Title: Amelioration of Cyclophosphamide-Induced Immunosuppression after Treatment with Polyherbal Formulation Extract

Running Title: Effect of PHF on CP-induced immunosuppression

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Abstract
The study evaluated the therapeutic potential of aqueous extract of polyherbal formulation (PHF) in cyclophosphamide (CP)-induced immunosuppression in rats. A total of 20 Wistar albino rats, 5 per group, were used. The animal was grouped into four: Normal control received 10 ml/kg BW of normal saline orally for 28 days. Negative control received 10 mg/kg BW of CP subcutaneously for three days. Treatment control received 1500 mg/kg BW of PHF orally for 28 days. The experiment group received 10 mg/kg BW of CP subcutaneously for three days, followed by immediate oral administration of 1500 mg/kg BW of PHF for 28 days. The rats were primed and challenged with 0.1 ml normal saline containing 100 μg/kg BW of KLH for delayed-type hypersensitivity (DTH) response. DTH response was assessed via paw edema using a vernier caliper. Keyhole limpet Hemocyanin (KLH)-IgG Antibody level and serum cytokines (IFN-γ, IL-4, and IL-10) were measured using sandwich enzyme-linked immunosorbent assay (ELISA). Histology of lymphoid organs was examined and reported. There is a significant difference between experimental and control groups in DTH (p<0.001), and the experimental group shows an increase in KLH-IgG antibodies compared to normal control groups. In experimental group, serum IFN-γ (p = 0.002) and IL-4 (p = 0.001) significantly increase after PHF-treatment compared to CP-treatment. Histology for normal control and experimental group shows a similar pattern. The PHF has demonstrated promising therapeutic ability in cyclophosphamide-induced immunosuppression in Wistar albino rats.

Keywords: Cyclophosphamide, immunosuppression, lymphoid tissue, polyherbal formulation.
1. **Introduction**

Herbal medicine, sometimes called traditional or natural medicine, existed in different cultures and civilizations and implied the utilization of various plant parts for medicinal purposes [1]. Some scientists argued that herbal formulation is safer than conventional medicines and can cure diseases [2]. Herbal medicines can stimulate both specific and non-specific immunity and modulate cytokines and chemokines expression [3].

Polyherbal formulations (PHF), commonly employed in herbal medicine [4], are a collection of plant therapeutic entities prepared and formulated to achieve synergy in efficacy and minimizing side effects [5]. Natural products provide unlimited opportunities to develop new drugs. The discovery and isolation of more specific immunomodulatory agents from plant origin possesses the potential to counteract the side effects and high cost of synthetic compounds.

There is growing interest among biomedical scientists in some natural products’ ability to stimulate immune cells’ production in immunosuppressed animal models. Several sources, including mushrooms, are being screened for immunomodulatory compounds that can be used to enhance cancer chemotherapy [6]. The serum concentration of various cytokines may give information on the presence or predictive value of inflammatory processes in various disease conditions. Optimal immunological functions need a balance between immunostimulation and immunosuppression [7]. Researchers view immunomodulation as the primary target for the treatment and prevention of several diseases [8].

Cyclophosphamide (CP) is a nitrogen mustard, an alkylating agent that suppresses bone marrow through DNA alkylation [9]. Animal studies had revealed lymphopenia and depletion of macrophages after the administration of CP [10], also increased DTH reaction and B cell depletion in lymphoid tissue when CP was given before immunization [11]. CP could suppress regulatory T
cells [12] and natural killer cells [13]. It can also raise the number of myeloid-derived suppressor cells (MDSCs) [12].

Our current study explored the therapeutic potentials of the aqueous extract of PHF in CP-induced immunosuppression in Wistar albino rats using immunomodulatory indices of total leukocytes count, DTH response, T- cells dependent antibody response (TDAR) against KLH antigen, Interferon-γ (IFN-γ), Interleukin-4 (IL-4), and IL-10 concentrations as well as histopathological studies of lymphoid organs and cytology of bone marrow to provide an insight into the effect of a high dose of the PHF in immunosuppressed condition. A previous study on this PHF demonstrated its potential immunostimulatory activity on neutrophils function, TDAR [14], and immunomodulatory activity on IL-12 [15].

