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IMMUNOMODULATORY ACTIVITY OF DICHCLOROMETHANE EXTRACT OF *SPINACIA OLERACIA* AND METHANOLIC EXTRACT OF *DESMODIUM TRIFLORUM*

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ABSTRACT

The dichloromethane extract of *Spinacia oleracea* leaves and methanolic extract of *Desmodium triflorum* leaves was studied for immunomodulatory activity. The immunomodulatory effect was studied in delayed type hypersensitivity response using SRBCs, phagocytic reponse using carbon clearance assay and cyclophosphamide induced myelosuppression. *Spinacia oleracea* leaves extract and *Desmodium triflorum* leaves extract showed an increase in the hypersensitivity response, produced a significant increase in the phagocytic index and protection against cyclophosphamide induced myelosuppression indicating its effect on cell mediated immunity. From the above result, it was concluded that the dichloromethane extract of *Spinacia oleracea* and methanolic extract of *Desmodium triflorum* has a significant effect on both cell mediated and humoral immunity.

KEYWORDS: analgesic and anti-inflammatory activity, anthelmintic action, anticonvulsant activity, antibacterial activity and antinociceptive activity.

INTRODUCTION

The leaves of *Spinacia oleracea* (Family: Chenopodiaceae) commonly known as Palak/Spinach and leaves of *Desmodium triflorum* (Family: Fabaceae) commonly known as Jangali methi is widely used in India for it various pharmacological effect.

Spinacia oleracea useful in diseases of blood and brain, asthma, leprosy, biliousness; causes "kapha" (Ayurveda). It has been used in the treatment of urinary calculi and have

hypoglycemic properties. Leaves are cooling, emollient, wholesome, antipyretic, diuretic, maturant, laxative, digestiblle, anthelmentic, useful in urinary concretion, inflammation of the lungs and the bowels, sore throat, pain in joints, thirst, lumbago, cold and sneezing, sore eye, ring worm scabies, leucoderma, soalding urine, arrest vomiting, biliousness, flatulence. And have been used in the treatment of febrile conditions. Seeds are useful in fevers, leucorrhoea, urinary discharges, lumbago, and diseases of the brain and of the heart. They have been used in the treatment of difficulty in breathing, inflammation of the liver and jaundice.[1,2]

Desmodium triflorum used as cooling, expectorant and galactagogue and used in vitiated condition of pitta, cough, bronchitis, wounds, abscess, sores, pruritus, dysentery, flatulence and burning sensation.[3] This plant is also used in ache (stomach), dermatosis, dysentery, abscess, diarrhea, ophthalmia, rheumatism, sore, tonic, diuretic and tumor.[4] Reported activities are antioxidant and antiproliferative activity.[5], analgesic and anti-inflammatory activity.[6], anthelmintic action.[7], anticonvulsant activity.[8], antibacterial activity.[9] and antinociceptive activity.[10]

Since these plants are widely used for treatment of various ailments, the present study was investigated its effect on cell mediated and humoral immunity in the experimental animal models.

MATERIALS AND METHODS

Plant Material: The leaves of *Spinacia oleracea* and *Desmodium triflorum* were collected from outfield medicinal garden near to Gwalior (M.P.) Plants were identified and voucher specimen deposited in Department of Pharmacognosy for future references. A voucher specimen number BU/Bot/10/05 for *Spinacia oleracea* and BU/Bot/10/06 for *Desmodium triflorum*. Fraction-4 of dichloromethane extract of *Spinacia oleracea* (F-4DESO) and Fraction-2 of methanolic extract *of Desmodium triflorum* (F-2MEDT) were used for pharmacological evaluation.

Animals: Albino wistar rats (150–200 g) either sex, were used for pharmacological evaluation. The animals were housed in standard environmental conditions of temperature (21 ± 2°C), humidity (55 ± 10%) and a 12-h light–dark cycle. Rats were supplied with standard pellet diet and water *ad libitum*. The animals were acclimatized to laboratory hygienic conditions for 10 days before starting the experiment. Animal study was performed

with the approval of the Ethical committee and CPCSEA approved animal house and Institutional Animal Ethics Committee (CPCSEA Reg. N0. 1039/PO/Re/S/07/CPCSEA).

Drugs and Chemicals: Indian ink (Camel India Pvt. Ltd.) colloidal carbon (pre-warmed at 37^0C), Cyclophosphamide (Biochem pharmaceutical, Mumbai), Levamisole of Khandelwal labs, Mumbai was used as reference standard drug. All other reagents and chemicals used were of analytical grade.

