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Evaluation of Immunomodulatory Activity of DCM Extract of *Nelumbo nucifera* on Mice.

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ABSTRACT

The objective of the present study was to carry out the extraction of Nelumbo nucifera rhizomes and evaluate the potential of alkaloid rich DCM extract on immunomodulatory effect on mice. The dried rhizomes were grinded and subjected to extraction with various solvents such as, petroleum ether, dichloro methane, chloroform, n-Butanol etc. and portion of the extracts were subjected to phytochemical analysis and toxicity study. The DCM extract of Nelumbo nucifera rhizomes (DCM-NN) was administered at the different doses of 100 mg/kg, 500 mg/kg and 1000 mg/kg p.o bw. The immunomodulatory study was done by performing Neutrophil adhesion test, Carbon clearance test, Haemagglutination antibody (HA) titer and Delayed hypersensitivity study (DTH). Phytochemical screening of different extracts established the presence of alkaloids and steroids. The oral administration of the DCM extract at median and high doses (500 and 1000mg/kg, p.o.) significantly increased in humoral immunity leading to increase in antibody titre, neutrophil adhesion, positive DTH response in mice when compared to normal control group. In conclusion, the DCM extract showed a potential effect as immunomodulator on immune system, which could be predicted by the presence of a considerable amount of alkaloids in the extract.

Keywords: HA titer, DTH, Nelumbo nucifera.

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INTRODUCTION

In the present world herbal components or herbal formulation are preferred than other system of medicine due to their minimum adverse effect and cost. Most of the herbal formulation available in the market for the treatment of cancer and immunological disorder are either few or lack of potency. Traditional or indigenous medicine stands for medical practice developed by local ethnic people by the use of natural herbs and minerals. Different parts of world has its own characteristic traditional medicine, for example, Ayurvedic medicine from Southeast Asia, Unani medicine in Arab countries/middle east, and acupuncture and traditional Chinese medicine (TCM) from China [1–3]. Herbal plants contain secondary metabolite like alkaloids, glycosides, terpene, steroids, flavonoids, tannins and others. Some chemical substances are phenols or their oxygen-substituted derivatives such as tannins while some may contain nitrogen or sulphur that are biologically active and useful for the prevention of disease and treatment of ailment and preserve well-being in humans and animals. For human, many of these herbal products and spices serve as useful medicinal source since ancient time period [4].

Body's natural resistance to disease can be increased by the use of traditional Indian system of medicines as suggested by Siddha, Ayurveda and Unani. Lots of Indian Medicinal plants and various "Rasayanas" have been claimed to possess immunomodulatory activity[5-7]. Immunomodulators are under development for the treatment of those diseases in which an abnormal immune response plays an important pathological role including cancer, auto immune disease and natural or acquired immune deficiency syndromes.

Clinically, these immunostimulants are used in immuno deficiency disorders such as AIDS, chronic infectious diseases, Cancer [8]. Additionally, medicinal plants employed for immunomodulation are interested to be promising choice against a wide range of diseases, particularly at the point when host defense system must be initiated under the situation of damaged immune reaction. It has been reported that plant metabolites like alkaloids, flavonoids, glycosides, polysaccharides, peptides have property to alter the immune response [9].

Nelumbo nucifera Geartn. is a seasonal aquatic plant, widely produced in the Eastern Asian countries. Almost all parts of N. nucifera, such as leaves, flowers, seeds, and rhizomes, found to have applications in either herbal medicine or food. Plants, belonging to the Nymphaea genus are believed to have properties such as antidepression, antidiabetic, antipyretic, anti-inflammatory, antiplatelet, anticancerous, antimicrobial, antiviral and anti-obesity properties[10-14]. However, to the best of our knowledge, the immunomodulatory activity of Nelumbo nucifera rhizomes extract has not yet been established scientifically. Therefore, an effort was made to evaluate the immunomodulatory effect of Dichloromethane extract of Nelumbo nucifera rhizomes (DCM-NN) in relation with its custom medicinal properties.