2. Materials and Method

2.1 Plants Collection and Identification
The plant materials were collected from Al-Mustakshif Medical Health Centre, Kano (RC: 1393615). The formulation consists of five plant materials, namely: Black cutch- Bark (Acacia polyacantha wild), orchid bush-Bark (Bauhinia rufescens lam), Gum Arabic tree-Bark (Acacia senegal), Baobab-leaves (Adansonia digitata), Garlic-Yellow-bulb (Allium sativum); each of the five plants was identified and assigned specimen voucher number (UDUH/ANS/0198, UDUH/ANS/0197, UDUH/ANS/0195, UDUH/ANS/0194, UDUH/ANS/0196) respectively, by the Taxonomist.

2.2 Polyherbal Preparation and Extraction
Each of the plant materials was separately washed under running tap water to remove dust and other pollutants, shade-dried and grounded into a coarse powder, and passed through a 40-mesh size to obtain a uniform size particle. The polyherbal preparation was made based on the herbalist's
instruction: a weighed quantity of the powder containing 20g of *Acacia polyacantha wild*, 10g each of *Bauhinia Rufescens*, *Acacia Senegal*, *Adansonia digitata*, and *Allium sativum* dissolved into 1 litre of distilled water. The preparation was left to soak for 24 hours in a water bath set at 40°C. The preparation was filtered using Whatman No. 1 filter paper. The resultant filtrate was concentrated to dryness at 40°C under reduced pressure [16]; about 0.1 g of the dried extract dissolved in 1 ml of distilled water, which served as stock. The stock was prepared in batches.

### 2.3 Experimental Animals

A total of 20 Wistar albino rats of 10-12 weeks old, weighed between 145-155 g of either sex (8 females, 12 males), were purchased from the Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The Wistar rats were housed in the animal house, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. The animal house has a normal ambient temperature and lighting period of about 12 hours daily; they were allowed to acclimatize for two weeks before the commencement of the experiment; they were fed with standard pelletized growers’ feed (Vital feed, Jos, Nigeria) and water *ad libitum* throughout the experiment. The rats were handled according to the Principles, Guidelines, and Methods of use of laboratory animals. The Animal Ethics Committee of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, approved our study (approval number PTAC/PF/AE/OT/11-19).

### 2.4 Animal treatment

The animals were randomly divided into four groups of five (5) rats each. Normal control (C) received 10 ml/kg BW of normal saline for 28 days orally; Negative control (NC) had 10 mg/kg BW of CP (purchased from Beijing Solar Bio Science and Technology Co., Ltd, China) for three days subcutaneously; Treatment control (TC) was given oral 1500 mg/kg BW of PHF for 28 days;
Experiment group (E) received 10 mg/kg BW of CP subcutaneously for three days, followed by oral administration of 1500 mg/kg BW of PHF for 28 days.

2.5 Inducement of Immunosuppression
Immunosuppression to animals in NC and the experiment group was done by administering 10 mg/kg BW of CP subcutaneously for three days. After the last dose, complete clinical signs of immunosuppression were observed. For us to ascertain immunosuppression, total leukocyte count was taken three times - before administration of CP (pre-treatment sample), immediately after the appearance of the clinical signs (i.e., the third day at NC and experiment group), and after all the treatments (post-treatment sample) using Genesis HA 6000 automated hematology analyzer (Perlong Medical Equipment, China).

2.6 Sample Collection and Processing
On the first day of the experiment, before any intervention, 1 ml of the blood sample was collected from each rat via cardiac puncture under mild chloroform anesthesia and divided into two (0.5 ml for total leukocyte count and the second 0.5 ml for cytokines analysis). All the animals were euthanized at the end of the experiment (after 28 days); the Liver and some lymphoid tissues (Bone marrow, Lymph node, Thymus, and Spleen) were then harvested and processed appropriately.

2.7 DTH Response
The rats were primed by injecting 0.1 ml of a suspension containing 100 μg/kg KLH (purchased from Beijing Solar Bio Science and Technology Co., Ltd, China) subcutaneously into the right hind footpad and an equal volume of 0.1 ml normal saline on the left hind footpad on the 21st day. The rats’ DTH response was determined by measuring the footpad thickness of both right and left hind footpad after 24, 48, and 72 hours using RD DC 706 digital vernier calipers (Raider Pro professional Tools Co. Ltd., China). The difference in the thickness of the right hind footpad and
the left hind footpad was used as a DTH reaction measure and was expressed as a mean increment in thickness [17].

