Original Article

In-vitro and *in-vivo* Immunomodulatory Effect of Polyherbal Suspension on Cyclophosphamide Induced Experimental Animal

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ABSTRACT

Aim: The aim of present investigation was to determine the efficacy of a polyherbal formulation. [Composed of *Nelumbo nucifera* (NN), *Euryale ferox* (EF) and *Trapa natans* (TN)]. **Materials and Methods:** The seeds of NN and EF and fruits of TN were extracted using hydroethanol (1:1) solvent. The dried extract of each plant was used for phytochemical investigation and DPPH radical scavenging activity to determine antioxidant activity. Polyherbal suspension (PHS) was prepared using dried extracts with other excipients. Acute toxicity study was performed for the PHS before *in vivo* experiment on animals. Albino Wistar rats were selected for the evaluation of immunomodulatory activity of PHS. Immunosuppression was induced by administration of cyclophosphamide subcutaneously on day 0 in all treated groups and treatment with test drug was continued for 14 days. **Results:** Results exhibited that extracts showed presence of various secondary metabolites including flavonoids, alkaloids, tannins, amino acids, carbohydrates and starch. The prepared PHS was found safe in acute oral toxicity study with no harm to animal's up to 2000 mg/kg dose. The percentage inhibition of DPPH radial by NN, EF and TN were 86.67%, 85.78% and 89.88%, respectively at the concentration of 200 μg/mL. PHS showed significant immunomodulatory activity by increasing neutrophil adhesion to 47.94% in 400 mg/kg dose with mean neutrophil percentage of 40.05%. Results of hematological parameters revealed that PHS restored all the levels of blood components in 400 mg/kg dose. **Conclusion:** Thus, PHS may be used as an effective and safe formulation in the treatment of immune deficiency.

Key words: Neutrophil adhesion, Phytochemistry, *Nelumabo nucifera*, *Euryale ferox*, *Trapa natans*.

INTRODUCTION

Immunodeficiency is a condition of weak immune system; it can be categorized as primary and secondary immune deficiencies.[1] Primary immunodeficiency (PID) arises from innate source, however, secondary immunodeficiency occurs due to iatrogenic and other physiological cause.[2] Deficiency of IgA is very common in PID along with severe infections, defects in regulation of T cells, abnormal cytokine production and autoimmune disorders.[3-5] Immunodeficiency is composed of several heterogenous disorders affecting innate and adaptive immunities.[6] Innate immunity, mediated by neutrophil granulocytes play a major role in defense against pathogens, tissues remodeling, chronic inflammation and cancer.[7] PID is also responsible for hematological changes in the body. It is associated with cytopenia, thrombocytopenia and neutropenia.[8] Several drugs are available to modulate the immunity either by increasing or decreasing the immune response. Immune-stimulant drugs are helpful for malnourished patients to boost the immunity and fight against diseases.[9] However, immune-suppressant drugs are required during organ transplantation and autoimmune diseases.[10] The conventional immune-

Submission Date: 02-07-2020;
Revision Date: 09-10-2020;
Accepted Date: 30-12-2020

DOI: 10.5530/ijper.55.1s.54

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stimulant organic synthetic drugs are associated with cost-effectiveness and safety concerns due to their adverse reactions.[11] These problems attract attention of researchers towards herbal medicines and search for natural products for the development of safe and potent immune stimulant drugs.

Ayurveda is an ancient traditional system of medicine in India.[12] It involves use of natural herbal products to restore body's essential nutrients and requirements for maintaining healthy lifestyle and eliminate the root cause of diseases.[13] Medicinal plants possess wide array of immune-stimulant property and influence on body's immune system to fulfill the nutritional deficiencies.[14] The phytochemicals (i.e. glycosides, alkaloids, flavonoids, saponins, resins, tannins and lactones) present in the plant have been reported for their immunomodulating properties.[15] *Nelumbo nucifera* Gaertn (Nymphaeaceae), *Euryale ferox* (Nymphaeaceae) and *Trapa natans* Roxb (Lythraceae) are highly nutritious and aquatic plants commonly found in Asian countries. *N. nucifera* is popularly known as 'Lotus'.[16] It has been traditionally used as diuretic, antidiabetic, astringent, hemostatic and emollient. In addition, *N. nucifera* has lots of nutritional value along with immune stimulation properties.[17,18]