Antigen: Fresh blood was collected from sheep sacrificed in the local slaughter house in a sterile bottle containing Alsever's solotion (2%dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride). Sheep red blood cells (SRBCs) were washed three times in large volumes of pyrogen free normal saline and adjusted to a concentration of 0.1ml of 0.5×10^9 cells/ml for immunization.[11]

Acute toxicity study and Dose selection: Acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guideline No. 423. Rats were kept for overnight fasting prior to drug administration. A total of three animals were used, which received a single oral dose in 2000 mg/kg, body weight of different extracts of plant *S.oleracea* and *D.triflorum*. The treated animals were monitored for 14 days for mortality and general behavior.

Delayed-type hypersensitivity (DTH) response: To evaluate hypersensitivity response Animals were divided in to four groups of six animals. Group 1 served as control, received 10 ml/kg normal saline, Group 2 received F-4DESO (100 mg/kg/p.o.), Group 3 received F-2MEDT (100 mg/kg/p.o), Group 4 received Standard drug, Levamisole (50mg/kg/ p.o), All the animals received drugs according to corresponding groups for 7 days, on 8^{th} day rats were immunized by injecting 0.5 ml of fresh sheep red blood cell suspension (SRBCs) (10^9 cells/ml suspended in normal saline) intraperitoneally. On 11^{th} day the thickness of right hind footpad was measured using vernier caliper. The rats were then challenged by injection of 20 µl of 1% SRBCs (suspended in normal saline) in right foot pad. Foot thickness was again measured after 24 hrs and 48 hrs of this challenge. The differences obtained for pre- and post challenge foot thicknesses were taken for the measurement of DTH and were expressed in mm.[11,12]

Phagocytic response by Carbon Clearance Assay: Animals were divided in to four groups of six animals. Animals were administered drug according to their respective groups orally for 10 days. On day 11th all the animals of the entire groups received the treatment of an intravenous injection of (0.3 ml per 30 g) Indian ink (Camel India Pvt. Ltd.) colloidal carbon (pre-warmed at 37^{0}C) via the tail vein. The blood samples (50µl) were collected from each animal by retro-orbital plexus at an interval of 2 and 10 min after the injection of ink dispersion. Blood samples were added to 4 ml of 0.1% sodium carbonate solution to lysed the erythrocytes. Absorbance of the samples was measured at 660 nm using spectrophotometer. The Phagocytic index (K) was calculated by using following formula:

$$\text{Rate of carbon clearance (K)} = \frac{\log OD2 - \log OD10}{T2 - T1}$$

Where OD2 is the log absorbance of blood at 2min; OD10 is log absorbance of blood at 10 min; *T*2 is the last time point of blood collection; *T*1 is the first time point of blood collection.[13]

Cyclophosphamide-induced myelosupression: Animals were divided in to five groups of six animals in each. Group 1 (control group) received the vehicle, group 2 (cyclophosphamide group) received 1% CMC, 0.25 ml each orally, group 3 received F-4DESO (100 mg/kg/p.o.), group 4 received F-2MEDT (100 mg/kg/p.o.) and group 5 received Levamisole (50 mg/kg/p.o.) for a period of 13 days. The animals of groups 2-5 were injected with cyclophosphamide (30 mg/kg/i.p.) on the 11th, 12th and 13th day, 1 hr after the administration of the respective treatment. On day 14th, blood samples were collected from the retro-orbital plexus of individual animals and analyzed for haematological parameters.[14]

RESULT AND DISCUSSION

Acute toxicity study and Dose selection: For toxicity studies the extracts were given in dose of 2000 mg/kg body weight. On 1000 mg/kg body weight dose, all the extracts didn't show any toxic symptoms. Hence 1/10th portion of 1000 mg/kg i.e. 100mg/kg was selected for further activity.

Delayed-type hypersensitivity (DTH) response by SRBC

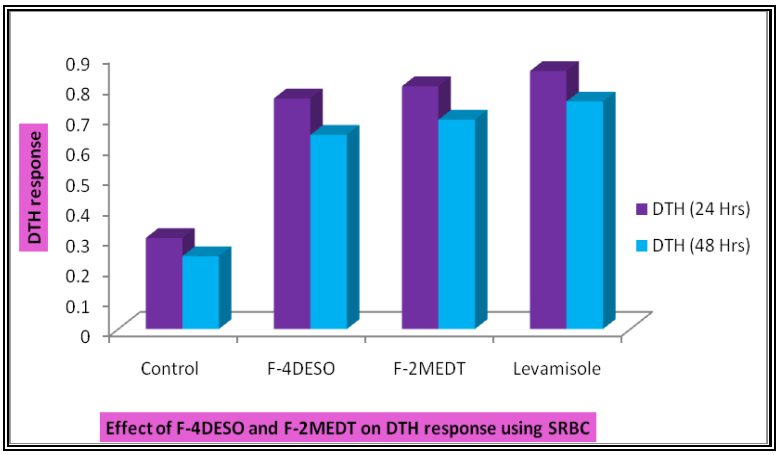
As shown in Table 1, the F-4DESO & F-2MEDT produced a significant, increase in DTH reactivity in rats. Increase in DTH reaction in rats in response to cell dependent antigen

