250 mg/kg extract + CPM and 500 mg/kg bw extract + CPM when compared with the cyclophosphamide administered group.

Table 1. Animals' body weight before and after administration of *Moringa oleifera* leaf extract and Cyclophosphamide

Groups	Weight before administration (g)	Weight after administration (g)
Control	120.0 ± 3.16	156.00 ± 5.01
CPM (30mg/kg)	122.50 ± 2.50	140.00 ± 4.08*
125mg/kg extract + CPM	121.25 ± 3.15	153.75 ± 3.75
250mg/kg extract + CPM	132.50 ± 7.50	155.00 ± 6.46
500mg/kg extract + CPM	130.0 ± 5.77	190.00 ± 5.77**

*All values are presented in mean ± SEM; n=5; *Values are statistically significant when compared to the control at p ≤ 0.05. **Values are statistically significant compared to Cyclophosphamide (Immunosuppressant) at p ≤ 0.05.*

N/B: CPM: cyclophosphamide

Table 2. Mean changes in Total WBC, RBC and Platelets following administration of *Moringa oleifera* leaf extract at different concentrations and Cyclophosphamide (Immune Suppressant)

Groups	WBC ($X10^9$/L)	RBC ($X10^{12}$/L)	Platelets ($X10^9$/L)
Control	20.28 ± 1.19	9.38 ± 0.18	285.00 ± 47.59
CPM (30mg/kg)	5.23 ± 1.26*	4.03 ± 0.62*	182.25 ± 49.90*
125mg/kg extract + CPM	19.13 ± 0.30**	9.34± 0.12**	259.75 ± 35.22**
250mg/kg extract + CPM	19.88 ± 2.95**	5.68 ± 1.49	268.25 ± 33.75**
500mg/kg extract + CPM	20.33 ± 1.48**	7.03 ± 1.30	277.33 ± 35.71**

*All values are presented in mean ± SEM; n=5; *Values are statistically significant when compared to the control at p ≤ 0.05. **Values are statistically significant compared to Cyclophosphamide (Immunosuppressant) at p ≤ 0.05.*

N/B: CPM: cyclophosphamide

Table 3. Percentage (%) changes in differential WBC following administration of *Moringa oleifera* leaf extract at different concentration and cyclophosphamide

Groups	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)	Total Lymphocyte Count (TLC)
Control	70.60 ± 5.29	18.00 ± 3.30	4.50 ± 0.93	3.00 ± 0.95	0.80 ± 0.37	1434.88 ±22.05
CPM (30mg/kg)	65.75 ± 3.68	11.75 ± 1.44*	3.00 ± 0.71*	2.50 ± 0.87	0.50 ± 0.29	344.95 ± 23.03*
125mg/kg + CPM	55.75 ± 3.28	18.00 ± 1.08	3.50 ± 0.65	2.75 ± 0.63	0.25 ± 0.25	1066.07 ± 55.66**
250mg/kg + CPM	69.50 ± 8.23	18.75 ± 0.75**	4.25 ± 0.85	2.75 ± 0.85	0.50 ± 0.29	1377.75 ± 45.05**
500mg/kg + CPM	70.33 ± 8.25	17.33 ± 1.86**	5.67 ± 0.33**	3.33 ± 0.88	1.33 ± 0.33	1452.33 ± 64.07**

*All values are presented in mean ± SEM; n=5; *Values are statistically significant when compared to the control at p ≤ 0.05. **Values are statistically significant compared to Cyclophosphamide (Immunosuppressant) at p ≤ 0.05.*

N/B: CPM: cyclophosphamide

A significant ($p<0.05$) increase in monocyte count was seen following administration 500mg/kg bw extract + CPM as compared with cyclophosphamide administered group.

4. DISCUSSION

The present study evaluated the reversibility capacity of aqueous extract of *Moringa oleifera leaf* on cyclophosphamide induced toxicity in male wistar rats. The results of the present investigation revealed that treatment with *Moringa oleifera* leaves significantly protected the animals from the toxic effects of cyclophosphamide. *Moringa oleifera* leaves are used as vegetables in Nigeria and Sub-Saharan Africa. Due to the deleterious effect of cyclophosphamide on blood, especially the immune system, there is an increasing interest in the development of preventive therapy for reducing cyclophosphamide toxicity in humans. *Moringa oleifera* is a safe natural antioxidant containing vegetable and is found as a potential source of four natural antioxidants such as total phenolics antioxidant, vitamin A, C and E [14]. It is also known to contain proteins, traces of carotenoids, saponins, alkaloids and phenolic constituents [10].

