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Research paper

3,4 Dihydroxycinnamic acid stimulates immune system function by modifying the humoral antibody response – An *in vivo* study

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ABSTRACT

The immunomodulatory property of 3,4-Dihydroxy-cinnamic acid (DCA) was studied under the normal and cyclophosphamide induced immunosuppressive conditions in animal models. The immunomodulatory activity was evaluated by studying the haematological parameters, humoral antibody, based on the plaque forming cell count, the bone marrow cellularity, the α-esterase's producing active cells, delayed type hypersensitive assay, phagocytic index assays. The chemopreventive effect of DCA was determined by cyclophosphamide induced immunosuppression in animals. From the response, DCA exhibits significant immunomodulatory activity by enhancing the humoral immune response. The histopathological investigation revealed the protective effect of DCA against cyclophosphamide induced immunosuppression. From the result it is observed that the DCA boosts up the immune system activity in a positive manner

1. Introduction

The body's immune system is involved in the physiological as well as pathophysiologic mechanism of many infections [1]. The immune response is triggered and facilitated by innate and adaptive immune systems [2]. Modulation of the immune system is a complex system tangled in the pathophysiological and etiologic of various infections by restraining the immune system. The two key mechanisms involved are immunostimulation and immunosuppression. Immunomodulatory agents can be used as stimulators to immune system to reduce the side effects of drug prompted immunosuppression [3] and can also be used as immunosuppressors under seditious circumstances [4]. The immunomodulators standardizes the immune cells function either by uplifting or conquering the immune reactions to maintain homeostasis in the body system [5].

Cancer leads to death in many developing nations. The majority of drawback due to cancer treatments lead to suppression of the immune [6]. The side effects of cancer treatment includes many such as nausea, ulceration, alopecia, cardiac and hepatic dysfunctions. One of the commonly used chemotherapeutic drug is cyclophosphamide (CP). The CP administration usually leads to immunosuppression and myelosuppression, it usually interferes with the proliferation of healthy immune cells [7].

Plants and plant products tend to boost the immune system and are used to treat various diseases from times immemorial [8]. Besides the plants as a direct source, recent research is focused on the plant derived compounds. The contribution of plants and plant derived products as an immunomodulatory agent has significant effect in cancer treatment [9]. Along with chemotherapeutic drugs natural immunomodulators are given to counteract the state of impaired immunity. It modulates the host defensive mechanism and activates it to maintain equilibrium and boosts immune system [10,11]. The hydroxycinnamic acids are the largest group of phenolic compounds, mentioned by caffeic, p-coumaric and ferulic acids [12,13]. 3,4-Dihydroxy-cinnamic acid (Caffeic acid) is the main hydroxycinnamic acid found in fruits (such as apples, berries, cherries, kiwis and plums), vegetables (potato and artichoke) and herbs (*Ilex paraguariensis* and *Achyrocline satureioides*) [14–16]. DCA and its derivatives are reported for its rich *in vitro* antioxidant and anticancer properties [17].

In order to overcome the drawbacks occurred due to cancer therapy there is always a need for substances that will boost the immune system. In this study, the main objective is focussed on determining the immunomodulatory properties of DCA under normal and CP induced conditions.

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2. Methodology

2.1. Chemicals

3,4-Dihydroxy-cinnamic acid and Cyclophosphamide was obtained from Sigma Aldrich, India. All chemicals and solvents used for the study were purchased from Hi-media, India and are of analytical grade.

2.2. Experimental animals

Male Swiss albino mice weighing from 20–25 g were procured from the College of Veterinary Science, Mannuthy (Kerala, India). The animals were given with good feed and water *ad libitum* and accustomed to usual standard laboratory conditions of 12 h light/-dark cycle and temperature 25 ± 2 °C. The investigational procedures were followed as per the guidelines and approved by the Animal Ethical Committee. (Reference No. IAEC/KU/BT/15/11).

2.3. Immunomodulatory activity

2.3.1. Preparation of Antigen for immunization

The sheep blood was collected freshly from the native authorized slaughter house and mixed with Alsever's solution. It was then washed with 0.9% normal saline twice and accustomed to 0.5×10^3 cells/ml concentration [18] for immunization studies.

2.3.2. Experimental grouping of animals

Four different groups of swiss albino mice each containing 6 animals were used for the study. The grouping of animals are mentioned below:

Group I: Control which received normal saline (15 ml/kg p.o.),
Group II: Negative control received CP (50 mg/kg p.o.)
Group III: Received DCA at the concentration of 50 mg/kg p.o.
Group IV: Received DCA at the concentration of 100 mg/kg p.o.