E. ferox is commonly known as 'Makhana' in India.[19] Its seeds are very nutritious and possess numerous biological activities such as immunomodulatory, antioxidant, hepatoprotective, antidiabetic, antihyperlipidemic, antidepressant and anti-cancerous properties.[20-27] *Trapa natans* is also called "Shingada" in India. It possesses several biological properties including immunomodulatory, antidiabetic, hepatoprotective, antioxidant, antimicrobial, antiviral, analgesic and anti-inflammatory activities.[28-30] According to Ayurveda, polyherbalism or multiple herbs formulation possesses greater therapeutic efficacy. The combination of herbs in desired ratio gives better therapeutic effect.[31]

Thus, present study was designed to formulate suspension a polyherbal formulation containing extract of plants (*N. nucifera*, *E. ferox* and *T. natans*) extract. Phytochemical investigation of the individual plant extracts was performed followed by their radical scavenging activity. Based on their remarkable bioactivity of the above plants, also evaluated the immune stimulation activity of poly herbs in an immune-compromised animal model.

MATERIALS AND METHODS

Collection, identification, authentication and extraction of plants

The plants parts used in the study were seeds of *Nelumabo nucifera* and *Euryale ferox* and fruits of *Trapa natans*. The plants were collected from the forest of Dhamtari district of Chhattisgarh, India. It was identified and authenticated by Dr. S. S. Chandravanshi, Krishi Vigyan Kendra, Sambalpur, Dhamtari and Chhattisgarh. The voucher specimens of the samples were deposited in the institute via Voucher no. 0242.

N. nucifera seeds (100 g) were dried, grounded and extracted with ethanol (1 L) and hydroethanol (50:50) as solvents maintained at 50°C in a water bath for 2 h. The extracts were allowed to evaporate under reduced pressure and stored in desiccator after drying till further use. *E. ferox* seeds (100 g) were dried, powdered and macerated individually with ethanol (1 L) and hydroethanol (50:50) solvents followed by placing in a mechanical shaker for 16 h. The extracts were filtered and evaporated using vacuum evaporator (700°C) followed by storage after drying in a desiccator.[32] *T. natans* fruits (100 g) were peeled, dried, grounded and macerated individually with ethanol (1 L) and hydroethanol (50:50) solvents using cold extraction process. The extracts were shaked for 15 h in a mechanical shaker then filtered and evaporated using vacuum evaporator. The exact was dried in room temperature and then stored in a desiccator till further use (Aidew and Buragohain, 2015).[33]

Qualitative phytochemical screening

Phytochemical screening was performed to determine presence of secondary metabolites in plant extracts by adopting standard procedure.[34-36]

In vitro activity of hydroethanolic extract of plants

DPPH radical scavenging activity

DPPH assay is performed to determine the radical scavenging activity of plant extracts. Briefly, the sample extracts (200 mg) were taken in centrifuge tubes were distilled water (200 µL) was taken as blank. DPPH solution was added to the centrifuge tubes containing samples and blank. The mixture was left at room temperature (25°C) for 30 min then centrifuged again at 4000 rpm for 10 min. The supernatant was separated and added in a tube containing ethanol (1 mL) followed by reading of absorbance at 517 nm using UV spectrophotometer (Shimadzu, Japan). Each sample was analyzed in triplicate.[37] The percentage inhibition was calculated using the following formula:

$I\% = A_{blank} - A_{sample} / A_{blank} \times 100$

where, A_{blank} = absorbance of the control reaction, A_{sample} = absorbance of the extracts,

Preparation of polyherbal suspension

The polyherbal formulation as suspension was formulated by trituration method in which dried extracts of individual plants were mixed with suspending agent along with other excipients. Polyherbal suspension

