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Research Article

SYNERGISTIC IMMUNOMODULATORY ACTIVITY OF AQUEOUS ROOT EXTRACT OF *ASPARAGUS RACEMOSUS* WILLD AND ETHANOL WHOLE PLANT EXTRACT OF *BOERHAVIA DIFFUSA* LINN

AMRITA KUMARI[1,2], MAINAK CHAKRABORTY[2]*, NILANJAN SARKAR[2], SEKHAR KUMAR BOSE[2], KALYAN ROY[3], GAUTHAMAN KARUNAKARAN[3]

[1]Department of Pharmacy, Ranchi College of Technology and Research Centre, Ranchi, Jharkhand, India. [2]Department of Pharmaceutical Technology, NSHM Knowledge Campus, Kolkata, Group of Institutions, West Bengal, India. [3]Department of Pharmacy, Government Pharmacy College, Sajong, Sikkim, India. Email: mainakchakraborty47@gmail.com

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ABSTRACT

Objective: On the basis of traditional use and Ethno pharmacological evidences *Boerhavia diffusa* whole plant and root part of *Asparagus racemosus* (Shatavari) both are widely used to enhance the immunity. But in combination there is no scientific evidence so current study was designed.

Methods: Laboratory based study, namely, carbon clearance, cyclophosphamide induced immune suppression and neutrophil adhesion was designed using mice as an experimental animals in different combination of aqueous whole plant extract of *B. diffusa* and methanol extract of root part of *A. racemosus* were used as a test drug in the ratio 1:1, 1:2, and 2:1 (100 mg/kg) against the established standard drug Ashwagandha.

Results: The results revealed that animals treated with combined extract (1:1, 1:2, and 2:1) at a dose of 100 mg/kg increase rate of carbon clearance from blood, there is significance alternation in blood parameter in cyclophosphamide group and also improve the Neutrophil adhesion when treated with different combination of polyherbal formulation treated groups.

Conclusion: The polyherbal formulation in different ratio showing good significant immunomodulatory activity as compare to standard.

Keywords: Immunomodulation, Carbon clearance, Neutrophil adhesion, Synergism.

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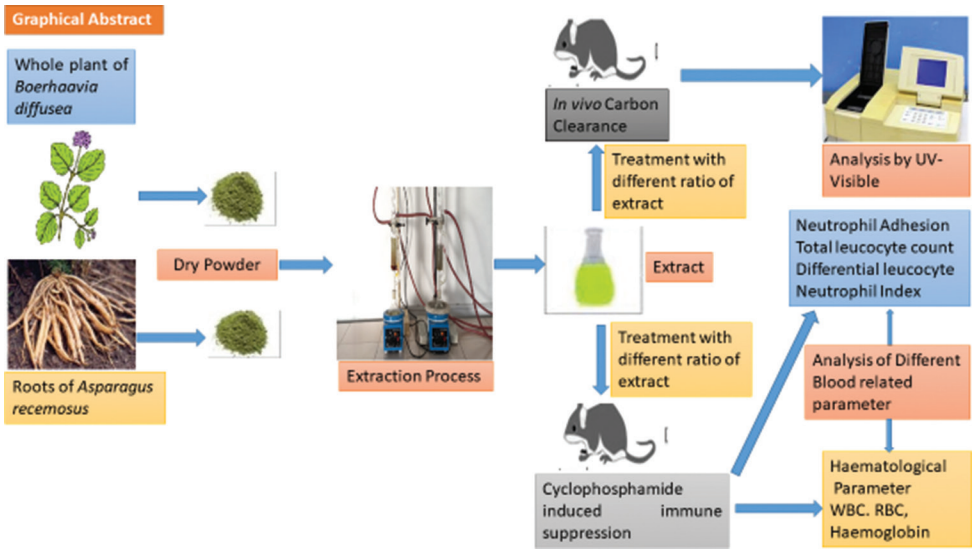
INTRODUCTION

Plants are an indispensable part of nature. They are composed with a specific purpose. Nature throws back the creative powers of a living supreme being. Nature always perches as a golden mark to represent the outstanding phenomena of symbiosis.

Herbal drugs are the major cure in traditional system of medicine. The practices continue in today's modern world because of its biomedical benefits as well as its place in traditional beliefs in many parts of world, have made a great contribution towards maintaining mankind [1]. It was found that almost 121 pharmaceutical products were formulated based on the traditional knowledge obtained from various sources [2].

Ayurveda, the ancient Indian traditional system of medicine lays significance on promotion of health for cure of diseases and strengthening of both physical and mental health [3]. The present work was done on the *Boerhavia diffusa* whole plant on the basis of



the literature obtained from the Ethno medicinal documentation. The common name is Punarnava. Keeping in view the tremendous ethno medicinal use of *B. diffusa* it was aimed to scientifically validate the immune modulatory property of the root part of *B. diffusa*.

