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Research Article

EVALUATION OF IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACTS OF *MORINGA CONCANENSIS* NIMMO IN MICE

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ABSTRACT

The present study was undertaken to evaluate immunomodulatory activity of ethanolic whole plant extract of *Moringa concanensis* and isolated compound from *Moringa concanensis*. Immunomodulatory activities were determined by *in vivo* models- delayed-type hypersensitivity (DTH), carbon clearance assay (phagocytic index) and cyclophosphamide induced immunosupression. The ethanolic extracts of *Moringa concanensis* and isolated compound, in the concentration range 5,10,25,50, and 100 µg were also tested for phagocytic effect on human neutrophils using the *in vitro* models–nitroblue tetrazolium (NBT) dye test. Mice treated with dose i.e. 170 mg/kg (Group III) and dose 1 g/kg (Group IV) increase response in foot pad edema was found to be statistically significant (*p<0.05), (**p<0.01) and showed statistically significant (p< 0.05) increase in WBC and platelets count and Phagocytosis index when compared to Cyclophosphamide (20 mg/kg) treated and control treated mice. In cyclophosphamide induced immunosupression, ethanolic roots extract *Moringa concanensis* and isolated compound showed significant (*P*<0.001) increase in total differential leucocytes count DLC and hematological parameters RBCs, Hb, Platelets and WBCs significant increases when compared with cyclophosphamide group. Ethanolic extract of *Moringa concanensis* and isolated compound in the concentration range 5, 10, 25, 50 and 100 µg, also showed significant (*P*<0.001) phagocytics effect on human neutrophils in the parameters studied. The present experimental finding demonstrated that isolated compound has superior immunomodulatory activity then ethanolic extract of *Moringa concanensis,* this effect was presumably due to greater ability of isolated compound to boost the innate and adaptive immune system.

Keywords: *Moringa concanensis* , Immunomodulatory, Ethanolic extract, Cyclophosphamide

1. INTRODUCTION

In recent years, there has been growing interest in the field of herbal medicines research and search for promising potential area of investigation of immunomodulatory agents from natural products. The immune system is designed to protect the host from invading pathogen and to eliminate disease. Plants are the essential and integral part in complementary and alternative medicine and due to this, they develop the ability for the formation of secondary, metabolites like proteins, flavonoids, alkaloids, steroids and phenolic substances which are in turn used to restore health and heal many diseases. Herbal drugs are believed to enhance the natural resistance of the body against infection and their immunomodulatory activities have been reported in numerous plants [1, 2].

Traditional and folklore medicines play an important role in health services around the globe. About three quarters of the world's population relies on plants and plant extracts for healthcare. India has an extensive forest cover, enriched with plant diversity. Several plants have been used in folklore medicine [3]. The rational design of novel drugs from traditional medicine offers new prospects in modern healthcare. Ayurveda, the traditional medicinal system in India, describes certain plants which strengthen the host immune system. *Ficus benghalensis* (Family Moraceae) is a very large tree, 20-30 meters high, with wide-spreading branches bearing aerial roots [4].

The immune system is a system of biological structures and processes within an organism that protects against disease. Disorders of the immune system can result in autoimmune diseases, inflammatory diseases, cancer and immunodeficiency [5].

Immunomodulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reaction, it is named as an immunostimulative drug

which primarily implies stimulation of non-specific system. Immunosuppressant implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors. Immunostimulation and immunosuppression both need to be considered in order to regulate the normal immunological functioning. Hence both immunostimulating agents and immunosuppressing agents have their own standing, so search for better agents exerting these activities is becoming the field of major interest all over the world. A number of Indian medicinal plants and various 'Rasayana' have been claimed to possess immunomodulatory activity [6].