All the four group of animals received treatment for 7 consecutive days and on the 7th day they were immunized with 100 μL of freshly prepared SRBC containing 0.5×10^9 cells [19].

2.3.3. The effect of DCA on humoral antibody titre

After the treatment period, the blood was collected in tubes and allowed to clot. The twofold of serum samples were mixed with 0.025 mL of normal saline and 1% SRBC was added. The serially diluted plates were mixed well and incubated at room temperature for 1 h. The reciprocal of the maximum concentration showing agglutination was considered [20].

2.3.4. Plaque forming cells assay

The spleen was collected aseptically on the seventh day after giving local anaesthesia. It was splashed thoroughly with RPMI medium and the cells were deferred at 1×10^6 cells/ml concentration in the same. 100 μL of the spleen cells and 50 μL of 5% SRBC were added in 0.5% agarose. It was then poured onto the glass petri plate and layered with 1.2% agarose and incubated for 2 h at 37 °C at 5% CO_2. After incubation, fresh rabbit serum was added and the plate was incubated for 20 min. The plaques formed were counted and expressed as 10^6 spleen cells [21,19].

2.3.5. Delayed type hypersensitivity (DTH) assay

The animals were primarily immunized after treatment period and the foot paw thickness was measured. The animals were injected with 20 μl of 0.5×10^9 SRBC cells in the right hind foot paw of the mice. Later to 24 h the foot thickness was measured DTH activity was expressed based on the observation [22].

2.3.6. Phagocytic index assay

The phagocytic index was studied by carbon clearance assay. After the treatment period Indian ink (0.1 ml/10 g) was given to the animals intravenously via the tail vein and blood was collected at 2 and 10 min. To 0.1% Na_2CO_3 solution the blood sample was added for erythrocyte lysis and later the absorbance were measured at 675 nm. The lymphoidal organs of the animals were weighed and the rate of carbon clearance and phagocytic index were calculated [23].

2.3.7. Bone marrow cellularity and α-esterase activity

The bone marrow cells were collected from the femur and made into single cell suspension after 24 h of the drug treatment. The number of cells were determined using haemocytometer. Bone marrow cells were smeared on a slide glass and stained with Harri's Hematoxylin to determine the non-specific α-esterase activity by the azodye coupling method [24].

2.4. Immunosuppression due to cyclophosphamide (CP) experimental design

Male swiss albino mice were randomly divided into four groups (n = 6).

Group I: Control received 0.9% saline (25 ml/ kg, orally).

Group II: Received 0.9% saline (25 ml/kg, orally.) and was induced with CP on last 3 days of the experiment.
Groups III: Received DCA at dose of 50 mg/kg and was induced with CP on last 3 days of the experiment.
Groups IV: Received DCA at dose of 100 mg/kg and was induced with CP on last 3 days of the experiment.

After the treatment period animals were sacrificed on the 14th day and the blood was collected for haematological analysis and the spleen and thymus were collected for histopathological studies [25].

2.4.1. Spleen and thymus indices and histopathological analysis

The animals were weighed after study period and sacrificed as per ethical guidelines. The spleen and thymus were weighted to calculate the spleen and thymus index [26]. The histopathological analysis for spleen and thymus performed with hematoxylin and eosin stain procedures.

2.4.2. Haematological analysis

To study haematological parameters like leukocytes (WBC), erythrocyte count (RBC), haemoglobin and platelet the blood sample was collected by cardiac puncture and analysed in auto-analyser.

2.5. Statistical analysis

The statistical analysis for the obtained data was analysed using SPSS software. The results were expressed as Mean ± S.D. The groups with significant difference of P value < 0.05 were considered significant.

3. Results

3.1. Antibody titre assay

The DCA treated groups showed a significant increased activity of circulating antibody titre in a dose dependent way. The dose of 100 mg/kg b.w amplified the concentration of antibody titre as compared with the control group, whereas the cyclophosphamide treated groups significantly decreased the antibody titre (Fig. 1).