2.8 T-cell dependent antibody response against KLH antigen

Each rat was immunized intramuscularly with 100 μg/kg BW of KLH contained in 0.1 ml normal saline on the 19th day; a second dose of KLH as above was given to elicit a secondary antibody response, and samples for IgG-KLH were collected on the 29th day [18] and determined using sandwich enzyme-linked immunosorbent assay (ELISA) purchased from Nanjing Pars Biochem Co., Ltd, China. According to the manufacturer’s instructions, the procedure was carried out with targeted assay sensitivity of 1.0 ng/L.

2.9 Serum cytokines concentration

Blood samples for serum cytokine analysis were collected at three different intervals in the experiment group only (i.e., Pre-treatment, CP-treatment, and PHF treatment). The serum concentration of cytokines (IFN-γ, IL-4, and IL-10) was determined using a sandwich ELISA purchased from Beijing Solar Bio Science and Technology Co., Ltd, China. The procedure was performed according to the manufacturer’s instructions, the assay sensitivity for IFN-γ, IL-4, and IL-10 was 19 pg/ml, 7 pg/ml, and 15 pg/ml, respectively.

2.10 Histopathological examination of lymphoid organs

The histopathological examination of the excised liver, lymph node, spleen, and thymus from the rats was processed and subsequently stained using Hematoxylin and Eosin (H and E); the bone marrow was processed and stained using Giemsa stain [19]. The processed sectioned slides and the stained bone marrow smear were examined microscopically by a Veterinary Histopathologist and presented in the results.
2.11 Statistical Analysis
The results obtained were entered into SPSS version 21 for analysis. Continuous variables were expressed as mean and standard deviation (SD). A test for normality was carried out to ascertain the normal distribution of the variables. Data were normally distributed based on tests of normality results Shapiro-Wilk, supported by the Q-Q plot. One way between-groups of analysis of variance (ANOVA) was to explore differences in variables across the groups for DTH and TDAR against KLH-IgG. In contrast, one-way repeated measures ANOVA was used to explore differences in samples collected at three different serum cytokines treatments. A Bonferroni was used as a post hoc test to compare between the groups or treatments. The p-value ≤ 0.05 was used to determine the level of statistical significance.

3. Results
3.1 The clinical signs of immunosuppression
On the second day of CP administration, mild clinical signs of immunosuppression manifested on the rats, with escalating signs from the third day of administration in the negative control and experiment group. The physical signs observed were weakness, weight loss, spontaneous loss of hair, and diarrhea.

3.2 Total Leukocyte count
The mean and standard deviation (SD) of leukocytes count for NC before CP treatment was 13.29 ×10⁹ cells /L (1.84), which changed to 3.17 ×10⁹ cells /L (0.32) after CP- administration and was 3.04 ×10⁹ cells /L (0.33) at the end of the experiment. The mean (SD) of leukocytes count of experiment group before, after treatment with 10 mg of CP and post-treatment was 13.06 ×10⁹ cells /L (2.21), 3.91 ×10⁹ cells /L (1.68), and 19.34 ×10⁹ cells /L (0.48), respectively as shown in Table 1.
### Table 1: Total Leukocytes Count Before and After Treatment with 10 mg of CP for 3 Days

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-treatment Mean count (SD)</th>
<th>CP-treatment Mean count (SD)</th>
<th>Post-treatment Mean count (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>13.29 (1.84)</td>
<td>3.17 (0.32)</td>
<td>3.04 (0.33)</td>
</tr>
<tr>
<td>E</td>
<td>13.06 (2.21)</td>
<td>3.91 (1.68)</td>
<td>19.34 (0.48)</td>
</tr>
</tbody>
</table>

CP = Cyclophosphamide, PHF = Polyherbal formulation, NC= Negative control, E=Experiment