M. concanensis Nimmo is a small tree indigenous to Northwest India resembling *M. oleifera* and it is abundant in Rajasthan, the dry hills of Konkan, Andhra Pradesh and is commonly found on recent alluvial land in or near the sandy beds of rivers and streams [7]. *Moringa concanensis* Nimmo is a medium sized deciduous tree with thick bark. Bark is fissured to 10cm deep, and is corky grey. The tree is hairless except younger parts and inflorescence. Leaves are bipinnate (very rarely tripinnate), 45 cm long. Primary pairs are 5-6, distant, 10-20 cm long. Leaflets are 4-6 pairs with an odd one, broadly elliptic to round, blunt at both ends, often notched at the tip, 2.5-3.8 cm long, 1.25-2.5 cm broad, pale beneath. Leaf fall is usually found in the month of January. Flowering occurs during February-April. Fruiting is from April onwards [8].

Herbal drugs are easily affordable and less potent than synthetic prescription immunomodulators and are also less likely to cause side effects. Therefore, there is a need to search for plants with immunomodulatory activity to offer a novel approach for the treatment of infectious disease.

The present work was aimed on evaluation parameters of selected herbs used in the treatment of immune disease, a major crippling disease in the world leading to huge economic losses.

2. MATERIAL AND METHOD

2.1. Plant Material

Whole plant of *Moringa concanensis* were collected from local Mandleshwar, Dist Khargone, Madhya Pradesh, India. The plant was identified and authenticated by Dr. S K. Mahajan, Ex. Professor Botany Department of Govt. P.G. College Khargone (M.P). A voucher specimen (Ref. No.: SKM/PGC/Herbarium/2017/A-1)) had been deposited at the departmental herbarium.

2.2. Preparation of Extracts

About 500g of the dried plant powder was placed in soxhlet apparatus (Perfit, India) and subjected to extraction using ethanol. Extracts was filtered and the filtrate was evaporated using vacuum evaporator (Perfit, India) under reduced pressure at $\leq 50^{o}C$ temperature. The crude extract obtained after evaporation was stored in desiccators. After extraction with solvent, remaining residue of plant was discarded and extract was weighed.

2.3. Experimental Animals

Albino mice (Swiss) of either sex were used in present study. The animals were fed with standard pellet diet, water *ad libitum* and maintained under standard environment condition employed. Animal were housed under standard conditions ($22\pm5^{o}C$ with 12 h of light/dark cycle). All experimental protocols have been approved by Institutional Animal Ethical Committee of BN College of Pharmacy, Bhupal Nobles University, Udaipur (Reg. No 870/PO/Re/S/05/CPCSEA) (IAEC Approval Number15/BNCP/IAEC/2018).

2.4. Antigen

Fresh Sheep blood was collected from local slaughterhouse in sterile Alsevar's solution (1:1 proportion). Sheep red blood cells (SRBCs) were washed three times in pyrogens free normal saline and centrifuged at 2500-3000 rpm for 10 minutes. The supernatant was removed with pasture pipette and suspended in normal saline. The concentration of 0.1 ml containing 1x $108/mm^3$ cells was adjusted by using improved Neubaur chamber for immunization and challenge.

2.5. Chemicals and reagents

Cyclophosphamide (Khandelwal Laboratories Ltd., Mumbai) and all other solvents used for experimental work were of analytical grade.

2.6. Acute toxicities

Acute oral toxicity studies of ethanolic extract of *Moringa concanensis* and isolated compound was carried out as per the OECD guidelines, draft guideline 423 adopted on 17th December, 2001, received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Govt.of India [9]. Administration of Methanolic extract of *Moringa concanensis* 50 mg/kg body weight up to the dose 1700 mg/kg body weight and administration of isolated compound 50 mg/kg body

weight up to 10 g/kg body weight, caused no considerable signs of the toxicity in the tested animals.