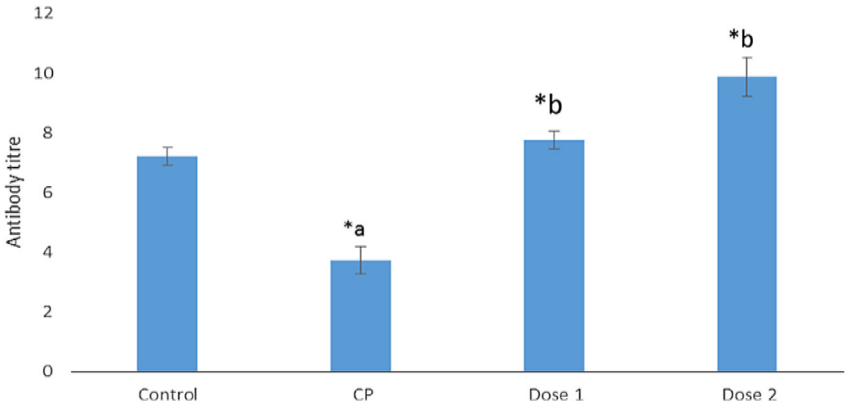

Fig. 1. Effect of 2,3 Dihydroxycinnamic acid (DCA) and cyclophosphamide (CP) on humoral antibody titer in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

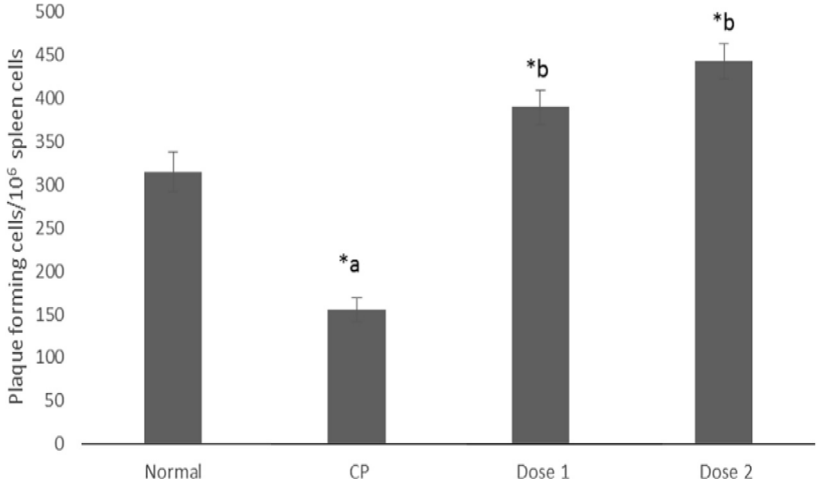

Fig. 2. Effect of 2,3 Dihydroxycinnamic acid (DCA) and cyclophosphamide (CP) on the plague forming cells in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

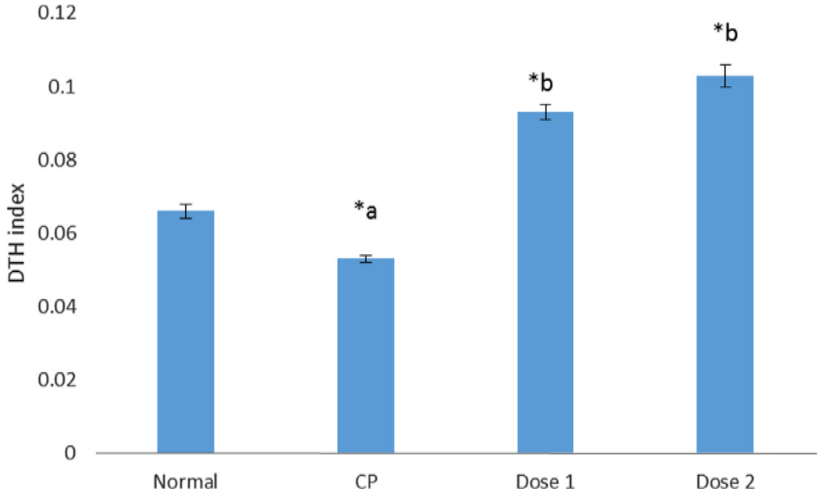

Fig. 3. Effect of 2,3 Dihydroxycinnamic acid (DCA) and cyclophosphamide (CP) on delayed type hypersensitivity in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

3.2. Plague forming assay

The DCA treated groups produced increasing antibody and it was measured based on the number of plaques formed. The maximum number of antibody producing cells were observed in the group treated with 100 mg/kg b.w (Fig. 2).