In combination with root part of *Asparagus racemosus* commonly known as Shatavari (*A. racemosus*). The plant has tiny white flowers, in small spikes. The roots are finger-like and clustered, and in tropical climates throughout India, Asia, Australia, and Africa [4].

In view of the above, the present investigation was undertaken to evaluate the immunostimulatory potentiality of *B. diffusa and A. racemosus* the ratio is 1:1, 1:2, and 2:1 the both by *in vivo* model system based on the evidence of ethno medicinal use of these plants in such diseases of immune suppression state in Kolkata.

METHODS

Plant materials

Ethanolic extract of the whole parts of *B. diffusa* and the aqueous extract of the root part of *A. racemosus* were used as test drug in these experiments. Plants were authenticated by Botanical Serve of India, Shibpur, Howrha, West Bengal India, Specimen no-CNH/26a/2012/Tech.II/7006.

Test compound formulations

Oral suspensions of the root and whole plant extract were prepared by suspending them in 1% solution of sodium carboxy methylcellulose to obtain suitable dosage forms the ratio of *A. racemosus* and *B. diffusa* (1:1, 1:2, and 2:1), respectively.

Animal used

Male Swiss albino mice weighing 18–22 g were taken. They were obtained from the institutional animal house, NSHM, Kolkata, India. The mice were grouped and housed in poly- acrylic cages (38 × 23 × 10 cm) with six animals per cage and maintained under standard conditions with (temperature 25±2°C and dark/light cycle 14/10 h). They were allowed to have free access to dry pellet diet and water *ad libitum*. The mice were habituate to laboratory conditions for 7 days before start of the experiment. All procedures described were viewed and approved by the Institutional Animal Ethical Committee.

Drugs and chemicals

EDTA, Phosphate buffered saline, sodium carbonate, solution, (Endoxan Injection German Remedies, India) Indian ink, Carboxy methyl cellulose (CMC), Cyclophosphamide 30 mg/kg b.w., and Ashwagandha 50 mg/kg b.w.

In vivo carbon clearance test

Mice were divided into four groups, each containing ten animals. Group I (Control) was given normal saline for 7 days, Groups II and III were administered different ratio of the methanol extract (50–100 mg/kg, p.o.), and Group IV was administered standard drug Ashawagandha 50 mg/kg, p.o.). At the end of 7 days, mice of all the groups were injected through the tail vein the carbon ink suspension (10 µl/g body weight). Blood samples were drawn (in EDTA solution 5 µl) from the cardiac puncture at intervals of 0 and 15 min, a 25 µl sample was mixed with 0.1% sodium carbonate solution of 2 ml and their absorbance was measured at 660 nm. The carbon clearance was calculated using the equation: (Loge OD1–Loge OD2)/15 where as OD1 and OD2 are the optical densities at 0 and 15 min, respectively [5].

Cyclophosphamide induced immunosuppression [6]

Dose calculation: Ethanolic extract of the whole parts of *B. diffusa* and the aqueous extract of the root part of *A. racemosus* were used as test drug the ratio is 1:1, 1:2, and 2:1 (100 mg/kg).

Treatment

Animals were divided into the six groups containing six animals in each group. Group1 (Control group) received CMC for 14 days and Group 2 (Challenge group) received CMC for 10 days, on 11th, 12th, and 13th day cyclophosphamide intraperitonially at a dose of 30 mg/kg b/w. Groups 3, 4, and 5 (Test group) received poly herbal formulation of extract (1:1, 1:2, and 2:1) at a dose of 100 mg/kg body weight orally for 14 days. On 11, 12, and 13th days, cyclophosphamide solution was given intraperitonially at a dose of 30 mg/kg b/w 1 h after the administration of the extract. Group 5 received the standard drug.

Hematological test

After the treatment, animals were light anaesthetized by using di-ethyl ether. The blood was collected from the cardiac puncture using heparin containing capillary tubes and hematological tests were carried out for white blood cell (WBC) count red blood cell (RBC) count and hemoglobin content.

Neutrophil adhesion test [7]

Total leukocyte counts (TLC) and differential leukocyte counts (DLC) both were studied by fixing the blood smears and staining it with Field stain I and II- Leishman's stain, blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37°C. Incubated samples were then again analyzed for TLC and DLC. The result of TLC and % neutrophil gives neutrophil index (NI) of blood sample. % of the neutrophil adhesion was calculated as shown below

Neutrophil adhesion can be calculated

$$NI\ u - NI\ t \times 100/NI\ u$$

Whereas,
NI u =NI of the untreated blood samples.
NI t = NI of the treated blood samples.