2.7. Experimental design

2.7.1. Delayed Type Hypersensitivity in mice [10]

Mice of either sex were divided into four, groups (n = 6), Group on 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×108 cells, i.p. Group I - Control; 1% Gum acacia suspension in saline, Group II received 30 mg/kg b.w. of standard, Cyclophosphamide, p.o. respectively (1 to 7 days), Group III received 170 mg/kg b.w. of EEMC (1-7 days), Group IV received 1 g/kg b.w. of isolated compound MC p.o. (1 to 7 days). On 7th day prior to injection, right hind footpad thickness was measured with digital vernier callipers (Mitutoyo). Then animals were challenged by injecting 1% SRBC (20 µL) into the right hind footpad. On 8th and 9th day footpad thickness was again measured. Difference between prior and post challenge footpad thickness was reported as DTH response.

2.7.2. Carbon clearance test for Phagocytic activity [11]

Mice were divided into four groups of six mice each. Control group I received vehicle only. Cyclophosphamide group II; 20 mg/kg, While animal of treatment group III EEMC; 170 mg/kg and group IV; ICMC 1g/kg were given test extract in 1 % Tween- 80 daily for 5 days , Cyclophosphamide were given to group II by i.p route at day 0 only. All the groups were given 0.1 ml of carbon ink suspension through the tail vein. After 48 hours of 5 days treatment, a Blood sample was collected from the retro- orbital plexus at 0 and 15 min immediately after the injection of carbon suspension. Blood (25µl) was lysed with 2 ml of 0.1 % sodium carbonate and the absorbance was measured spectrophotometrically at 675 nm for determination of optical densities.

The rate of carbon clearance, termed as phagocytic index (K), was calculated by using equation:

$$K = (\ln OD1 - \ln OD2) / t2 - t1$$

Where, OD1 and OD2 are the optical densities at times t1 and t2, respectively [12].

2.7.3. Cyclophosphamide induced immunosuppression in mice [13]

Mice were divided into four groups (n = 6), Group I : Control, 1% Gum acacia suspension in saline, Group II : received Cyclophosphamide (CP) 30 mg/kg, intraperitonealiy (i.p.) for 10 days, Group III Received EEMC 170 mg kg^{-1}, orally daily for 14 days and CP (30 mg kg^{-1}, i.p.) for 10 days, respectively. Group IV received ICMC 1 g kg^{-1}, orally daily for 14 days and CP (30 mg kg^{-1}, i.p.) for 10 days, respectively. On day 14, blood was collected by retro-orbital plexus under mild ether anaesthesia. DLC, Total RBCs and Hb (haemoglobin) platelets and WBCs were determined in laboratory.

2.7.4. Nitroblue Tetrazolium NBT Test [14]

2.7.4.1. Prepration of test solution

Stock solution for *in vitro* studies were prepared by dissolving ethanloic extract of *Moringa concanensis* and isolated compound in 0.5 dimethyl sulfoxide (DMSO) and with phosphate buffer salt solution according to concentration range from 5 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml.

2.7.4.2. Evaluation of nitroblue test

The leukocyte suspension (5×10^6 ml) of 0.5 ml and 0.25 ml of freshly prepared NBT (90.15%) were incubated at 37°C in an incubator for 20 min. After incubation the leukocyte suspension was centrifuged gently at 400 g for 3 minutes. The supernatant was discarded and precipitate of cells was resuspended with phosphste buffer saline (pH 7.2). The film was prepared on microscopic slide, fixed gently by heating and counter stained with dilute corbol fusion. Then slides were washed with running water and percentage of Nitro blue tetrazolium positive cell counting blue deposition were counted. This procedure was repeated for different concentration of the extracts in given table. For control 0.25 ml of leucocyte suspension and 0.2 ml of freshly prepared nitro blue tetrazolium (0.15%) were incubated

2.8. Statistical Analysis

The data expressed as mean±standard error mean (SEM). The significance of differences among the groups was assessed using one way analysis of variance (ANOVA) by prism software The test was followed by Dunnett's; *P<0.5, *P<0.01, ***P<0.001 were considerd as significance

3. RESULTS

3.1. Effect of *Moringa concanensis* ethanolic extract and isolated compound on mean foot paws oedema in DTH model and haematological parameters