MATERIALS AND METHODS

Plant material

The collected rhizomes of Nelumbo nucifera from local area of Kamrup district, Assam, India were authenticated by Dr. P. P. Baruah (HOD, Department of Botany, Gauhati University, India). The voucher specimen of Nelumbo nucifera rhizomes (GIPS/NN/2014/010) were kept at the Department of Pharmacognosy, Girijananda Chowdhury Institute of PharmaceuticalSciences, Assam. The plant material was subjected to washing under running water, cut into small pieces, dried under shade for 12-15 days and stored in air tight containers until used.

Preparation of Extract

Dried rhizome were mechanically grinded and about 1 kg of crude powder of dried rhizomes was subjected to extraction, carried out by using cold maceration process for 72 hrs with petroleum ether for defatting. The next extraction of the plant material was carried out with various solvents such as Dichloro methane, Chloroform, n-nutanol, Ethyl acetate for about 48 hrs followed by frequent shaking up to 6 hrs. The extract was then collected and concentrated on water bath and finally dried in lyophiliser. A portion of the

extracts were subjected to phytochemical analysis and dried aqueous extract was preserved in refrigerator for further toxicity study.

Preliminary phytochemical screening

The quantitative chemical evaluation of different extracts were performed as per the standard procedures available in the literature to identify the presence of different metabolites (alkaloids, flavonoids, saponins, steroids, carbohydrates, glycosides and tannins) [4].

Experimental Animal

The guidelines set by CPCSEA and were approved by the Institutional Animal Ethical Committee (GIPS/IAEC/Phd/2015/01) were followed throughout experimental works. Male and female Swiss albino mice weighing between 20-25 gm were housed following the standard laboratory conditions as per the CPCSEA guideline such as 22±3 °C of temperature, 50±10% humidity, 12 hrs interval of light and dark phase and were fed with standard pellet diet throughout the entire experiment..

Acute toxicity study and dose optimization

The acute toxicity study was evaluated according to the OECD guideline 425. Acute toxicity studies of all the extracts were tested at different dose levels of 100, 500, 1000, 1500 and 2000 mg/kg p.o. to overnight fasted experimental mice as suggested in OECD Guidelines 425. The animal behavioral changes, abnormalities and mortality were observed in the next 24 hrs and then recorded up to 14 days [9,10]. From the toxicity studies depending on the safety, the doses for animal experiment were optimized.

Experimental method

Neutrophil adhesion test

Animals were divided into five groups with six animals (n=6) in each group. Group I served as normal control received 10 ml/kg p.o. of normal saline. Group II served as control received levamisole (50 mg/kg p.o) for 14 days; Group III, IV and V were administered with DCM-NN extract at the dose of 100 mg/kg p.o., 500 mg/kg p.o., 1000 mg/kg p.o. respectively for two weeks. After completion of last dose on the 14th day, all the animals were anesthetized and blood samples were withdrawn from all the groups by puncturing the retro-orbital plexus and stored in disodium EDTA vials. The total leukocyte count (TLC) and differential leukocyte count (DLC) were analyzed and then incubated for 15 min at 37 °C along with nylon fibre (80 mg/ml of blood sample). TLC and DLC were analyzed again with the incubated blood sample to get to neutrophil index (NI) [11].

Percent (%) of neutrophil adhesion was calculated as follows

$$\text{Neutrophil Adhesion (\%)} = \frac{NI_u - NI_t}{NI_u} \times 100$$

Where,

NIu = Neutrophil Index before incubation with nylon fibre.
NIt = Neutrophil Index after incubation with nylon fibre.

Carbon Clearance test (CCT)

The study was performed to evaluate the phagocytic cell activity of macrophase system which was measured by elimination of carbon of macrophase system by carbon clearance test. For this test, the grouping of animals and drug treatments were performed in similar way as describe in the neutrophil adhesion test respectively for 7 days. After 2hrs of last dose on day 7th all the mice were injected via tail vein with carbon ink suspension (10 µl/gm bw). Blood sample were collected about 25 µl (in EDTA solution 5 µl) from retro orbital plexus under mild ether anesthesia at 0 and 15 min interval and which was then mixed with 2 ml of 0.1% sodium carbonate solution and the absorbance was determined at 675 nm [15].