### 3.3 DTH Response

The results of DTH response across the groups at 24 hours, 48 hours, and 72 hours showed a statistically significant difference ($p < 0.001$), as depicted in Table 2; Post-hoc group comparison using Bonferroni tests indicated that after 24 hours, there was a significant difference between C vs. E, NC vs. E and C vs. NC ($p<0.001$). However, there was no statistically significant difference between TC vs. E ($p > 0.05$). After 48 hours, the difference between C vs. E, NC vs. E ($p < 0.001$), as well as C vs. NC (p=0.004), was statistically significant. However, there was no statistically significant difference between TC vs. E ($p > 0.05$). After 72 hours, there was still a significant difference between C vs. E ($p=0.01$), NC vs. E (p=0.002), and C vs. NC (p=0.03). However, no statistical difference was observed between TC vs. E.

### Table 2: Effect of aqueous extract of PHF on DTH response measured after 24, 48, and 72 hours

<table>
<thead>
<tr>
<th>Group (N=5 per group)</th>
<th>Dose (kg BW)</th>
<th>DTH response (mm) Mean Difference (SD)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Normal saline 10 ml</td>
<td>0.43(0.13)</td>
<td>0.40(0.0)</td>
<td>0.50(0.04)</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>CP 10 mg</td>
<td>0.17(0.01)***</td>
<td>0.27(0.01)***</td>
<td>0.15(0.05)***</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>PHF 1500 mg</td>
<td>0.88(0.05)***</td>
<td>1.71(0.15)***</td>
<td>1.94(0.64)***</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CP 10 mg + PHF 1500 mg</td>
<td>0.88(0.03)***</td>
<td>1.73(0.20)***</td>
<td>1.99(0.60)***</td>
<td></td>
</tr>
</tbody>
</table>

$p$-value <0.0001 <0.0001 <0.0001

CP = Cyclophosphamide, PHF = Polyherbal formulation, a= C vs E, b= NC vs E, c= C vs NC, *p<0.04, **p<0.005 ***p<0.0001, ANOVA, C= Normal control, NC= Negative control, TC= Treatment control, E=Experiment
3.4 T-cell dependent antibody response against KLH antigen

As depicted in Table 3, there was a statistically significant difference in mean serum concentration of KLH-IgG response across the groups (p < 0.0001). When compared, there was a statistically significant difference between C vs. E (p < 0.0001) and the NC vs. E group (p < 0.0001). However, there was no statistically significant difference between TC and E (p > 0.05).

Table 3: Effect of aqueous extract of PHF on serum concentration of KLH-IgG

<table>
<thead>
<tr>
<th>Group (N=5 per group)</th>
<th>Dose (kg BW)</th>
<th>KLH-IgG (ng/L)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean (SD)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Normal saline 10 ml</td>
<td>125.5 (1.98)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NC</td>
<td>CP 10 mg</td>
<td>74.93 (1.09)</td>
<td>b*** c**</td>
</tr>
<tr>
<td>TC</td>
<td>PHF 1500 mg</td>
<td>187.7 (2.88)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>CP 10 mg + PHF 1500 mg</td>
<td>192.2 (5.27)</td>
<td>a***</td>
</tr>
</tbody>
</table>

a= C vs E, b= NC vs E, c=C vs NC, **p<0.001 ***p<0.0001, ANOVA; C= Normal control, NC= Negative control, TC= Treatment control, E=experiment

3.5 Serum cytokines concentrations

A one-way repeated measure ANOVA was conducted to compare the mean serum concentration of IFN-γ, IL-4 and IL-10 in experiment group at Pre-treatment (A), CP-treatment (B), and PHF-treatment (C). As depicted in Table 4. There was a significant effect for PHF treatment (C), IFN-γ (Wilks’ Lambda = 0.001, F = 768.41, p = 0.026) and IL-4 (Wilks’ Lambda = 0.001, F = 718.24, p = 0.026). While for IL-10, there was no significant effect for CP-treatment (B), nor for PHF-treatment (C) (Wilks’ Lambda = 0.333, F = 4.0, p = 0.184).