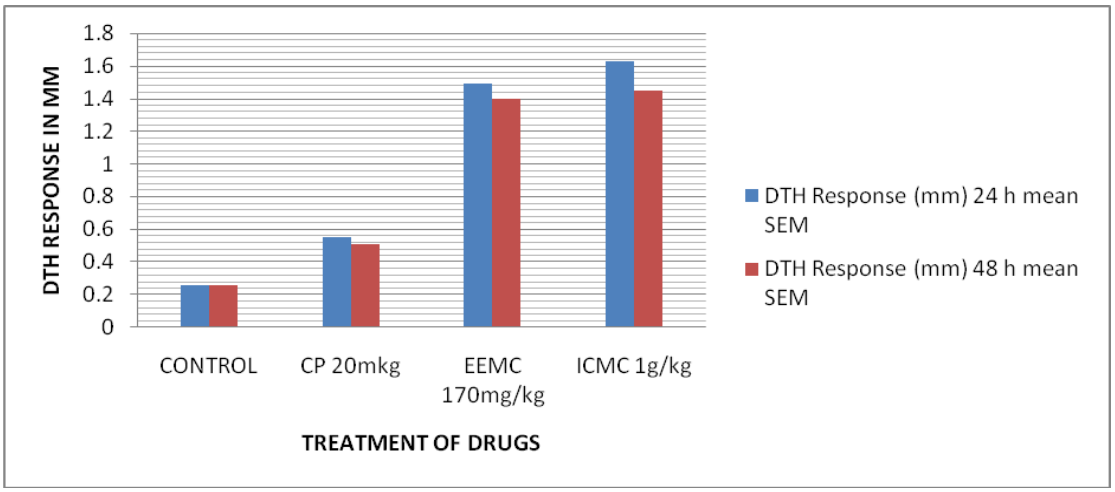
The results obtained in the DTH model are given in table 1. The immune response was determined by DTH response *i.e.* increase in foot pad thickness using vernier

caliper. The observations reported in table1, indicate that mice treated with dose *i.e.* 170 mg/kg (Group III) and dose 1 g/kg (Group IV) increase response in foot pad edema was found to be statistically significant (*p <0.05), (**p<0.01), when compared to Group I (Control) and also with Group II (Cyclophosphamide).

Table 1: Delayed type hypersensitivity of ethanolic extract of *Moringa concanensis* and isolated compound

Groups	Treatment	DTH Response (mm) 24 h mean SEM	DTH Response (mm) 48 h mean SEM
I	Control	0.2670±0.0109	0.2670 ±0.01097
II	Cyclphosphamide (30mg/kg)	0.5537±0.5537*	0.5160±0.01658
III	EEMC (170mg/kg)	1.4923±0.0088**	1.404±0.0006**
IV	ICMC (1 g/kg)	1.5354±0.0607**	1.4513±0.0117**

*Values are expressed as meanμ SEM, (n=6). All the groups were compared with control and standard groups using ANOVA followed by Dunnett's t- test. Significant values are expressed as (**p<0.01)*



*Data is expressed as Mean +SEM **P <0.01, *P <0.05 considered*

Fig. 1: Effect of *Moringa concanensis* extract on DTH Response

Table 2: Effect of *Moringa concanensis* ethanolic extract and isolated compound on WBCs and Platelet count in DTH Model

Groups	Treatment	Haematological Parameters	
		WBC count (Thousand/mm^3)	Platelet count (Thousand/mm^3)
I	Control	7.150±0.6198	448.8±75.52
II	Cyclphosphamide (30mg/kg)	6.513±0.3469	425.0±56.12
III	EEMC (170mg/kg)	8.245±0.6343*	676.7±67.20*
IV	ICMC (1 g/kg)	8.828±0.4190*	782±25.00*

Data is expressed as a Mean + SEM, n=6; using one way analysis variance (ANOVA) followed by Dunett s test. P<0.05 was considered as statistical significant, n=6 in each group

3.2. Results of *Moringa concanensis* ethanolic extract and isolated compound on Haematological Parameters

The results obtained on haematological parameters in the DTH model are given in table 2. Administration of methanolic extract of *Pavonia odorata* and isolated compound at both the levels showed statistically significant (*p<0.05) increase in RBC count and WBC count when compared to Cyclophosphamide (30 mg/kg) treated and control treated mice.