RESULTS

The results are presented in Table 1 showed the immune stimulatory activity of poly herbal formulation in mice. The results revealed that

Table 1: *In vivo* carbon clearance test

Group number	Treatment mg/kg	Carbon clearance
I	Control	0.069±0.014
II	Extract (1:1) 100	0.094±0.014 *
III	Extract (1:2) 100	0.139±0.17*
IV	Extract (2:1) 100	0.135±0.27*
V	Ashwagandha 50	0.158±0.014

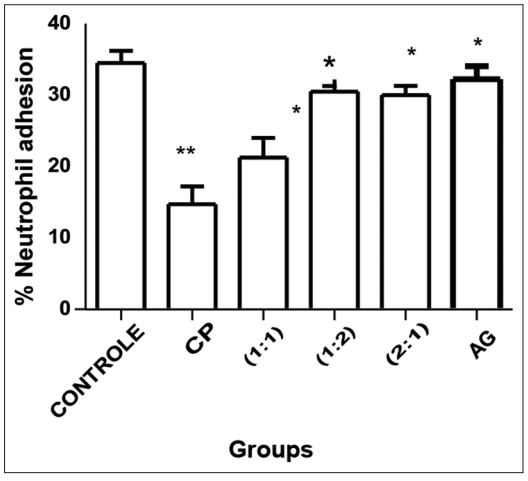


Figure 1 : Effect of poly herbal formulation at various ratio on cyclophosphamide induces neutrophils adhesion. All data were expressed as mean ± SEM (n = 6). Where **p < 0.0001 (with respect to Normal) and *p < 0.001(with respect to cyclophosphamide) are considered to be highly significant and significant.

Table 2: Haematological Parameter

Parameters	Normal saline (5ml/kg)	CP(30 mg/kg)	CP+Test drug (1:1) (100 mg/kg)	CP+Test drug (1:2) (100 mg/kg)	CP+Test drug (2:1) (100 mg/kg)	CP+Std.drug (50 mg/kg)
RBC (cell×106/mm^3)	6.29±0.20	4.63±0.09	4.83±0.08	5.53±0.25**	5.19±0.20**	5.19±0.20**
WBC (cell×103/mm^3)	4.98±0.32	2.84±1.13	3.70±0.62**	4.25±0.65**	4.09±0.33**	5.09±0.33**
Hb. (g/dL)	11.48±0.38	6.23±0.14	7.9±0.12**	10.35±0.21**	8.4±0.46**	10.75±0.46**

animals treated with combined extract (1:1, 1:2, and 2:1) at a dose of 100 mg/kg increase rate of carbon clearance from blood. Administration of test drug 100 mg/kg resulted in significant increase in the rate of carbon clearance compared with control group. On the other hand, the rate of carbon clearance value of 1:2 ratio was found to be slightly lower than that of the standard compound Ashwagandha. The effectiveness against chemical-induced immunosuppression after Administration of Cyclophosphamide (30 mg/kg, i.p) produced a significant decrease in the total leukocyte. The count is from 6.2±0.081 to 3.08±0.214, and the RBC count from 4.91±0.116 to 2.9±0.152, and % of hemoglobin (hb) from 16.60±0.081 to 10.32±0.153 ($p<0.01$) (Table 2). It was found to be consistent with earlier studies which state that cyclophosphamide induces immune dysfunction through reactive intermediate- induced damage to the cells of the immune system [8]. Evaluation of effect of the ethanol extract of the whole parts of *B. diffusa* and the aqueous extract of the root part of *A. racemosus* were used as test drug in these experiments on cyclophosphamide induced immune suppression indicated good protection by increasing all the hematological parameters. WBC count, RBC count, and % hb values observed were better than untreated control groups.

Neutrophil adhesion test

This test is an indicative of the marginalization of phagocytic cells in the blood vessels, i.e. an indication of immune stimulation. The % neutrophil adhesion in control group animals was, 34.5±1.7 in CP treated group was 14.74±2.46 in 1:1 treated group it was 21.25±2.78, 1:2 is 30.50±1.70, 2:1 is 30±1.29 and standard 32.25±1.79 (Fig. 1). The results of neutrophil adhesion test indicating that there was significant ($p<0.05$) increase in neutrophil adhesion after administration of poly herbal extract at various ratio when compare to cyclophosphamide treated groups.

Statistical analysis

All the data are given as the mean±SEM of three individual measurements. Data on all the experiment were analyzed using analysis of variance (ANOVA) and the group means were compared by Dunnett's by Graph Pad Prism software, version 4.03. A probability of $p<0.05$ was considered as significant.

Statistical significance (p) calculated by one-way ANOVA between Cyclophosphamide control group and the treated groups followed by Dunnett's test (**$p<0.05$). Each point represents the mean±SEM (n=6 mice per groups).