revealed the stimulatory effect of F-4DESO and F-2MEDT on T cells. Both fractions had shown a highly significant (P<0.001) activity in DTH response activity.

Table No 1. Effect of F-4DESO and F-2MEDT on DTH response using SRBC

S No.	Groups	Treatments	Dose	DTH Response (mm) 24 Hrs	DTH Response (mm) 48 Hrs
1	Group I	Control	(10 ml/kg vehicle)	0.30±0.05	0.24±0.06
2	Group II	F-4DESO	100 mg/kg	0.76±0.04***	0.64±0.03***
	Group III	F-2MEDT	100 mg/kg	0.80±0.06***	0.69±0.08***
3	Group IV	Levamisole	50 mg/kg	0.85±0.02***	0.75±0.05***

Values are expressed as mean±SEM, n=6 in each group; ** p<0.01, compared to control. *** p <0.001, compared to control.



Graph No 1. Effect of F-4DESO and F-2MEDT on DTH response using SRBC

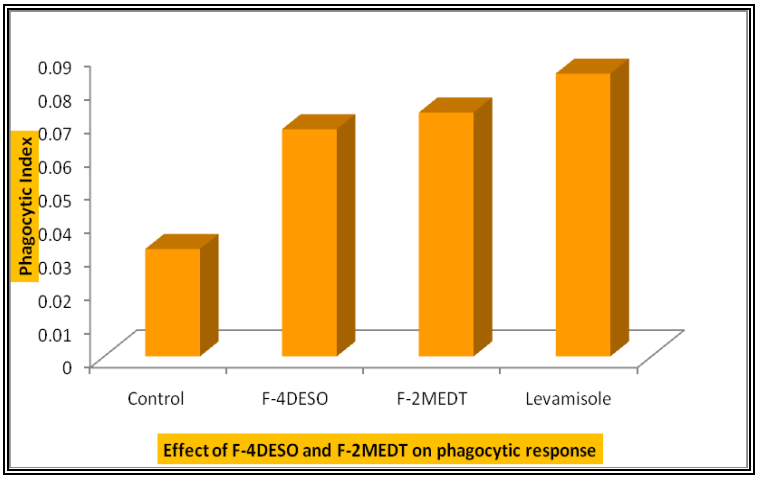
Phagocytic response by Carbon Clearance Assay

Both doses of F-4DESO and F-2MEDT showed significant increase in the phagocytic index when compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs. However, the clearance was best with high doses of both compounds. F-2MEDT shown highest activity as compared to F-4DESO.

Table No 2. Effect of F-4DESO and F-2MEDT on Phagocytic Index

S No.	Groups	Treatments	Dose	Phagocytic Index
1	Group I	Control	(10 ml/kg vehicle)	0.0321±0.0018
2	Group II	F-4DESO	100 mg/kg	0.0679±0.0088***
	Group III	F-2MEDT	100 mg/kg	0.0729±0.0017***
3	Group IV	Levamisole	50 mg/kg	0.0985±0.0032***

Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to control ** p<0.01, compared to control. *** p <0.001, compared to control.



Graph No 2. Effect of F-4DESO and F-2MEDT on phagocytic response

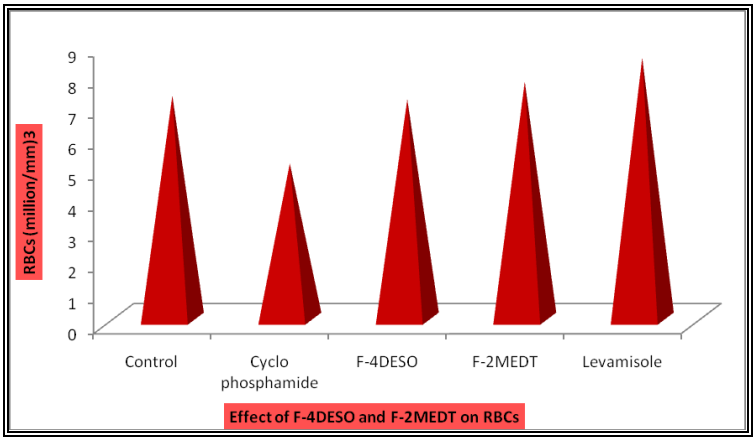
Cyclophosphamide-induced myelosupression

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the haemoglobin, RBCs and WBCs count. In case of cyclophosphamide induced myelosupression, there was decrease in the WBCs count in the control group. In treatment groups, the WBC count was found to be increased with $p<0.01$ and $p< 0.001$ on 11^{th} day. There was no alteration in other haematological parameters like RBCs and HB.