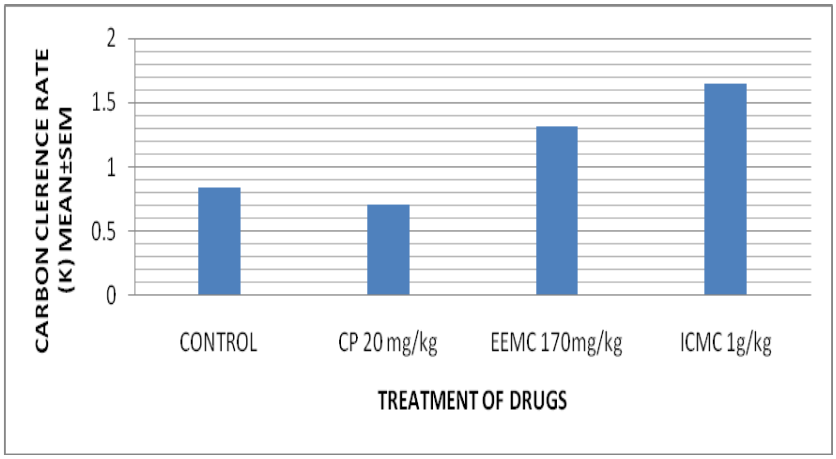
3.3. Effect of *Moringa concanensis* ethanolic extract and isolated compound on Carbon clearance test for phagocytic activity in Swiss albino mice

The results were as shown in table 3. The phagocytic activity is generally measured by the rate of removal of carbon particles from blood stream. The phagocytic index of both the levels of extract of *Moringa concanensis* and isolated compound increase (1.319 and 1.654) and was found to be statistically significant ($p<0.05$) when compared to phagocytic index (0.849) of vehicle treated Group I mice (Control) and also with Group II (Cyclophosphamide).

Table 3: Effect of *Moringa concanensis* doses on Carbon clearance test for Phagocytosis index

Groups	Treatment	Phagocytoic Index
I	Control	0.8499 ±0.007027
II	CP (30 mg/kg)	0.7128±0.024
III	EEMC (170mg/kg)	1.319±0.010**
IV	ICMC (1 g/kg)	1.654±0.013***

*Data is expressed as a Mean + SEM, n=6; using one way analysis variance (ANOVA) followed by Dunetts test. *P<0.05 and ***P<0.001 was considered as statistical significant compare with vehicle and standard*



*Data is expressed as Mean +SEM **P <0.001, *P <0.01 considered*

Fig. 2: Effect of *Moringa concanensis* ethanolic extracts on carbon clearance rate in mice

3.4. Cyclophosphamide induced immunosuppre--ssion in mice

Cyclophosphamide at the dose of 30 mg/kg. *i.p.* (Group II) caused a significant reduction in total DLC, as compared to control group (Table 4 & 5). Ethanolic extracts of *Moringa concanensis* and Isolated compound showed highly significant (P <0.001) increase, DLC and significant when compared with cyclophosphamide group (Group II) which was dose dependent. However, the increase in the hematological parameters was more with the extract EEMC and ICMC as compared to the standard group at the mentioned doses, but both dose showed similar levels of significance (Tables 4).