When the mean serum concentration of IFN-γ were compared using Bonferroni tests it shows significant difference between Pre-treatment sample vs. CP-treatment (p = 0.005), CP-treatment sample vs PHF treatment sample (p = 0.002) and Pre-treatment sample vs. PHF-treatment sample (p = 0.006). When mean serum concentration of IL-4 was compared it indicate significant different between Pre-treatment sample vs CP-treatment (p = 0.033), CP-treatment sample vs. PHF treatment sample (p = 0.001) and Pre-treatment sample vs. PHF treatment sample (p = 0.003).
Table 4: Comparison of mean serum concentration of cytokines in the experiment group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (SD)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treatment (A)</td>
<td></td>
<td>19.99 (0.58)</td>
<td>129.06 (0.98)</td>
<td>1009.0 (1.12)</td>
</tr>
<tr>
<td>CP-treatment (B)</td>
<td></td>
<td>10.50 (0.12)</td>
<td>119.56 (4.61)</td>
<td>1019.6 (2.88)</td>
</tr>
<tr>
<td>PHF-treatment (C)</td>
<td></td>
<td>39.88 (1.38)</td>
<td>269.63 (3.56)</td>
<td>1086.6 (7.60)</td>
</tr>
<tr>
<td>Wilks' Lambda value</td>
<td>0.001</td>
<td>0.001</td>
<td>0.333</td>
<td></td>
</tr>
<tr>
<td>p-value</td>
<td>0.026</td>
<td>0.026</td>
<td>0.184</td>
<td></td>
</tr>
</tbody>
</table>

a= A vs B, b= B vs C, c= A vs C, *p<0.05, **p<0.007, Repeated measure ANOVA

3.6 Histopathological Features of Lymphoid Organs

Histology of the liver showed the distribution and orientation of hepatocytes and the central vein.

The histology of Bone marrow displayed the distribution and proliferation of immune cells (Figure 1). The histology of the thymus exhibit the distribution of follicles with the orientation of germinal centers in the cortex. Histology of the spleen demonstrated the cellular orientation of red and white pulps. The histology of lymph nodes depicts the distribution of lymphatic follicles and germinal centers’ orientation in the cortex with medullar (Figure 2).
Figure 1: Photomicrograph of the Liver. (A) showing normally distributed hepatocytes (Black arrow), the normal orientation of sinusoid (Green arrow) with normal clear CV. (B) showing highly disorientation of hepatocytes (black arrow), highly congested sinusoid with a high level of necrotic cells (Red circle area) with cellular infiltrated CV. (C) showing normally distributed hepatocytes (Black arrow), the normal orientation of sinusoid (Green arrow) with normal clear CV. (D) showing normally distributed hepatocytes (Black arrow), the normal orientation of sinusoid with normal clear CV. A= Normal control, B= CP treatment, C= PHF treatment, D= CP + PHF treatment, CV= Central vein. Photomicrograph of Bone marrow. (A) Showing normal distribution of different defensive cells small lymphocytes (Black arrow), large lymphocytes (Green arrow), and neutrophils (Yellow arrow) with normal clumps of platelet cells (red circled area). (B) Showing scanty distribution of different defensive cells, few lymphocytes (Red arrow), Fat cells (Green arrow), monocytes (Yellow arrow), minimal clumps of platelets cells (red circled area) with few aggregates of undifferentiated basophils and eosinophils (Black arrow). (C) Showing normal distribution of different defensive cells, small and large lymphocytes (Red arrow), Fat cells (Green arrow), neutrophils (Black arrow), severe distribution of platelets (red circled area) with much aggregate of undifferentiated basophils, eosinophils, and monocytes. (D) Showing normal distribution of different defensive cells, small and large lymphocytes (Red arrow), neutrophils (Black arrow), mild distribution of platelets (Green arrow) with undifferentiated basophils, eosinophils, monocytes, and fat cells. A= Normal control, B= Negative control, C= Treatment control, D= Experiment group. First-row Liver (H & E x 250), Second raw Bone Marrow (Giemsa x 450).