Changes in the body weight after extract administration have been used as valuable and growth index of *M. oleifera* leaf [9]; and thus, have been justified in this study that the reported changes in the animal's body weight following administration of cyclophosphamide were considered to be related to both growth and valuable index, since the decrease were dose dependant and significant ($p<0.05$). This indicates that the leaf extract of *Moringa oleifera* has growth potentials and agrees with the study conducted by Nwanjo [15]; which confirms the growth potentials of some medicinal plants. We observed that white blood cell count, red blood cell count and platelet count were significantly

reduced in the rats treated with cyclophosphamide compared with the control. The reduced white blood cell count, red blood cell count and platelet count observed in the study is in agreement with the previous results of cyclophosphamide - induced immunosuppressant in balb/c mice [13]. In mammals, half the neutrophils in circulation are detectable in the blood, while the rest adhere to vessel walls as the marginating pool [13]. Thus, the increase in the neutrophils count in rats administered with the aqueous leaf extract of *Moringa oleifera* may suggest localized tissue inflammation that may be due to the action of bioactive constituents of the extract – flavonoids and vitamins A, B, C, and E that might have increased the demand for neutrophils production by the endothelial cells of the inflamed tissue.

However, this study recorded a significant increase in white blood cell count and platelet count; and also recorded significant increases in lymphocyte, monocytes and Total lymphocyte count following co – administration of graded doses of *Moringa oleifera leaves* and cyclophosphamide; and a significant increase in red blood cell count at low dose of *Moringa oleifera leaves* co – administered with cyclophosphamide compared with cyclophosphamide induced toxicity group. The trend observed in this study is consistent with the previous report of protective effect of extracts on cyclophosphamide – induced immunosuppression [16]. Although IL – 2 assay was not done in this study, its physiologic role has been implicated in haematopoiesis; hence, several studies reported that IL-2, which plays a major role in reported that IL – 2, which plays a major role in maturation / development of lymphocytes and monocytes and stimulates natural killer (NK) cell of secretion of interferon – gamma (IFNy) [17]; which in turn stimulates macrophage activation and t – cell differentiation is impacted by cyclophosphamide treatment [18]. Similiarly, granulocyte / megakaryocyte colony stimulating factor (GM –CSF), a haematopoietic growth pivotal for the regulation of bone marrow progenitor cells proliferation was also shown to be affected by cyclophosphamide treatment [18]. Other studies have shown that flavonoids / phenolics can stimulate leukocyte proliferation and increase the activity of macrophages and helper T – cells.

Flavonoids in combination with chemoprotective agents have been proven to increase the effectiveness of cyclophosphamide as well as to reduce the toxicity of the drug to normal cells [19]. The significant increase in lymphocytes suggests presence of lymphocytosis in the treated rats. This may be as a result of immune response of the rats to the administration extract, which led to the mobilization of immune competent cells. Also, increase in lymphocyte might be indicative that the plants leaves enhanced the animal's ability to wade off infection and this may account for the plants' antimicrobial activity [17]. The reportedly increase in the platelet count may suggest coagulating potential of the extract. This is because the primary function of platelets is to detect damaged blood vessel endothelium, which accumulates at the site of injury and then cause blood clotting to close the wound. Also, they are part of adaptive immune and innate system, and play a role in the initiation of inflammation by interacting with leukocytes, and are further involved in atherosclerosis and tumor growth. Thus this reason may be the root cause of the increased level of platelets [20].

This increase in the platelets count also suggests that there was no anaemic capability of the extract as suggested by the results of the RBCs count (Table 2). Thus, Anaemia has been reported in cases of reduced number of platelets (Topley, 1998).

Therefore, the results strongly suggest that *Moringa oleifera* leaves have potential effect as a naturally derived immunostimulant for the immunosuppression induced by cyclophosphamide. Combined administration of *Moringa oleifera* leave extract and cyclophosphamide modulate cellular immunity and could be beneficial in intracellular bacterial and viral infections.

5. CONCLUSION

The results here suggest that *Moringa oleifera* leave could be used to increase white blood cell count and total leukocyte counts as well as possible proliferation and differentiation of bone marrow cells following exposure to cyclophosphamide. Similarly, reversals of effects of cyclophosphamide on the host total lymphocyte, white blood cell and neutrophil count indicate the ability of the extract to help reverse the immunotoxicity induced by cyclophosphamide. Further studies using isolated compounds of the extract are recommended to identify the active agent with its exact mechanism of action.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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