Table 4: Effect of ethanolic extracts of *Moringa concanensis* and isolated compound on differential leucocyte counts in cyclophosphamide-induced immunosuppression in mice

Group No	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	Basophils (%)	Monocytes (%)
Control	19.84±0.58	69.83±1.37	2.60±0.08	1.65±0.08	1.63±0.07
CP (30mg/kg)	10.94±0.77	59.17±1.17	0.65±0.07	0.34±0.04	0.31±0.16
EEMC (170mg/kg)	15.59±0.33***	64.00±0.87**	1.14±0.09***	0.41±0.04	040±0.07
ICMC (1g/kg)	16.03±0.26***	67.32±1.35***	1.51±0.06***	0.44±0.04*	0.64±0.07**

*Values are expressed as Mean±SEM. using one-way ANOVA followed by Dunnett's 't' test *P < 0.05, *** P < 0.001 considered significant compared to group II*

Table 5: Effect of ethanolic extracts of *Moringa concanensis* and isolated compound on cyclophosphamide induced myelosupprwssion in mice.

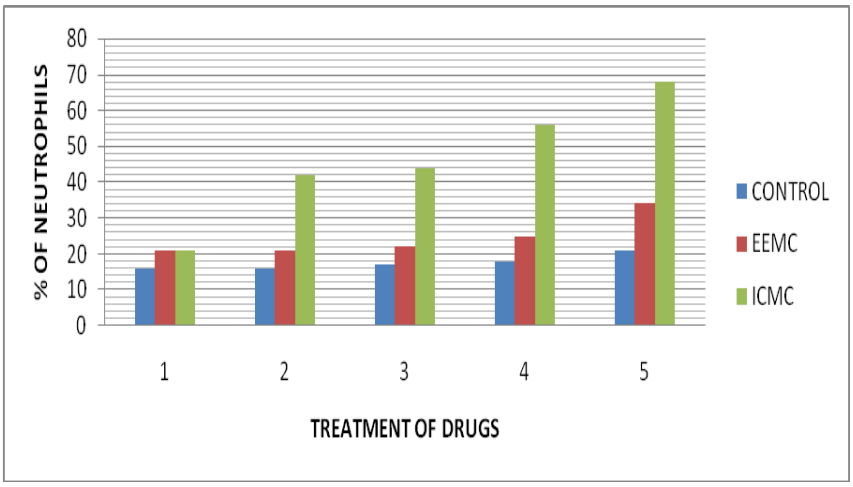
Group No	Group	RBC (106/mm^3)	Hb (g%)	Platelets (105/mm^3)	WBC (103/mm^3)
I	**Control**	6.43±0.06	9.62±0.08	6.57±0.07	4.52
II	**CP (30mg/kg)**	5.02±0.09	8.12±0.12	4.73±0.05	2.28
III	**EEMC (170 mg/kg)**	5.77±0.13*	9.13±0.06*	5.70±0.14**	3.14**
IV	**ICMC (1 g/kg)**	5.74±0.13*	9.20±0.09*	5.74±0.13**	3.67**

*Values are expressed as mean ±SEM *P < 0.01**P < 0.01as compared to Group II (Cyclophosphamide) standard.*

Table 6: Nitroblue Tetrazolium (NBT) qualitative test on ethanolic extracts of *Moringa concanensis* and isolated compound

Concentration (µg/ml)	Normal Control	EEMC	ICMC
5	16.00±0.057	21.00±0.57	21.67±0.33
10	16.00±0.057	21.00±0.57	42.00±0.57***
25	17.67±0.88	22.67±0.33	44.00±0.57***
50	18.33±0.33	25.00±0.57**	56.00±0.57***
100	21.00±0.57	34.00±15.04**	68.00±0.57***

*Values are expressed as Mean ± SEM., **P < 0.01,***P<0.001 (n=3) by ANOVA followed by Dunett t test.*



*Data is expressed as Mean +SEM ** P <0.01 considered*

Fig. 3: Effect of *Moringa concanensis* ethanolic extract and isolated compound on neutrophils

3.5.Nitroblue Tetrazolium (NBT) Dye Test

The results indicate that the ethanolic extracts of *Moringa concanensis* and isolated compound have stimulated phagocytosis of NBT dye by the neutrophils at concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, 10 µg/ml, and 5 µg/ml. The ethanolic extract of *Moringa concanensis* and isolated compound showed significant (P<0.01) activity compared to the normal control. The activity of both the extracts was comparable at all concentrations (Table 6).