The phagocytic index (K) was calculated by using the equation:

$$K = \frac{\ln OD1 - \ln OD2}{T2 - T1}$$

Where, OD1 and OD2 are the optical density at time T1 and T2 respectively.

Haemagglutination antibody (HA) titer

This test also carried out in similar way as describe in CCT for 7 days. The extract was administered orally on three days before and three days after of immunization including the day of immunization (i.e -3, -2, -1, 0, +1, +2, +3). All the mice of different groups were immunized with 0.1 ml of 20% SRBC in normal saline i.p. and challenged on day 0. On day 7th blood was withdrawn from retro-orbital plexus from all antigenically sensitized and challenged mice respectively. Blood was centrifuged to separate serum. Antibody levels were determined by the haemagglutination technique. From each group the equal volumes of individual serum samples were pooled. Two fold serial dilutions with pooled serum samples were prepared in 25 µl volumes of normal saline in microtitration plates were added to 25 µl of 1% suspension of SRBCs in saline. The plates were incubated at 37°C for one hour and then observed for haemagglutination under microscope. The highest dilution giving haemagglutination was taken as the antibody titre [16–19].

Delayed type hypersensitivity (DTH) response:

The drug treatment was provided exactly the same as HA titre technique. On the day 0 the animals of different groups from I to V (n=6 in each group) were immunized with SRBCs (0.1ml of 20% SRBC i.p.) in normal saline. On 8th day the thickness of the right hind footpad was measured by using a vernier caliper and animals were challenged with 0.03 ml of 20% SRBCs in subplantar region of right hind paw. After 8, 24 and 48 hrs of challenge the footpad thickness was measured. The difference between the pre- and post challenge footpad thickness were calculated to measure the DTH response was expressed in mm and the mean values of all treatment groups were compared with that of control group [14,20]

Statistical analysis

All the data are expressed as Mean±S.E.M. (n=6) and the analysis of variance for evaluation of percentage neutrophil adhesion, HA titre and carbon clearance assay was done by one way ANOVA followed by Dunnett's post test for comparison against control group. However, the data for Delayed type hypersensitivity (DTH) response was evaluated by two way ANNOVA followed by Bonferroni post test. The difference was considered to be significant when *p<0.05.

RESULTS

Preliminary phytochemical screening:

The preliminary phytochemical screening of different extract Nelumbo nucifera rhizomes showed the presence of phytocostituents like alkaloids, flavonoids, saponins, Steroids, carbohydrates, glycosides and tannins (Table 1).

Table 1: Phytochemical Screening of various fractions of Nelumbo nucifera

Sl.No.	Bioactive materials	Pet. Ether	Dichloro-Methane	Chloroform	n-Butanol	Ethyl Acetate
1	Alkaloid	-	+	+	+	+
2	Flavonoids	-	-	-	-	-
3	Saponins	-	-	-	-	-

4	Steroids	+	+	+	+	+
5	Tannins	-	+	-	-	-
6.	Carbohydrates	-	-	-	-	-
7.	Glycosides	-	-	-	-	-
8.	Phenolics	-	+	+	-	-

Indications: [+] denotes present; [-] denotes absent

Acute toxicity studies

After performing the acute toxicity studies it was found that there was no abnormality nor any mortality rate was observed at the define tested dose up to 2000 mg/kg p.o. So the drug was found to be safe within the tested dose. Therefore, the dose optimization was done at 100 mg/kg, 500 mg/kg and 1000 mg/kg for the experimental study.

Neutrophil adhesion test

Oral administration of levamisole (50 mg/kg p.o) for 14 days in animals when compared to normal control group showed an increased level of neutrophil adhesion percentage by 65.10%. Animals treated with DCM-NN (100, 500 and 1000 mg/kg, p.o.) showed a significant increased in the percentage of neutrophil adhesion by 44.76%, 51.29% and 59.26% respectively.