Cyclophosphamide, Test drug-Combined poly herbal formulation of *B. diffusa* and *A. racemosus* (1:1), (1:2) and (2:1) Std.drug- Standard drug. Statistical significance (p) calculated by one-way ANOVA between cyclophosphamide control group and the treated groups followed by Dunnett's test (**$p<0.05$). Each point represents the mean±SEM (n=6 mice per groups).

DISCUSSION

Immunity is the ability of the body to defend itself against specific invading agents such as bacteria, toxins, viruses and foreign tissues [9]. An immunomodulator is any substance that helps to regulate the immune system. This "regulation" is a normalization process, so that an immunomodulator helps to optimize immune response. Immune dysfunction is responsible for various diseases like ulcerative colitis, asthma, allergy, cancer and infectious diseases [10]. The extend up to which the patient becomes abnormally susceptible to infections by environment depends on the extent of immunosuppression. This immunosuppression allows pathogens to submerge the host to cause secondary infection [11]. This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs [12]. Many medicinal plants are known to have immunomodulatory properties and maintain organic resistance against infection by re- establishing the body's immune system such as *Azadirachta indica* [13] *Terminalia chebula* [14] *Lawsonia alba* [15]. The phytochemical constituents such as diterpenoids, steroids, proteins, and tannins [16] are considered to exhibit immunomodulatory property.

The use of medicinal plants to cure human illnesses has been practiced from time immemorial. Some of these drugs are believed to enhance the natural resistance of the body to infection [17]. In fact, one of the therapeutic strategies in Ayurvedic medicine is to provide protection by increasing body's resistance to disease [18]. The idea of immunomodulation refers to a non-specific trigger to the immune system. It implies non-antigen dependent activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells, lymphocytes, and also the production of various effector molecules by activated cells (paraimmunity). After being non-specific, it is highly expected to provide protection against different pathogens including bacteria, fungi, viruses, etc., and add upto another or adjunct to conventional chemotherapy.

The present study is aimed at studying the immunomodulatory properties of two selected immunomodulator plant at different ratio. In this study, we report on the effect of combined polyherbal formulation on different aspects of immunity such as carbon clearance from blood, total RBC, WBC count, and Hb content neutrophils adhesion test.

Phagocytosis act for as an important innate defense mechanism against devour foreign materials [10]. In the following carbon clearance test, rate of clearance of carbon from blood by phagocytic cells is governed by phagocytic index(K). In this study an increased in phagocytic index at administration of poly herbal formulation at different ratio, but the 1:2 ratio shown the very good result. The phagocytic index of 1:2 is near about standard drug Ashwagandha. The increased level carbon clearance from blood is may be due to the increased production of phagocytic cell stimulated by administration of poly herbal formulation at different ratio.

Bone marrow is a site of continued proliferation and turnover of blood cells and is a source of cells involved in immune activity. Bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs. Loss of stem cells and inability of bone marrow to regenerate new blood cells will result in thrombocytopenia and leucopenia [10]. Immune activation is an effective as well as protective approach against emerging infectious diseases [19].

Immunomodulatory activity of poly herbal formulation at different ratio was explored by evaluating their effects on cyclophosphamide induced bone marrow suppression in mice at combined at formulation of three ratio at 100 mg/kg dose levels. Results of the study revealed the ratio dependent counteracting effect of the extracts to the cyclophosphamide induced bone marrow activity suppression, that is, myelosuppression, as indicated by increase in RBC, total WBC platelet counts, Hb%, and DLC in the test drug treated groups (Groups III, IV, V, and VI), when compared to cyclophosphamide treated group (Group II). The potentiated activity of test drug may be due to presence of flavonoids, alkaloids, tannins, and steroids. The results

shows the modulation of bone marrow activity, namely, – suppression when used cyclophosphamide alone and stimulation to counteract the cyclophosphamide induced myelosuppression in pretreated with test drug and standard drug.

CONCLUSION

After performing the experiment by the ethanolic extract of whole plant of *B. diffusa* and aques extract of *A. racemosus* root part having the three ratio 1:1, 1:2, and 2:1, respectively, (100 kg/body wt) the combined effect of ethanolic extract of *B. diffusa* and aqueous extract of *A. racemosus* having ratio 1:2 is found effective. However this is a preliminary research work and the precise mechanism(s) of immunomodulatory action influenced by potent bio-active constituents of ethanolic and aqueous extracts of root and whole plant of *B. diffusa and A. racemous* against cyclophosphamide induced immunesuppression needs to be investigated.

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AUTHORS CONTRIBUTION

Amrita Kumari, Sekhar Bose and Mainak Chakraborty are responsible for study design, protocol development and study conduction, manuscript writhing. Nilanjan Sarkar, Kalyan Roy and Gouthaman Karunakaran are responsible for critical evaluation data interpretation and manuscript evaluation.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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