4. DISCUSSION

In the present study, *Moringa concanensis* and isolated compound showed an overall stimulatory effect on the immune function in mice. Stimulatory effects were observed on both *in vivo* and *in vitro* models.

DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines [15]. These in turn increase vascular permeability, induced vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity and increased concentration of lytic enzyme for more effective killing [16-17].

The effect of ethanolic extract of *Moringa concanensis* and isolated compound on the foot pad thickness and hematological data such as WBC and Total Platelet counts of antigenically challenged mice in (tables 1 & 3 and fig. 1) showed, the (*P<0.05) significant decrease in the foot pad thickness, WBC and Total Platelet counts in cyclophosphamide group as compared to control group. While in extract-treated group animals showed (P<0.05) significant increase. General characteristics of DTH are an invasion of immune cells at site of injection and induction became apparent within 24 to 48 hrs. Increase in paw edema after 24 hrs of challenge was observed in all extract treated groups when compared to control. Increase in DTH response of animals revealed the stimulatory effect of EEMC on T lymphocytes *i.e.* cell mediated immunity or specific immunity. The result indicates that there was significant difference in the foot paw thickness at doses of 170 mg/kg b.w. (p<0.01) and 1 g/kg b.w. (p<0.01), ethanolic extract *M concanensis* and isolated compound treated group when compared against normal control and standard.

In view of the pivotal role played by the macrophages in coordinating the processing and presentation of antigen to β-cells. Phagocytic index (Table 3) were (*P<0.05 and ***P<0.001) significantly increased as compared to control and Cyclophosphamide group. Hence, increased clearance rate of carbon particles from circulation in animal reflects the enhancement of phagocytic function of mononuclear macrophage and non-specific immunity. Phagocytosis by macrophages is important against pathogenic microorganisms and its effectiveness is markedly enhanced by opsonisation of parasite with antibodies and complement C3b leading to more rapid clearance of parasite from blood. The modulation of immune response using medicinal plant products as a possible therapeutic measure has become a subject of active scientific investigations [18]. Based on results obtained, ethanolic extract of *M concanensis* and isolated compound was found to have a promising immunostimulant potential.

Immunomodulatory activity of ethanolic extract of *M. concanensis* and isolated compound was explored by evaluating their effects on cyclophosphamide induced myelosuppression (table 4 & 5) in mice at 2 dose levels of 200 mg/kg and 370 mg/kg p.o. The results revealed the dose dependent effect of the extracts in counteracting the myelosuppression induced by cyclophosphamide as indicated by increased RBC, total WBC, platelet counts, Hb%, and DLC in the extract treated groups (Group III, IV,), when compared to cyclophosphamide-treated group (Group II). The isolated compound was found to be more effective than the methanolic extract at the mentioned doses.

Immunomodulatory activity of ethanolic extract *M concanensis* and isolated compound was also evaluated on human neutrophils using the parameters, viz., NBT assy (table 6). From these parameters, the process of immunomodulation (immunostimulation) of the ethanolic extract *M concanensis* and isolated compound at various concentration range can be assessed by observing stimulation (opsonization) of neutrophils (NBT assay), Both the ethanolic extracts and isolated compound demonstrated potent *in vitro* immunomodulatoty activity with the isolated compound showing more activity than the ethanolic extracts.

5. CONCLUSION

Ethanolic extract of the of *Moringa concanensis* and isolated compound showed potential effect on haemopoetic system. Immunomodulatory potential of *M. concanensis* attributed for the presence of hexadecanoic acid may modulate and potentiate humoral as well as cellular immunity. This emphasizes the future scope of this study.

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