Table 2: Study of DCM extract of Nelumbo nucifera rhizome (NENR) on carbon clearance assay, HA titre test and

% of Neutrophil adhesion test

Group	Treatments (Dose)	% of neutrophil Adhesion	HA titre	Carbon clearance assay
I	Normal control	13.2±2.41	83.2±3.8	0.0112±0.0017
II	Standard (Levamisole 50 mg/kg)	37.9±2.27*	491.7±11.8*	0.0401±0.0015*
III	100mg/kg (DCM-NN)	23.9±1.42*	124.6±4.9*	0.0169±0.0013*
IV	500mg/kg (DCM-NN)	27.1±0.92*	249.6±7.2*	0.0206±0.0014*
V	1000mg/kg (DCM-NN)	32.4±0.54*	244.1±4.2*	0.0237±0.0011*

Values are expressed as mean±SEM, (n=6). One way ANOVA followed by Dunnett's test *p<0.05, ns = not significant when compared with normal control group.

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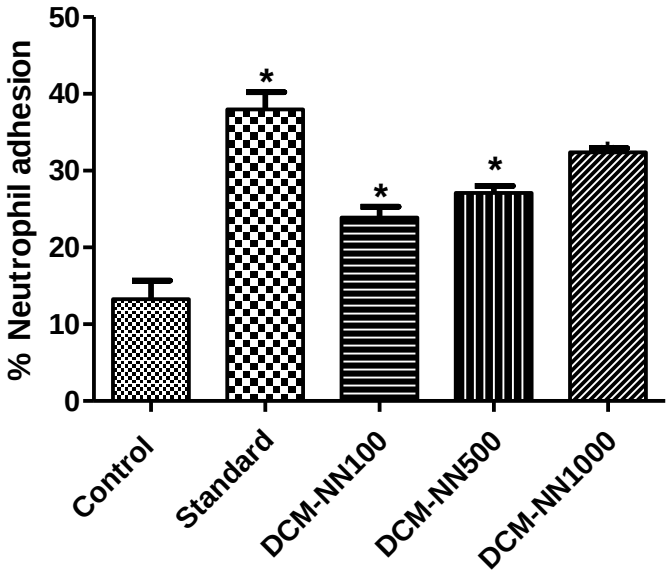

Fig 1: Effect of (DCM-NN) (100-1000 mg/kg, p.o.) against the percentage of Neutrophil adhesion. Values are expressed as mean±SEM, (n=6)

Haemagglutination antibody (HA) titer

The humoral response against SRBC could be established by determining Haemagglutination antibody titer. It was observed that there is a six fold increased (83.10%) in the amount of HA titre after 1 hour of incubation with SRBCs when compared with normal control. However, animals treated with NENR (100, 500 and 1000 mg/kg, p.o.) also showed a significant proportional increase in HA titre by 33.22%, 66.67% and 65.91% respectively. This justified that there was a significant effect of the immunostimulatory property of (DCM-NN) extract through humoral immunity.

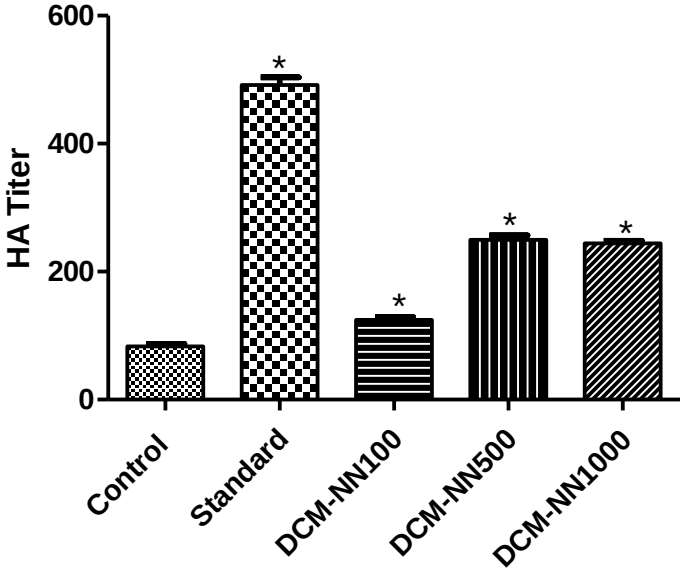

Fig 2: Effect of DCM-NN (100-1000 mg/kg p.o.) on HA titre. Values are expressed as mean±SEM, (n=6)

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Carbon Clearance test:

The faster removal of carbon from blood circulation is being considered as the cause to enhance in phagocytic activity. It was observed that the maximum and enhanced removal of carbon was seen with standard levamisole treated group with as high as up to 72.10% clearance. However, animals administered with DCM-NN with dosage of 100, 500 and 1000 mg/kg showed significant increase in phagocytic index with a percentage value of 27.81%, 45.63% and 52.74% respectively, but lesser as compared to standard control (Table 2, Fig. 3).