Figure 2: Photomicrograph of Thymus. (1) Showing normal distribution of follicles (A), the normal orientation of germinal centers in the cortex (black arrow) with normal clear connective tissue fibers (green arrow). (2) Showing normal distribution of follicles (A), the normal orientation of germinal centers in the cortex (black arrow) with severe proliferative connective tissue fibers (green arrow) with severe cellular infiltrative cells (C), and zone of severe necrotic cells (red circled area). (3) Showing normal distribution of follicles (A), the normal orientation of germinal centers in the cortex (black arrow) with moderate proliferative connective tissue fibers (green arrow) with moderate cellular necrotic cells (red circled area) zone of synergistic follicular communication (red rectangular area). (4) Showing normal distribution of follicles (A), the normal orientation of germinal centers in the cortex (black arrow) with severe proliferative connective tissue fibers (green arrow), and moderate cellular infiltrative cells (C). Photomicrograph of Spleen tissue. (1) Showing normal cellular orientation with two resting zones Red pulp (B) and white Pulp (A). (2) Showing normal cellular orientation with two demarcated zones, Red pulp (B) and white Pulp (A),
the zone of necrotic cells (Red circle zone), and moderate toxic degenerative changes (cracks). (3) Showing clear cellular disorientation with no zonal demarcation between Red pulp and white Pulp (A), proliferative mitotic cells (Black arrow), numerous disorganized connective tissue (Green arrow), prominent zones of regenerative defensive cells (Red circle zone), and minimal toxic degenerative changes (cracks). (4) Showing normal cellular orientation with two demarcated zones Red pulp (B) and white Pulp (A), numerous disorganized connective tissue (Green arrow), prominent zones of regenerative proliferative mitotic defensive cells (Red circle zone) with no distinct, prominent trabeculae arrangement, and mild toxic degenerative changes (cracks). 

Photomicrograph of Lymph node.  
(1) Showing normal distribution of lymphatic follicles (A), the normal orientation of germinal centers in the cortex (Red circle area) with normal clear medullar (B). (2) Showing normal distribution of lymphatic follicles (A), the normal orientation of germinal centers in the cortex (Red circle area) highly filled with lymphocyte (green arrow) with severe degenerative medullar (B). (3) Showing scattered distribution of lymphatic follicles (A), the diffused orientation of germinal centers in the cortex is surrounded with lymphocytes (B), and the medullar’s normal appearance. (4) Showing scattered distribution of lymphatic follicles (A), diffused orientation of germinal centers in the cortex (B) surrounded with lymphocytes (green arrow), and normal appearance of the medullar. 1= Normal control, 2= Negative control, 3= Treatment control, 4= Experiment group.  First raw Thymus, Second raw Spleen, Third raw Lymph node (H & E x 250).

4. Discussion

Cyclophosphamide is a common cytotoxic drug that can inhibit immune function by causing atrophy and weight loss of the immune organs and disturbing peripheral blood leukocytes composition [20]. The CP target is rapidly dividing cells, including cancerous cells, normal immune cells, and other cells in the body [21].

This study revealed a moderate increase in DTH response among the experiment group from 24 hours up to 72 hours, thereby suggesting the potential role of the PHF in the gradual improvement in the DTH response. Interestingly, a comparison between the experiment group and that of the treatment control group on the DTH response showed indifference; this justifies the formulation’s efficacious role in ameliorating and rehabilitating the immunosuppressed condition of the rats. Indeed, some formulations exhibit a significant increase in cell-mediated immune response [22]. A study reported an increase in paw size due to boosting the immune system after treatment with Methanolic leaf extract of *Moringa oleifera* [23]; several other studies [24-26] reported a significant increase in DTH response.
The increase in DTH response by the PHF may be associated with the formulation’s ability to activate lymphocytes and other cells associated with a cell-mediated immune response such as macrophages. Since activation of T cells can lead to the release of lymphokines, which causes the activation and accumulation of macrophages, increases vascular permeability, induces vasodilatation, and produces inflammation [23].

The current study findings revealed an increase in serum concentration of KLH-IgG among the experimental group compared with the normal control group; this signifies the formulation’s potential role to improve or restore the humoral immune response against the KLH antigen. Comparing the experimental group and treatment control further suggests the formulation’s therapeutic potential to ameliorate CP’s effect on the humoral immune response. This study finding is in line with Allam and colleagues, which reported that ellagic acid tends to increase IgM and IgG-mediated humoral immune response among the murine model of *Schistosoma mansoni* infection [24]. Study mice express higher IgG1, IgG2b, and IgG3 against hen egg-white lysozyme (HEL) when fed with glycoprotein extract of *Atractylodes macrocephala* Koidz, *Helianthus annuus* L., *Scutellaria barbata* D, and *Hedyotis diffusa* [27].