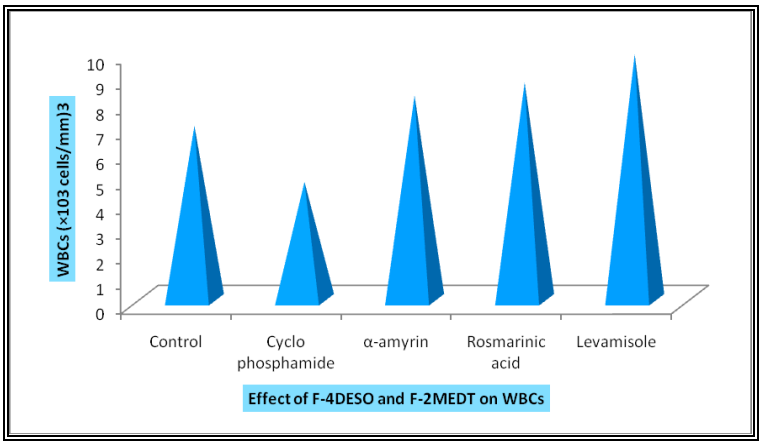
Table No 3. Effect of F-4DESO and F-2MEDT on RBCs, WBCs and HB

S No	Treatments	RBCs (million/mm)^3	WBCs (10^3cells/mm)^3	HB (g/dl)
		11^{th} Day	11^{th} Day	11^{th} Day
1	Control 10 ml/kg normal saline	7.25±0.15	6.96±0.19	10.6±1.63
2	Cyclophosphamide (30 mg/kg/ i.p)	5.05±0.22**	4.72±0.21***	7.53±0.36**
3	Cyclophosphamide 30 mg/kg/ i.p+F-4DESO (100 mg/kg/p.o.)	7.14±0.66**	8.18±0.77**	8.99±0.42*
4	Cyclophosphamide 30 mg/kg/ i.p+F-2MEDT (100 mg/kg/p.o.)	7.71±0.78**	8.70±0.33***	9.35±0.61**
5	Cyclophosphamide 30mg/kg/i.p+Levamisole (50 mg/kg/p.o.)	8.49±0.22**	9.83±0.31***	10.39±0.88***

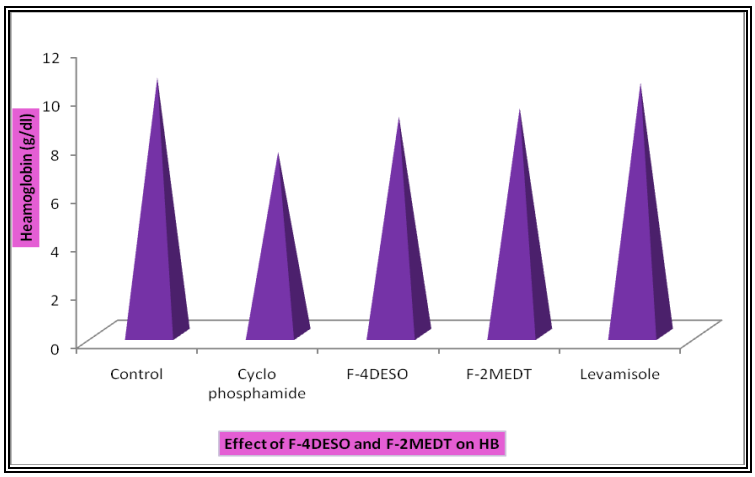
Values are expressed as mean±SEM, n=6 in each group; * p <0.05, compared to control ** p<0.01, compared to control. *** p <0.001, compared to control.