(PHS) was prepared by weighing extracts (1 g of each plant extract), sodium carboxy methyl cellulose (0.5%, w/v), methyl paraben (0.2%, w/v), Tween-80 (0.1%, w/v) and water to make up to 100 mL. Briefly, extracts and methyl paraben were triturated using motor-pastle followed by addition of Tween-80 and small amount of sodium carboxy methyl cellulose (sCMC) to form a uniform paste. Rest amount of sCMC was added into the paste and again triturated to form slurry. It was rinsed with water to make up the volume (100 mL) and transferred into a beaker. Mechanical stirrer (500 rpm) was used to form a homogenous suspension. The prepared PHS was stored in refrigerator till further use.[38]

Evaluation of polyherbal suspension (PHS)

The prepared PHS was evaluated for yield, organoleptic characteristics, viscosity and pH.

Determination of percentage yield

The percentage yield of each extract was calculated by using following formula:

Percentage yield = (Weight of extract/Weight of powder drug taken) × 100

Color, odor and taste

The organoleptic characters of PHS including color, odor and taste were evaluated according to the method described by Kamath and Shah, 2014.[39]

Viscosity

Viscosity of PHS was determined using viscometer (Brookfield) at room temperature.[40]

pH

pH meter was used to determine the pH of PHS. pH meter was calibrated using buffer and distilled water until constant reading were noted.[41]

Acute toxicity study

The toxicity of orally administered polyherbal formulation was determined according to OECD guideline 423 (OECD, 2001). The female Wistar rats were selected for the study for administration of test drug in the doses of 50, 100, 150, 300, 1000 and 2000 mg/kg. The animals were observed very keen at 4 h and 24 h after drug administration. Then the animals were observed on a daily basis for 14 days for their change in behavior, fur color, feeding habits and lethargy.

In vivo immunomodulatory activities

Experimental animals

Albino Wistar rats of both sexes weighting 180-200 g were selected for the study. Institutional Animals Ethical Committee of Columbia Institute of Pharmacy approved the present protocol. The animals were free access to feed and water *ad libitum*. They were maintained at laboratory condition of temperature 25±2°C and relative humidity 75-85%.

Experimental protocol

Animals were randomly grouped into five groups each containing six animals. Group 1 was control group received water, Group II was toxic group received cyclophosphamide (200 mg/kg), Group III was reference group received levamisole (50 mg/kg), Group IV to V were test groups received 200 and 400 mg/kg dose of *Nelumbo nucifera* ethanolic seed extract, respectively. Immunosuppression was induced by administration of cyclophosphamide subcutaneously on day 0 in all treated groups. However, treatment was started once daily (p.o.) from day 1 and continued for 14 days.

Neutrophil adhesion test

It was performed by collecting the blood sample of animals after 14 days of treatment with the test drugs. Total Leukocyte Count (TLC) of blood collected from retro-orbital route of animals was determined using Medonic M20 cell counter (POCT Services Pvt. Ltd., New Delhi, India). Differential Leukocyte Count (DLC) was determined on blood samples by preparing smear with leucofine stain and then calculated neutrophil content. Neutrophil index was evaluated by analyzing TLC and DLC, which was previously incubated with nylon fibers (80 mg/mL) for 15 min at 37°C.[42,43] Neutrophil adhesion percentage was calculated using the formula below:

Percentage neutrophil adhesion = NIu - NIt × 100/NIu

Where, NIu = neutrophil index of untreated blood samples, NIt = neutrophil index of treated blood samples.

Hematological analysis

The blood was collected from 12 h fasted animals from retro-orbital plexus puncture. The collected blood was used to measure neutrophils (NEUT), basophils (BAS), white blood cell count (WBC), monocytes (MON), red blood cell count (RBC), lymphocytes (LYM), eosinophil (EO), platelet count (PLT) through automatic haemoanalyser.[44]

RESULTS

Phytochemical investigation of different plants extracts

Table 1 represents phytoconstituents present in the different plants extracts. It was revealed that all the extracts

showed presence of steroids, phenolic compounds, alkaloids, proteins, flavonoids, tannins, amino acids, carbohydrates and starch. However, saponins were absent in all the plants extracts. Glycosides were present in *N. nucifera* seed extract only.