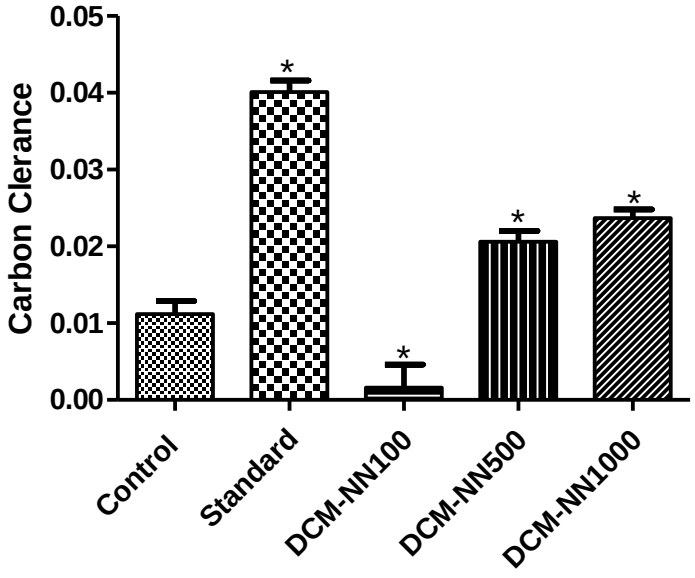

Fig 3: Effect of DCM-NN (100-1000 mg/kg p.o.) on carbon clearance Values are expressed as mean±SEM, (n=6)

Delayed type hypersensitivity (DTH) response:

Foot pad reaction (DTH reaction) has been conducted by evaluating the cell mediated immune response of DCM-NN. The DCM-NN at high doses of 500 and 1000 mg/kg, p.o., respectively showed a significant increase in DTH reactivity with increase in dose when compared with normal control group. The thickness of right foot pad was measured after 8, 24 and 48 hrs. The enhancement of DTH reaction in mice by SRBC proved the experimental study which established the modulatory effect of DCM-NN extract. (Table 3) (Fig. 4)

Table 3: Effect of DCM-NN on DTH response using SRBCs as an antigen

Group	Treatments (Dose)	Mean Right footpad thickness (mm)		
		After 8 hrs	After 24 hrs	After 48 hrs
I	Normal control	0.0510±0.024	0.0574±0.030	0.0819±0.029
II	Standard control (Levamisole 50 mg/kg)	0.131±0.049^{ns}	0.4007±0.0378*	0.5248±0.0447*
III	100mg/kg (DCM-NN)	0.0749±0.031^{ns}	0.2273±0.0248*	0.3893±0.0476*
IV	500mg/kg (DCM-NN)	0.0797±0.016^{ns}	0.2661±0.0194*	0.3273±0.0167*
V	1000mg/kg (DCM-NN)	0.109±0.085^{ns}	0.3168±0.0434*	0.4804±0.0715*

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Values are expressed as mean±SEM, (n=6) Two way ANOVA followed by Bonferroni post test *p<0.05, significant; ns = not significant when compared with control group.