3.3. Delayed type hypersensitivity assay

The observation in DCA treated groups showed that the foot paw edema formation was observed in the mice as compared with the control group in a significant manner. The foot paw thickness was measured and reported after 24 h of treatment with SRBC.

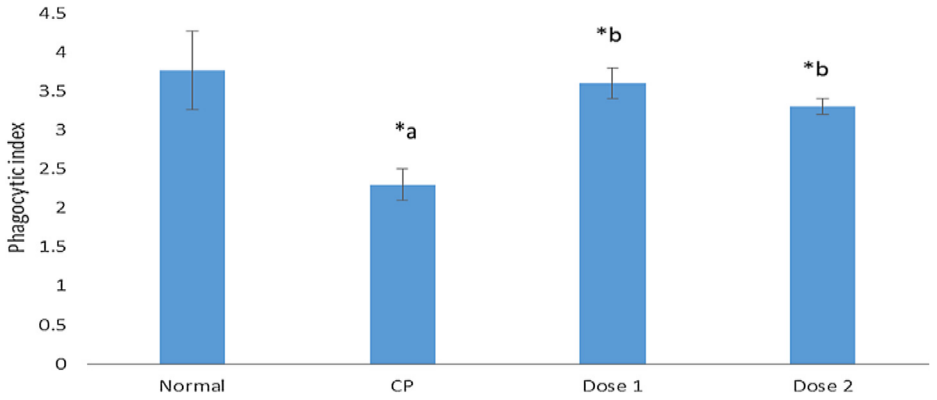

Fig. 4. Effect of 2,3 Dihydroxycinnamic acid (DCA) and cyclophosphamide (CP) on the phagocytic index in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

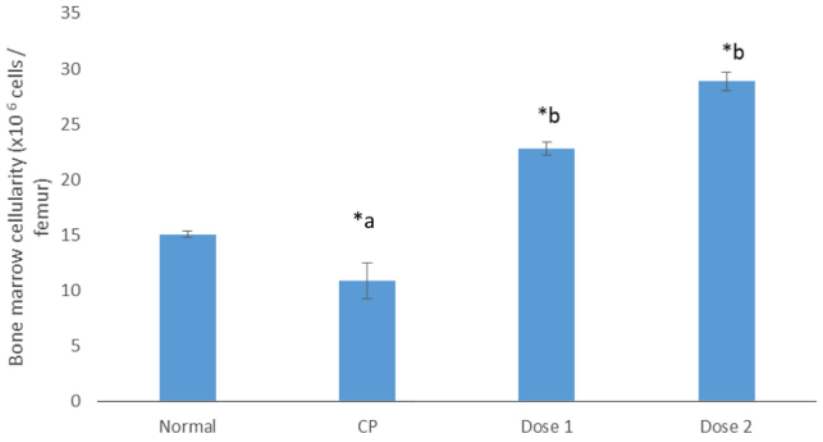

Fig. 5a. Effect of 2,3 Dihydroxycinnamic acid (DCA) and cyclophosphamide (CP) on the bone marrow cellularity in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

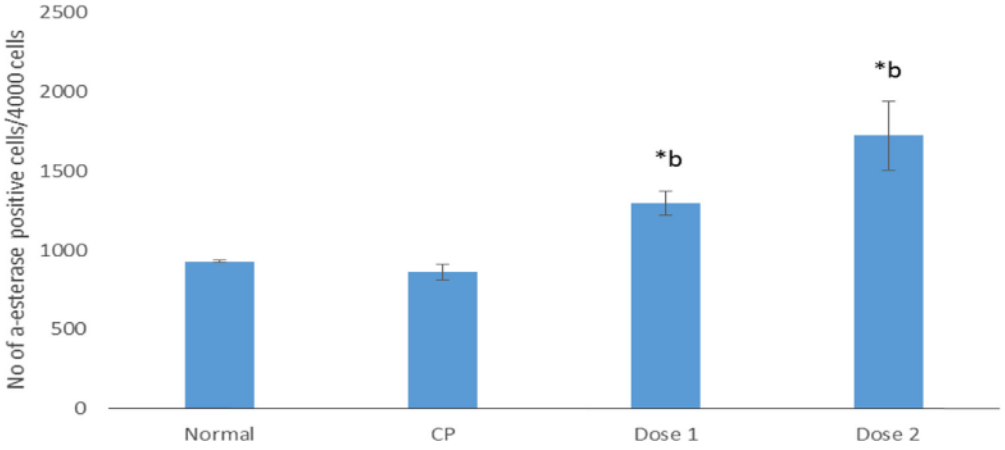

Fig. 5b. Effect of 2,3 Dihydroxycinnamic acid (DCA) and cyclophosphamide (CP) on the α-esterase producing cells in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

The CP treated groups also showed a reduction in the paw edema as compared to control groups (Fig. 3).