Evaluation of polyherbal formulation

The obtained extracts were further concentrated on water bath to evaporate excess solvent for getting percentage yield of extract. The percentage yield of *N. nucifera, E. ferox* and *T. natans* was found to be 5.38%, 9.37% and 8.22%, respectively. The organoleptic characteristics revealed that PHS was brown in color, pleasant in odor and bitter in taste. The viscosity and pH were found to be 49.38 cps and 6.51 pH, respectively.

DPPH radical scavenging activity

Table 2 shows the DPPH radial scavenging activity of different plants extracts. Results exhibited dose-dependent radical scavenging activity of plants extracts. DPPH radical scavenging activity of ascorbic acid was found to be 93.00%. The percentage inhibition by different groups was achieved in a dose dependent manner. It was 86.67%, 89.88% and 85.78% by *N. nucifera, T. natans* and *E. ferox*, respectively at the concentration of 200 µg/ mL. The IC_{50} values for different plants extracts were 133.96 µg/mL, 132.32 µg/mL and 137.37 µg/mL for *N. nucifera, T. natans* and *E. ferox*, respectively.

Table 1: Phytochemical analysis of extracts of different plants.

S. No.	Chemical Test	*Nelumbo nucifer*	*Trapa natans*	*Euryale ferox*
1.	Alkaloids	+	+	+
2.	Saponins	-	-	-
3.	Steroids	+	+	+
4.	Proteins	+	+	+
5.	Phenolic compounds	+	+	+
6.	Flavonoids	+	+	+
7.	Tannins	+	+	+
8.	Amino acids	+	+	+
9.	Carbohydrate	+	+	+
10.	Glycoside	+	-	-
11.	Starch	+	+	+

+, presence; - absence

Acute toxicity study

Results exhibited that the prepared polyherbal nutraceutical formulation was non-toxic to the animals. No any change in behavior, locomotor activity, color of fur and eyes were detected in 14 days study. Thus, lower doses of formulation were selected for immune-stimulant studies on animals.

Immune-stimulant activity of polyherbal neutraceutical formulation in cyclophosphamide induced immunocompromised rats

Table 3 shows the effect of plants extracts on percentage neutrophil adhesion on Wistar rats. The percentage neutrophil adhesion was abruptly decreased due to administration of cyclophosphamide (200 mg/kg). It was significantly raised with the treatment of polyherbal suspension. The mean neutrophil percentage after treatment with PH1 (200 mg/kg) and PH2 (400 mg/kg) was 29.90% and 40.05%, respectively. The other extracts treated groups were also effective in normalizing mean percentage neutrophil in a dose-dependent manner. The percentage of neutrophil adhesion was highest for PH2 i.e. 47.94% in comparison to control group (Figure 1). Administration of cyclophosphamide reduced the neutrophil adhesion to 13.71%. The reference group was also effective in normalizing neutrophil (41.13%) and restoring neutrophil adhesion (31.78%).

Effect of different polyherbal neutraceutical formulation plants extracts on hematological profile of cyclophosphamide induced immunocompromised rats

Table 4 shows the effect of polyherbal suspension (PHS) on hematological parameters of cyclophosphamide

Table 2: DPPH radical scavenging activity of different plants extracts.

S. No.	Concentration (µg/ ml)	Inhibition (%)			
		Ascorbic acid	*Nelumbo nucifera*	*Trapa natans*	*Euryale ferox*
1	50	93.00±0.24	16.84±1.54	15.75±1.25	13.25±1.45
2	100	-	25.13±0.29	23.12±0.75	22.45±1.75
3	150	-	56.26±2.23	52.25±1.25	54.25±1.75
4	200	-	86.67±0.20	89.88±0.80	85.78±0.30
	**IC_{50} (µg/ml)	10.67	133.96	132.32	137.37

All data are expressed as the mean ± SD (n=3)

Table 3: Efficacy of different plants extracts in neutrophil adhesion of cyclophosphamide induced immunocompromised rats.