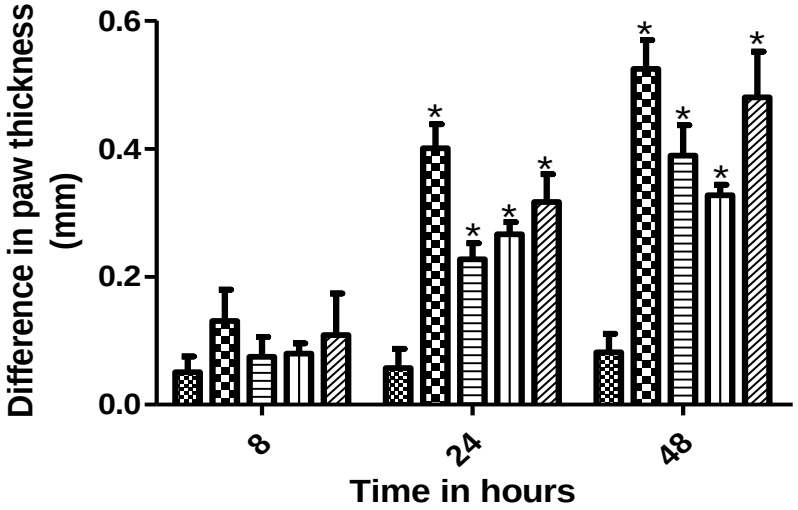

Fig 4: Effect of DCM-NN on DTH response using SRBCs as an antigen after 8, 12, 24 hrs against normal group.

DISCUSSION

Nelumbo nucifera rhizomes proved its traditional value scientifically. Phytochemical screening of Nelumbo nucifera rhizomes different extracts showed the presence of various phytochemical classes such as alkaloid, steroids and phenolic compounds. DCM-NN extract was found to possess a major quantity of alkaloids and steroidal components which can be justified for the possible pharmacological activity of the plant over immunomodulatory effect.

Neutrophils which circulate around the body through blood have an important role in immune system. If an infectious agent is present, they are the first line of defense to move to the site of the infection and start eliminating the microorganism by formation of oxygen radicals [19,21]. This study showed, a significant increase in neutrophils, which correlates the increase in percent neutrophils at high dose (500 and 1000 mg/kg, p.o.). This could have been significantly help in increasing the immunity of the body system. The initial focal point of the immunomodulatory compound is accepted to be macrophages which have a major role in modulating the immune system[22]. The effect on reticulo-endothelial cell mediated phagocytosis, carbon clearance assay was performed. Dichloromethane extract of Nelumbo nucifera rhizomes (DCM-NN) at a dosage of 500 and 1000 mg/kg enhanced the phagocytic capacity by showing the improvement in the clearance rate of carbon. To establish the humoral response against SRBC heamagglutination antibody titer was evaluated. Antibody particles, a result of B lymphocytes and plasma cells, are fundamental to humoral insusceptible reactions; IgG and IgM are the significant immunoglobulins which are included in the supplement enactment, opsonization, balance of toxins and so forth. DCM extract of Nelumbo nucifera rhizomes showed an increased humoral immunity to SRBCs which was observed by a rise in the antibody titre in animals (Table 2). This signify the role of T and B lymphocyte in the antibody synthesis[23]. In addition, the high values of haemagglutinating antibody titer was also observed when the animals were treated with DCM-NN, this signify an increased activity of humoral immune response which is directly proportionate to immunostimulation. Cell-mediated immunity (CMI) reactions play a vital role to defense the body physiological system against infectious agents, infection of foreign matters and delayed-type hypersensitivity reactions. One of the major defense mechanisms against various intracellular pathogens is delayed hypersensitivity and it occurs in transplant rejection and tumor immunity. In the present study, an attempt was also made to evaluate the delayed type hypersensitivity reactions by DCM-NN extract and it was found that the extract has the capability to increase the difference in paw thickness which is proportionate with increase in time. Therefore, enhancement in the DTH response in mice in light of Immune system microorganism subordinate antigen uncovered the stimulatory impact of DCM extract of Nelumbo nucifera rhizomes on white blood cells.

CONCLUSION

From the present investigation, it can be concluded that the immune response showed a significant increase when the animals were treated with DCM-NN extract. This can be predicted due to the presence of various chemical constituents such as alkaloid and steroidal components in the rhizome part. In conclusion, both medium dose (500 mg/kg) as well as high dose (1000 mg/kg) of the DCM extract of Nelumbo nucifera stimulates immune system by acting through cellular and humoral immunity in experimental models of immunity in animals. Therefore the study suggested that the DCM extract is a potent immunostimulant on both specific and nonspecific immune mechanisms which might serve as an effective natural immunomodulatory agent.

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