

REVERSIBILITY CAPACITY OF AQUEOUS EXTRACT OF *MORINGA OLEIFERA* LEAF ON CYCLOPHOSPHAMIDE INDUCED TOXICITY IN MALE WISTAR RATS

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 **in this study.**

Method: Twenty five (25) wistar rats were used, which were divided into 5 groups of 5 rats each. Group I served as standard control and received water and normal feed. Group II served as negative control and received 30mg/kg cyclophosphamide (a potent immune suppressant) only. Groups III-V served as test groups and were administered with 125mg/kg 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, Neutrophil 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, non significant 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.

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 *Moringaceae* 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, "*Moringaceae*" ^[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 of 10–25 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.

MATERIALS AND METHODS

Plant collection and preparation of aqueous leaf extract of *Moringa oleifera*

Fresh matured leaves of *Moringa oleifera* were collected before sun rise in June at Rumuji area, Emohua L.G.A, Rivers State. 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 750g of the leaves was obtained. The dried leaves were suspended in distilled water at room temperature.

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

90

91 **Experimental Animals**

92 Twenty five (25) male wistar rats weighing (120-200g) were used for this study. The animals were
93 purchased from animal farm of University of Nigeria Nsukka, Enugu State, and kept at the animal
94 house, Department of Human Physiology, College of Health Sciences, University of Port Harcourt,
95 Rivers State, Nigeria. The animals were housed in a spacious and well ventilated cage with suitable
96 temperature, relative humidity under 12 hour house light and dark cycle at ambient temperature of 27
97 $\pm 1^\circ$ for acclimatization for two (2) weeks. They were allowed free access to feed diet (Top feeds,
98 Broiler grower-product of Eastern Premier feed mill Ltd) and water *ad libitum* but starved for 12 hours
99 prior to commencing of the experiment.

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

104

105 **Experimental Design**

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

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110 Group I: Served as standard control, and received water and normal feed only

111 Group II: Served as **negative control**, and received 30mg/kg cyclophosphamide for the last 
112 4 days (24 – 28th days).

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114 Group III: Received 125mg/kg aqueous extract of *Moringa oleifera* + 30mg/kg of
115 cyclophosphamide

116 Group IV: Received 250mg/kg aqueous extract of *Moringa oleifera* + 30mg/kg of
117 cyclophosphamide

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119 Group V: Received 500mg/kg aqueous extract of *Moringa oleifera* + 30mg/kg of
120 cyclophosphamide

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122 The different animal groups received 1ml of the respective doses of the extract using an oral gavage
123 once daily (9am-10am), for a period of 28 days. The doses of the extract chosen were based on the
124 results of acute toxicity (LD₅₀) study ^[12], which was obtained for aqueous leaf extract of *Moringa*
125 *oleifera* as 1g/kg.

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

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134 Statistical Analysis

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

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142 Results

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

			144
Groups	Weight before administration (g)	Weight after administration (g)	145
Control	120.0 $\pm$ 3.16	156.00 $\pm$ 5.01	146
CPM (30mg/kg)	122.50 $\pm$ 2.50	140.00 $\pm$ 4.08*	147
125mg/kg extract	121.25 $\pm$ 3.15	153.75 $\pm$ 3.75	148
+ CPM			149
250mg/kg extract	132.50 $\pm$ 7.50	155.00 $\pm$ 6.46	150
+ CPM			151
500mg/kg extract	130.0 $\pm$ 5.77	190.00 $\pm$ 5.77**	152
+ CPM			153
			154

155 All values are presented in mean $\pm$ SEM; n=5; *Values are statistically significant when compared to the control
156 at $p \leq 0.05$. **Values are statistically significant compared to Cyclophosphamide (Immune Suppressant) at $p \leq$
157 0.05.

158 N/B: CPM: cyclophosphamide

159 Animals' body weight before and after administration of *Moringa oleifera* leaf extract and
160 cyclophosphamide is as shown in table 1. There was a significant ($p < 0.05$) reduction in body weight of
161 animals administered with cyclophosphamide when compared with control group. A significant
162 ($p < 0.05$) increase in the animals' body weight was recorded when compared with the
163 cyclophosphamide – induced group.

164

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

Groups	WBC ($\times 10^9/L$)	RBC ($\times 10^{12}/L$)	PLATELETS ($\times 10^9/L$)
Control	20.28 $\pm$ 1.19	9.38 $\pm$ 0.18	285.00 $\pm$ 47.59
CPM (30mg/kg)	5.23 $\pm$ 1.26*	4.03 $\pm$ 0.62*	182.25 $\pm$ 49.90*
125mg/kg extract + CPM	19.13 $\pm$ 0.30**	9.34 $\pm$ 0.12**	259.75 $\pm$ 35.22**
250mg/kg extract +	19.88 $\pm$ 2.95**	5.68 $\pm$ 1.49	268.25 $\pm$ 33.75**

CPM			
500mg/kg extract + CPM	20.33 ± 1.48**	7.03 ± 1.30	277.33 ± 35.71**

168 All values are presented in mean ± SEM; n=5; *Values are statistically significant when compared to the control
 169 at p ≤ 0.05. **Values are statistically significant compared to Cyclophosphamide (Immune Suppressant) at p ≤
 170 0.05.