3.4. Phagocytic index

The phagocytic function of macrophages is determined by this method. The phagocytic index was retained in the DCA treated groups as similar to the control. Whereas the macrophages activity in the induced group is very less. Phagocytic index was observed to be more in the DCA treated groups (Fig. 4).

Table 1
The effect of DCA and CP on the spleen indices and thymus indices in experimental animals. Values are the mean ± SD for six animals in each group (N = 6).

	Spleen indices (%)	Thymus indices (%)
Control	3.25 ± 0.03	4.72 ± 0.05
CP	1.77 ± 0.06*[a]	3.34 ± 0.02
Dose 1	2.37 ± 0.1	3.8 ± 0.65
Dose 2	3.63 ± 0.06*[b]	5.24 ± 0.2*[b]

Values are statistically significant at *P < 0.05; statistical significance was compared within the groups as follows.
[a] CP induced mice were compared with normal mice.
[b] 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

3.5. The cellularity in bone marrow and α-esterase activity

The result of DCA treatment on the bone marrow cellularity and α-esterase positive cells are given in Figs. 5a and b. The DCA showed to significantly increase the bone marrow cellularity as compared with the CP treated groups. The α-esterase positive cells were also found to significantly increase as compared with the other groups.

3.6. Cyclophosphamide induced immunosuppression

The spleen and thymus weight of the DCA treated groups and the cyclophosphamide treated groups were measured. The weight of organs in cyclophosphamide treated groups were observed to be reduced as compared with the normal group animals. The treatment with DCA tend to increase the lymphoidal (spleen and thymus) indices in a dose dependent way (Table 1) as related with other groups. This shows the protective effect of DCA against the myelosuppression due to CP. The histopathology of spleen and thymus tissues revealed that the morphology was altered in the cyclophosphamide treated group. The treated groups showed a quiet improvement in the tissue morphology, indicating the protective effect of DCA against tissue damage (Figs. 6 and 7).