Graph No 3. (a) Effect of F-4DESO and F-2MEDT on RBCs



Graph No 3. (b) Effect of F-4DESO and F-2MEDT on WBCs



Graph No 3. (c) Effect of F-4DESO and F-2MEDT on HB

CONCLUSION

An immunomodulator can be defined as a substance, biological or synthetic, which can stimulate or modulate any of the components of the immune system including both innate and adaptive arms of the immune responses. These substances have been described to possess

pharmacological properties like Immunostimulant, tonic, antiaging, antistress, antirheumatic, adaptogenic anticancer, antibacterial etc. Therefore the fraction-4 of dichloromethane extract of *Spinacia oleracea* (F-4DESO) and fraction-2 of methanolic extract of *Desmodium triflorum* (F-2MEDT) was pharmacologically evaluated for Immunomodulatory activity by delayed-type hypersensitivity reactions (DTH) model, Phagocytic index model using carbon clearance assay and Cyclophosphamide induced myelosupression model.

Delayed type Hypersensitivity required the specific recognition of given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. DTH is a part of the process of graft rejection, tumor immunity and most important, immunity to many intracellular microorganisms. It can also be due to activation of complement, release of reactive oxygen or nitrogen species by activated phagocytes and pro-inflammatory cytokines.[15] Delayed type hypersensitivity (DTH) is antigen specific and cause erythema induction at the site of antigen infection in immunized animals. Cell-mediated immunity (CMI) involves effectors mechanisms carried out by T lymphocytes and their products (lymphocytes). CMI responses are critical to defense against infectious organisms, infection of foreign grafts, tumor immunity and delayed-type hypersensitivity reactions.[16] Therefore, increase in DTH reaction in animals in response to T cell dependent antigen revealed the stimulatory effect of F-4DESO and F-2MEDT on T cells. The F-4DESO and F-2MEDT produced a significant, increase in DTH reactivity in animals. Increase DTH reaction in animals in response to cell dependent antigen revealed the stimulatory effect of F-4DESO and F-2MEDT on T cells. Both compounds had shown a highly significant (P<0.001) activity in DTH response activity.

The carbon clearance assay was used to evaluate the effect on reticulo-endothelial cell mediated phagocytosis.[17,18] When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. Rate of clearance of (carbon particles) ink from blood is known as phagocytic index. The extract produced an increased in phagocytic index suggesting its effect on reticulo-endothelial system. Stimulation of phagocytosis is influenced by the activation of macrophages, the activated macrophages secrete a number of cytokines, which in turn stimulate other immune cells.[19] Phagocytosis is the process by which certain body cells, collectively known as phagocytes, ingests and removes microorganisms, malignant cells, inorganic particles and tissue debri.[16] Both fraction F-4DESO and F-2MEDT showed significant increase in the phagocytic index when

compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs. F-2MEDT shown highest activity as compared to F-4DESO.

Cyclophosphamide suppresses humoral, cellular, non-specific and specific cellular immune response. When animal was treated with cyclophosphamide then haemoglobin (Hb), RBC counts, WBC count, are reduced significantly.[20-22] The suppressive effect of cyclophosphamide was protected by the administration of F-4DESO and F-2MEDT. Triterpenoids and flavonoids in biological systems tend to adhere with the molecules of cyclophosphamide this causes to increase the size of the molecules and prevent its entry to the stem cells. As already stated that such compounds are detected in the plant extract besides this some more compounds are there which are not only negating the effect of cyclophosphamide, but also accelerating the total WBC and haemoglobin count. The F-4DESO and F-2MEDT significantly produces the changes in WBCs, RBCs and hemoglobin levels. This suggests that the constituent of the plant preventing the access of cyclophosphamide to the stem cells so that synthesis of haemoglobin, WBC and RBC is not inhibited. Another point is that the compound as are reutilizing this immunosuppressant before it could act upon haemopoeitic and myeloid tissue. Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the haemoglobin, RBCs and WBCs count. In case of cyclophosphamide induced myelosupression, there was decrease in the WBCs count in the control group. In treatment groups, the WBC count was found to be increased on 14th day. There was no significant alteration in other haematological parameters like RBCs and HB when compared with control.

On the basis of the results obtained in the present study it can be concluded that *Spinacia oleracea* and *Desmodium triflorum* has the potential to stimulate the humoral immune response and cell-mediated immune response and it may be a potential candidate in several immuno-suppressed clinical conditions. Therefore α-amyrin containing fraction-4 of dichloromethane extract of *Spinacia oleracea* (F-4DESO) and Rosmarinic acid containing fraction-2 of methanolic extract of *Desmodium triflorum* (F-2MEDT) having imminostimulant activity.

Besides from the obvious therapeutic importance, these fractions (F-4DESO and F-2MEDT) would be useful in understanding the mechanism of diseases with higher levels of cellular and molecular level. The present findings are significant for the development of alternative,

inexpensive and perhaps safer strategies for the treatment of diseases. A detailed study is also required on structure determination of the compounds from bioactive fractions in order to find the structure activity relationship.

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