171 N/B: CPM: cyclophosphamide

172 Table 2 depicts mean changes in total wbc, rbc and platelets following administration of *Moringa*
 173 *oleifera* leaf extract at different concentrations and Cyclophosphamide (Immune Suppressant).
 174 It is reported that cyclophosphamide induces a decrease in white blood cells and lymphocyte in mice
 175 [13]. In this albino rat model there was also significant (p<0.05) reduction in total white blood cell count,
 176 red blood cell count and platelet count was observed following cyclophosphamide administration when
 177 compared to the control. Interestingly, co – administration of graded doses of *Moringa oleifera* leaves
 178 and cyclophosphamide (125mg/kg extract + CTX, 250mg/kg bw extract + CTX and 500mg/kg bw
 179 extract + CTX) produced a dose dependent significant (p<0.05) increase was recorded in the total white
 180 blood cell count, and platelet count compared with the cyclophosphamide administered group.
 181 The lower dose of the extract and simultaneous administration of cyclophosphamide (CTX) recorded a
 182 significant (p<0.05) increase in red blood cell count compared with cyclophosphamide administered
 183 group.

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197 Table 3: Percentage (%) changes in Differential WBC following administration of *Moringa oleifera*
 198 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**
250	69.50 ± 8.23	18.75 ± 0.75**	4.25 ± 0.85	2.75 ± 0.85	0.50 ± 0.29	1377.75 ± 45.05**

mg/kg + CPM						
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**

199 All values are presented in mean ± SEM; n=5; *Values are statistically significant when compared to the control
200 at $p \leq 0.05$. **Values are statistically significant compared to Cyclophosphamide (Immune Suppressant) at $p \leq$
201 0.05.

202 N/B: CTX: cyclophosphamide

203 The percentage (%) changes in white blood cell differentials following administration of *Moringa*
204 *oleifera* leaf extract at different concentration and cyclophosphamide are as shown in table 3. A
205 significant ($p < 0.05$) reduction in lymphocyte count, monocyte count and total lymphocyte count was
206 seen following cyclophosphamide induction when compared with control. A dose dependent significant
207 ($p < 0.05$) increase was observed in the total lymphocyte count following the administration of
208 125mg/kg bw extract + CPM, 250mg/kg bw extract + CPM and 500mg/kg bw extract + CPM when
209 compared with the cyclophosphamide administered group. A significant ($p < 0.05$) increase in the
210 lymphocyte count was observed following administration of 250mg/kg extract + CPM and 500mg/kg
211 bw extract + CPM when compared with the cyclophosphamide administered group.
212 A significant ($p < 0.05$) increase in monocyte count was seen following administration 500mg/kg bw
213 extract + CPM as compared with cyclophosphamide administered group.

215 DISCUSSION

216 The present study evaluated the reversibility capacity of aqueous extract of *Moringa oleifera* leaf on
217 cyclophosphamide induced toxicity in male wistar rats. The results of the present investigation revealed
218 that treatment with *Moringa oleifera* leaves significantly protected the animals from the toxic effects of
219 cyclophosphamide. *Moringa oleifera* leaves are used as vegetables in Nigeria and Sub-Saharan Africa. 
220 Due to the deleterious effect of cyclophosphamide in the human body, especially the immune system,
221 there is an increasing interest in the development of preventive therapy for reducing cyclophosphamide
222 toxicity in humans. *Moringa oleifera* is a safe natural antioxidant containing vegetable and is found as a
223 potential source of four natural antioxidants such as total phenolics antioxidant, vitamin A, C and E ^[14].
224 It is also known to contain proteins, traces of carotenoids, saponins, alkaloids and phenolic constituents
225 ^[10].

226 Changes in the body weight after extract administration have been used as valuable and growth index
227 of *M. oleifera* leaf ^[9]; and thus, have been justified in this study that the reported changes in the
228 animal's body weight following administration of cyclophosphamide were considered to be related to
229 both growth and valuable index, since the decrease were dose dependant and significant ($p < 0.05$). This
230 indicates that the leaf extract of *Moringa oleifera* has growth potentials and agrees with the study
231 conducted by Nwanjo ^[15]; which confirms the growth potentials of some medicinal plants. We observed
232 that white blood cell count, red blood cell count and platelet count were significantly reduced in the rats
233 treated with cyclophosphamide compared with the control. The reduced white blood cell count, red
234 blood cell count and platelet count observed in the study is in agreement with the previous results of
235 cyclophosphamide - induced immunosuppressant in balb/c mice ^[13]. In mammals, half the neutrophils
236 in circulation are detectable in the blood, while the rest adhere to vessel walls as the marginating pool
237 ^[13]. Thus, the increase in the neutrophils count in rats administered with the aqueous leaf extract of

238 *Moringa oleifera* may suggest localized tissue inflammation that might have increased the demand for
239 neutrophils production by the endothelial cells of the inflamed tissue.

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

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

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

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

275 CONCLUSION

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

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