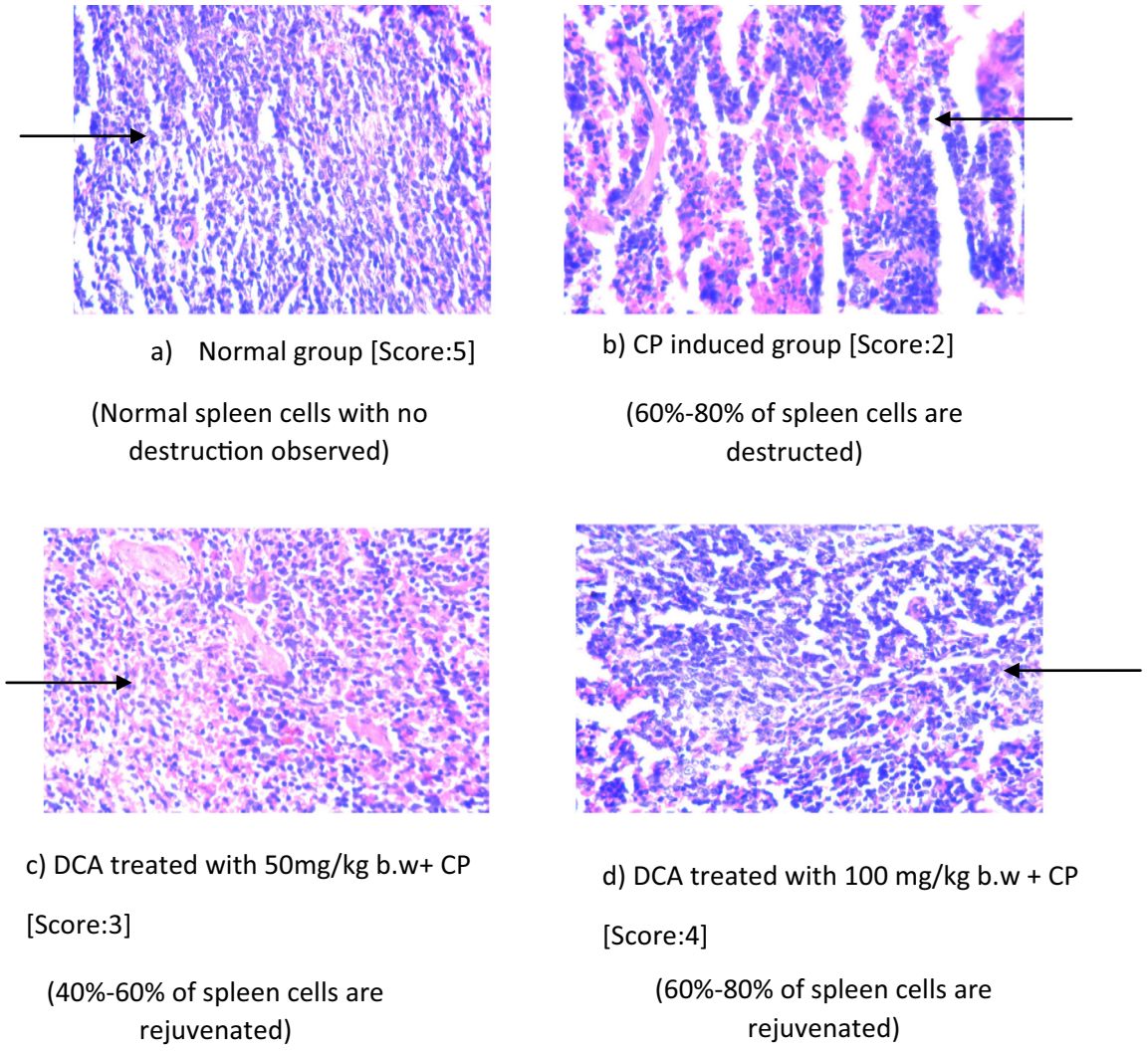

Fig. 6. Histopathological section of spleen showing the protective effect of DCA on normal and CP induced tissues section observed under light microscope with 40x magnification. Scoring rate: 0%-20% (1); 20%-40% (2); 40%-60% (3); 60%-80% (4); 80%-100% (5)