Groups	Dose (mg/kg)	Mean neutrophil adhesion	
		Before treatment	After treatment
Control	-	20.73±2.60	19.55±2.82
Toxic	200	27.73±1.26	26.85±1.61
Reference	50	50.71±0.31	41.13±2.8
NN1	200	37.65±0.94	29.90±1.28
NN2	400	49.68±0.60	43.06±0.41
EF1	200	35.65±0.94	27.90±1.28
EF2	400	47.58±0.60	41.05±0.41
TN1	200	37.65±0.94	29.90±1.28
TN2	400	47.58±0.60	40.05±0.41
PH1	200	37.65±0.94	29.90±1.28
PH2	400	47.58±0.60	40.05±0.41

Values are presented as mean±SEM (n=6); significantly different at $^{a}p<0.05$ in comparison to control group; Control - Normal saline, Toxic - Cyclophosphamide, Reference - Levamisole, NN - *Nelumbo nucifera*, EF - *Euryale ferox*, TN - *Trapa natans*, PH - Polyherbal suspension.

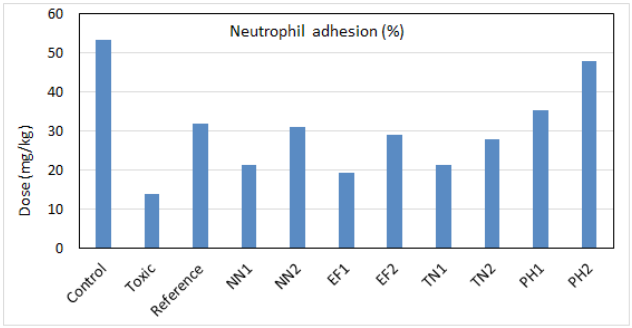

Figure 1: Effect of polyherbal formulation on percentage neutrophil adhesion of cyclophosphamide-induced toxic rats.

induced immunocompromised rats. Cyclophosphamide caused toxicity to the animals and disturbed the normal level of blood components. All the treatment groups were effective in restoring the normal level of blood parameters in a dose-dependent manner as compared to control group. Reference group (levamisole) was significantly revered the toxic effect of cyclophosphamide. PH restored the level of WBC, NEUT, LYMP, MON, EO, BAS, RBC, HGB, HCT and PLT to 12.62, 9.27, 9.06, 1.44, 0.58, 0.03, 7.37, 11.75, 36.40 and 1147, respectively in the dose of 400 mg/kg in comparison to control group.

DISCUSSION

In the present study, the immune-stimulant ability of a polyherbal formulation composed of *Nelumabo nucifera,*

Table 4: Effect of polyherbal formulation on hematology of cyclophosphamide induced immunocompromised rats.