Moreover, Ogawa and coworkers reported decreased serum anti-KLH IgM and IgG concentrations among CP treated rats [28]. Plants constituents such as alkaloids increase immune-bioactivities, thus enhance the circulating antibody [29], flavonoids increase the production of immunoglobulin [30], vitamins, glycosides, and terpenoids also increase antibody production [31].

Significant differences were observed when Pre-treatment, CP-treatment, and Post-treatment samples were compared on serum concentrations of IFN-γ and IL-4 in the experiment group; this difference indicated further the potential role of the PHF in neutralizing the effect of CP. The increase in cytokine concentration at the post-treatment level signifies the formulation’s
effectiveness on immune cells responsible for the secretion of IFN-γ and IL-4. However, the PHF has no significant effect on the serum concentration of IL-10, suggesting that the formulation does not have therapeutic activity on the cells responsible for the cytokine’s secretion.

In comparison with other studies, the findings of this study agreed with Jin and coworkers [32], which reported that aqueous extract of hi-ka-ron, a polyherbal formulation induces an increase in IFN-γ production. Furthermore, this study's findings align with Shin and colleagues that reported that a polyherbal formulation called PG 201 increases IL-4 production [33]. However, contrary to these study findings, Wang and coworkers reported that aqueous extract of Shen-fu-tang, a polyherbal formulation decreases IFN-γ production [34]. Kurokawa et al. also reported contrary to these findings [35].

Interferon-γ is regarded as a proinflammatory cytokine associated with Th1 cells stimulating macrophage function and cytotoxic T-cell function [36]. IFN-γ is one of the most vital cytokines that induced immune shift towards Th1 [37] and play a crucial role in clearing pathogens and preventing allergic inflammation [38]. IL-4 is associated with Th2, which is known to stimulate a humoral immune response [39]; it is critical for initiating humoral immunity against extracellular pathogens. It has been suggested that specialized T cells that express NK cell markers and secrete large amounts of IL-4 immediately after T cell receptor stimulation produce the IL-4 that promotes Th2 cell differentiation [40].

The photomicrograph results revealed amelioration and restoration of pathological changes of the immunosuppression in the histology for lymphoid organs of the study animals (Figures 1 and 2). Indeed, indifferences between the normal control and experiment and treatment control groups suggest that the PHF extract possess potential therapeutic activity against CP-induced histopathological alterations. The potentiated activity of the PHF extracts may be due to many
bioactive compounds present in the formulation (16). Studies indicated that some plant constituents, such as saponins, could neutralize the myelosuppression induced by CP in mice [10, 41].

Duggina et al. (2015) reported that *Centella triterpene* saponin extract significantly reversed CP-induced histopathological alterations in Spleen. Bergenin was reported to confer a protective role on Spleen and Thymus, where it maintains a healthy balance for both cell-mediated immunity (Th1/Th2) and humoral immune response against CP-induced immunosuppression [42]. A study showed that herbal formulation could reverse the immunosuppressive effect in lymph nodes and other organs in dexamethasone-induced immunosuppressed animals [43].

We have previously reported the presence of secondary metabolites including alkaloids, terpenoids, steroids, cardiac glycosides, flavonoids, saponins, tannins, polyphenols, carbohydrates, phytosterols, proteins, and vitamins in the aqueous extract of this polyherbal formulation [15] and some of the above metabolites could have contributed in the observed biological activity. Further study will be required to provide information on the specific cells responsible for the cytokines and immunoglobulins’ secretion.

5. **Conclusion**

The PHF has effectively improved and restored the DTH response, T-cell dependent antibody response as well as increase in serum concentration of IFN-γ and IL-4 among the CP-induced immunosuppressed rats. The typical histological appearance and cellular orientation of lymphoid organs among the CP-induced immunosuppressed rats compared to the experimental group depicted the effectiveness of PHF on the histology and cytology of the lymphoid organs. The polyherbal formulation has potential therapeutic potential against CP-induced immunosuppression; this could improve immune responses, especially in immunosuppressed conditions due to aging or immune deficiency.
Conflict of interest

The authors declared that there is no conflict of interest.

Funding

This paper is not funded.
References