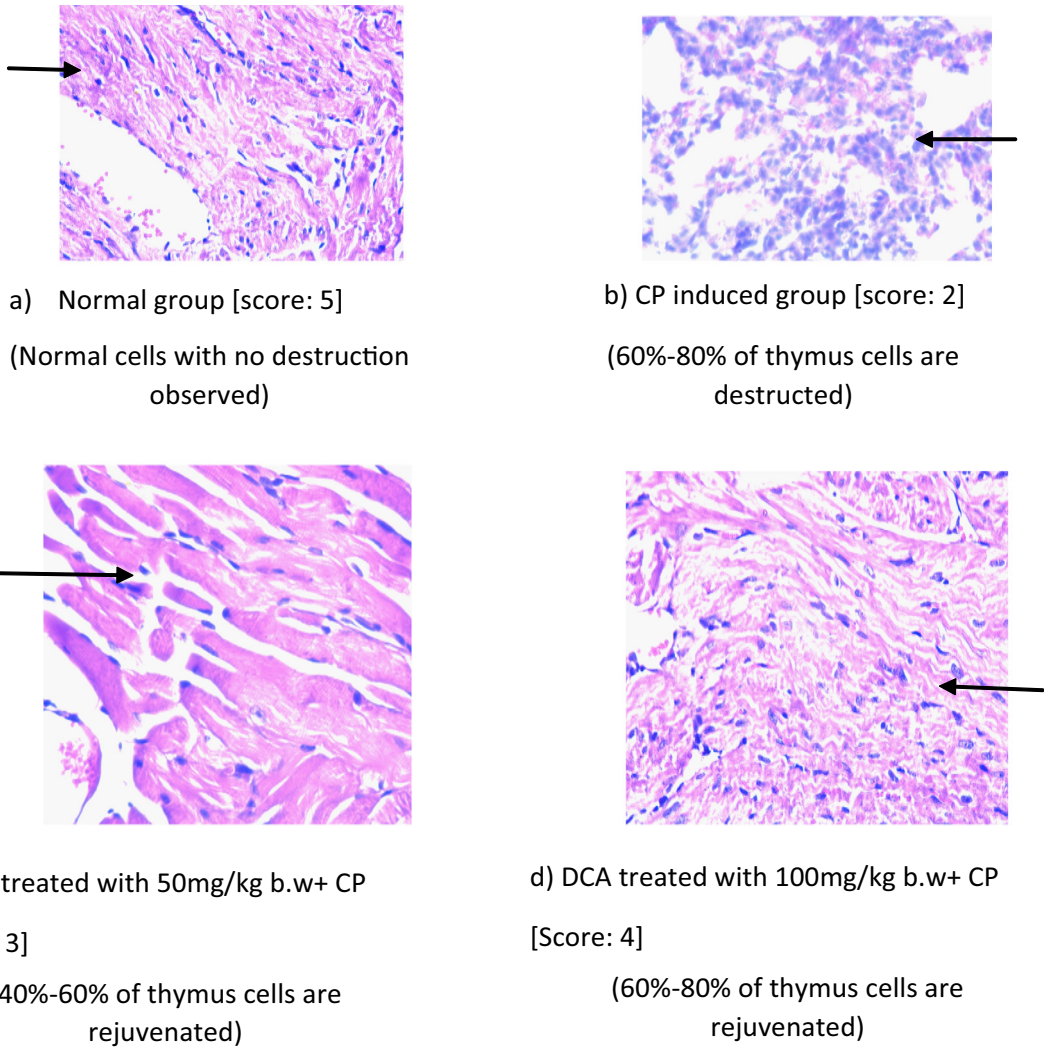

Fig. 7. Histopathological section of thymus showing the protective effect of DCA on normal and CP induced tissues section observed under light microscope with 40x magnification. Scoring rate: 0%-20% (1); 20%-40% (2); 40%-60% (3); 60%-80% (4); 80%-100% (5)

3.7. Haematological analysis

A significant variation was observes with various treated group of animals. The RBC, WBC, Haemoglobin, platelets was found to be increased in the group of animals treated with 50 mg/kg b.w and 100 mg/kg b.w (Figs. 8a and 8b).

4. Discussion

In order to overcome the side effects occurred due to conventional therapy, plants can be used as such from the natural source. It may help to maintain the proper immune system and its functions. The compounds that activate the host defence mechanisms due to weakened immune responses can provide sympathetic therapy to conventional therapy [27].

The antibodies are secreted by the plasma cells and B-lymphocytes and they play as a key elements in humoral immune responses [28]. The hemagglutination titre was adopted to study the effect of DCA on humoral immune response. The increase in the antibody titre against SRBC among the treated groups show that DCA is capable of activating the plasma cells that will secrete the antibodies [29]. The antibodies activity was confirmed by plaque forming assay to analyse the ability of the B lymphocytes to differentiate themselves into antibody producing cells [30]. A dose dependent increase in the hemagglutination antibody titre and the number of antibody producing cells from B lymphocytes confirms that the compound DCA has the ability to play a role in humoral immunity.

The T lymphocytes and the lymphokines are associated with the immune cells responses [28]. The cell mediated immune response of DCA was studied by DTH assay. The paw edema was measured in the normal and DCA treated groups. The administration of DCA boosts the phagocytic activity and thus increases the foot pad thickness in the mice. The reduction in paw volume was also observed in the CP treated group showing its inhibitory effect on the T lymphocytes [31]. The carbon clearance test was performed to determine the effect of DCA on the reticuloendothelial cell mediated phagocytosis. The majority of phagocytic cells are the macrophages, functioning to remove the foreign antigens from entering the systemic circulation. The phagocytic index was evaluated based on the clearance of the colloidal particles from the circulatory system [32]. An increased phagocytic index was observed proving the active phagocytosis of the macrophages in the respective body function. The main role of neutrophils as stated in Badway and Karnovski (1980) [33] and many other research is that it serves as a first line defence mechanism involving phagocytosis. The increase in the α-esterase positive bone