Parameters	Control group	Toxic group	Reference group	*Nelumabo nucifera*		*Euryale ferox*		*Trapa natans*		Polyherbal suspension	
				200 mg/kg	400 mg/kg	200 mg/kg	400 mg/kg	200 mg/kg	400 mg/kg	200 mg/kg	400 mg/kg
WBC (103/µL)	12.05±1.40	7.77±1.44	11.52±0.6a	6.36±0.21	7.02±0.90a	7.23±0.21	8.41±0.90a	8.36±0.21	9.42±0.90a	11.56±0.21	12.62±0.90a
NEUT (103/µL)	9.20±0.23	3.27±0.43	9.30±0.04a	6.27±0.02	7.37±0.49a	6.77±0.02	7.27±0.49a	5.27±0.02	7.37±0.49a	8.77±0.02	9.27±0.49a
LYMP (103/µL)	9.31±1.39	0.45±0.04	9.14±0.29a	5.86±1.05	7.56±0.34a	5.76±1.05	7.06±0.34a	4.86±1.05	6.56±0.34a	8.76±1.05	9.06±0.34a
MON (103/µL)	1.53±0.12	0.58±0.33	1.47±0.01	1.20±0.02	1.30±0.09	1.22±0.02	1.31±0.09	1.28±0.02	1.30±0.09	1.39±0.02	1.44±0.09
EO (103/µL)	0.53±0.09	0.11±0.10	0.52±0.00	0.41±0.00a	0.51±0.09	0.35±0.00a	0.49±0.09	0.39±0.00a	0.41±0.09	0.46±0.00a	0.58±0.09
BAS (103/µL)	0.01±0.00	0.01±0.00	0.04±0.01a	0.02±0.00	0.02±0.01a	0.02±0.00	0.03±0.01a	0.01±0.00	0.02±0.01a	0.02±0.00	0.03±0.01a
RBC (103/µL)	6.84±0.48	2.55±0.18	7.48±0.72a	6.08±0.23	6.55±0.69	6.18±0.23	6.27±0.69	6.28±0.23	6.35±0.69	6.88±0.23	7.37±0.69
HGB (g/dL)	12.28±0.71	6.06±0.19	12.12±0.19a	10.05±0.31	10.35±0.95	10.22±0.31	10.75±0.95	10.39±0.31	10.55±0.95	10.75±0.31	11.75±0.95
HCT (%)	37.68±2.54	14.23±0.23	36.37±1.11a	31.45±0.55a	33.00±2.65	31.85±0.55a	34.40±2.65	32.45±0.55a	33.00±2.65	35.65±0.55a	36.40±2.65
PLT (103/µL)	1207.0±63.39	502.50±80.52	1103±128a	1040±259.9	1065±300.0a	1040±259.9	1087±300.0a	1010±259.9	1020±259.9	1137±300.0a	1147±300.0a

Values are presented as mean±SEM (n=6); significantly different at ap<0.05 in comparison to control group; Control - Normal saline, Toxic - Cyclophosphamide (200 mg/kg), Reference - Levamisole (50 mg/kg); WBC, white blood cell count; NEUT, neutrophils; LYM, lymphocytes; MON, monocytes; EO, eosinophil; BAS, basophils; RBC, red blood cell count; PLT, platelet count.

Euryale ferox and *Trapa natans* extracts was evaluated in an immunocompromised rat model. Results revealed that polyherbal formulation was significantly restored the immune functions with normal hematological profile of animals as compared to control group. Deficient nutritional level in an immunocompromised animal breaks the immune functions due to suppression of immune system.[45] It has been reported that medicinal plants possess immune-stimulant property.[46,47] Medicinal plants extracts possesses several nutritional bioactive compounds that are useful in boosting immunity and treatment of various diseases.[48-50]

N. nucifera, E. ferox and *T. natans* are the potential nutraceutical sources that possess lots of secondary metabolites and active substances in different parts of the plants that have pharmaceutical value.[16,30,51-53] Phytochemical study of selected herbs showed presence of different herbal metabolites including alkaloids, glycosides, steroids, proteins, phenolic compounds, flavonoids, tannins, amino acids, carbohydrates and starch. These compounds are very nutritional and beneficial in different diseased conditions. The antioxidant potential of these herbs may be due to presence of secondary metabolites in herbal extracts.[54] Our results were in agreement with the findings of Khan *et al.* 2019, reporting antioxidant potential of herbal extract.[55]

The immune system works as a defensive system with ability to protect body from broad range of toxins and allergens.[56] Some non-specific substances that stimulates immune system are neutrophils and macrophages. These substances increase the defense capability of phagocytic cells.[57] Immunodeficiencies are commonly associated with disturbance in hematological values.[58] Administration of cyclophosphamide decreased percentage neutrophil adhesion in rats; additionally, disturbed normal hematology profile of the animals. Results showed that percentage neutrophil adhesion was significantly raised with the treatment of polyhebral formulation. However, individual herbs were also effective in restoring normal levels of percentage neutrophil adhesion in immunocompromised and diseased animals. Similar results were represented by Vinothapooshan and Sundar, 2011, for their immunomodulatory work on *Adhatoda vasica* Linn. in experimental animals.[59] The polyherbal formulation attenuated cyclophosphamide induced toxicity and restored normal level of blood parameters in a dose-dependent manner. A polyherbal formulation of nutritional medicinal plants possess immune-stimulant and chemoprotective properties that can restored hematological profile of cyclophosphamide induced toxic animals.[60] Therefore, the use of this polyherbal suspension may be effective for malnourished immunocompromised patients to boost immunity and fight against diseases. No such formulation is available till date. This novel PHS will contribute in improving immune system cost effective approach.