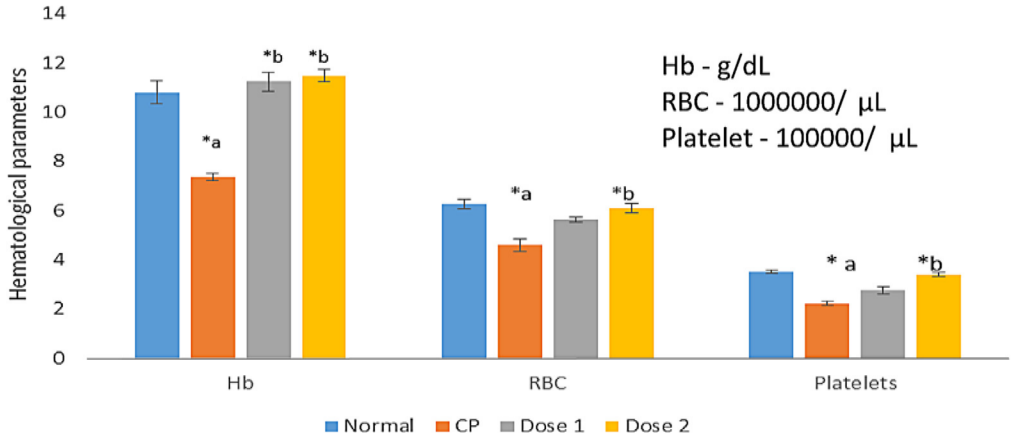

Fig. 8a. The effect of DCA and CP on the haematological parameters (Hb, RBC and Platelet) in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

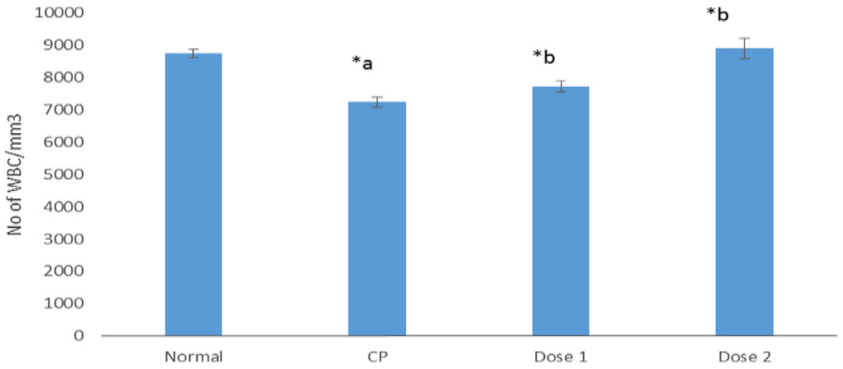

Fig. 8b. The effect of DCA and CP on the WBC count expressed per mm^3 in experimental animals. Values are the mean ± SD for six animals in each group (N = 6). Values are statistically significant at $^{*}P < 0.05$; statistical significance was compared within the groups as follows. a CP induced mice were compared with normal mice. b 50 mg/kg bw abd 100 mg/kg bw DCA treated mice were compared with CP induced mice.

marrow cells indicates that DCA could also boostup the productivity of the stem cells.

Cyclophosphamide is used in combination with chemotherapy agents for treatment of lymphomas, [34] and some solid tumours [35]. It is also reported to serve as a carcinogen [36]. The present study revealed that DCA exhibits significant immunomodulatory as well as chemo protection against CP induced mice models. The primary lymphoidal organs will be affected due to CP treatment [37]. The protective effect of DCA was highly proven by determining the organ indices and the data from histophathological observations. The DCA treated groups effectively improved the spleen and thymus indices in a dose dependent manner. The increase in mass of spleen and thymus indicate that the DCA could possibly stimulate the production of immune cells. From the histophathological observations it is noted the DCA protects the spleen cells and thymus from the damage caused due to CP administration. The function of CP is that it interferes with the DNA synthesis thus affecting the immune cells as well as the hematopoietic cells, thereby affecting the haematological parameters [38]. In this study the administration of DCA increased the RBC, WBC, Haemoglobin, Platelets count in a dose dependent manner, whereas the CP treated groups decreased the count of such Haematological parameters. This increase in the haematological parameters signifies that the DCA possess immunomodulatory properties. Thus the present study showed that the administration of the compound 3,4 Dihydroxycinnamic acid protects the damage caused to immune system due to administration of Cyclophosphamide and thereby improves the blood cell count and the antibody function.

5. Conclusion

3,4 Dihydroxy-cinnamic acid is a potent phenolic compound found usually in many berries. It serves as antioxidant rich source and has a wide range of pharmaceuticals applications. In the present study the treatment of DCA shown to serve as a good immune stimulator agent with enhanced activation of the immune cells function. The administration of drug against the CP induced immunosuppression in mice may also possess to have chemo protection activity which may require the further confirmation studies. The present investigation suggest that DCA can serve as a better immune stimulator and its parameters are discussed under normal and CP induced conditions.

Conflict of interest statement

We declare that we have no conflict of interest.

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