CONCLUSION

The polyherbal suspension was found to be quite effective in the treatment of immunocompromised animals. The findings of the study indicated the immune-stimulant activity of polyherbal suspension of *Nelumabo nucifera, Euryale ferox* and *Trapa natans* extracts and suggest its use in the condition where immune-stimulant property is required for any therapeutic purposes. This polyherbal suspension may have application in immunodeficiency diseases, allergic conditions and as a vaccine adjuvant in combination therapy with antibiotics. Further mechanistic investigations of prepared formulations are required to determine immune-stimulant properties.

ACKNOWLEDGEMENT

The authors are thankful to the DST /FIST/ (Department of Science and Technology)/SR/FST/ college/418/2018, the Department of Science and Technology, New Delhi for financial assistance for the research work. We are also showing our gratitude towards Columbia Institute of Pharmacy, Raipur for providing all facilities for easy conducting of the research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

NN: *Nelumbo nucifera;* **EF:** *Euryale ferox*; **TN:** *Trapa natans*; **PHS:** Polyherbal suspension; **PID:** Primary immunodeficiency; **TLC:** Total Leukocyte Count; **DLC:** Differential Leukocyte Count; **NEUT:** Neutrophils; **BAS:** Basophils; **WBC:** White blood cell count; **MON:** Monocytes; **RBC:** Red blood cell count; **LYM:** Lymphocytes; **EO:** Eosinophil; **PLT:** Platelet count.

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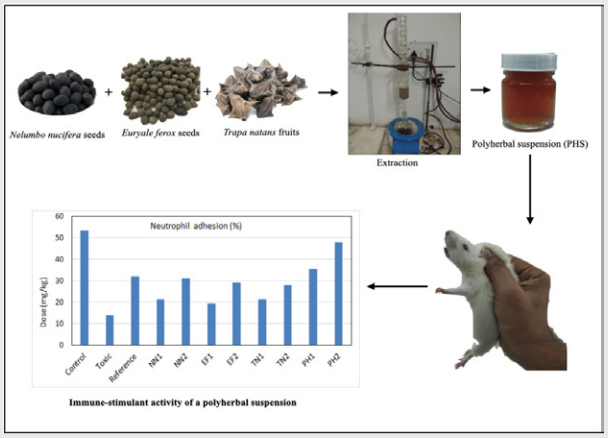
PICTORIAL ABSTRACT



SUMMARY

In the present study, a polyherbal formulation [Composed of *Nelumbo nucifera* (NN), *Euryale ferox* (EF) and *Trapa natans* (TN)] was prepared to determine its immunomodulatory activity in Albino Wistar rats. Polyherbal suspension (PHS) did not produce any sign of toxicity during oral administration for 14 days. PHS produced significant immunomodulatory activity by increasing neutrophil adhesion it may be due presence of different secondary metabolites in the formulation. PHS significantly reduced free radicals and restored hematological profile of animals. Thus, the prepared PHS was found safe and effective in immunestimulation of cyclophosphamide induced immunesuppresed rats. It may be used as an effective and safe formulation in the treatment of immune deficiency.

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Cite this article: Majumdar A, Shukla SS, Pandey RK. *In-vitro* and *in-vivo* Immunomodulatory Effect of Polyherbal Suspension on Cyclophosphamide Induced Experimental Animal. Indian J of Pharmaceutical Education and Research. 2021;55(1s):s225-s232.