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Immunomodulatory Activity of Different Extracts of Stem Bark of *Alstonia scholaris* Linn

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ABSTRACT

Aim- The main of the study is to evaluate the immunomodulatory activity of different extracts of stem bark of *Alstonia scholaris*. **Material & Methods-** Different extracts were prepared by successive solvent extraction methods according to polarity of solvents. Preliminary phytochemical screenings were performed for the presence of various active phytochemicals. For the dose determination of different extracts, an OECD guideline was used and 200, 400 mg/kg were used for activity. for immunomodulatory activity, SRBC, phagocytic index and cyclophosphamide model were used. **Result-** The cell-mediated immune response of different extracts of *Alstonia scholaris* were assessed by DTH reaction, i.e. foot pad reaction. The chloroform and methanolic extract of *Alstonia scholaris* produced a significant, dose-related increase in DTH reactivity in rats. Chloroform and methanolic extracts (400 mg/kg) showed highest phagocytic index 6.44±0.57 & 6.67±0.48 respectively. The phagocytic index of control (group I) was 3.78±0.22. In case of cyclophosphamide induced myelosupression, there was decrease in the WBCs count in the control group. In treatment groups, the RBCs, HB and WBC count was found to be increased with $p<0.01$ and $p< 0.001$ at 200 & 400 mg/kg dose respectively on 11th day. **Conclusion-**On the basis of the results obtained in the present study it can be concluded that stem bark of *Alstonia scholaris* had 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.

Keywords: DTH Response, *Alstonia scholaris*, Stem Bark, Carbon Clearance, Phagocytic Index

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INTRODUCTION

Immunity is a homeostatic process, a sequence of delicately balanced complex, multicellular and physiologic mechanisms that allow an individual to make a distinction foreign material from"self" and neutralize and/or eliminate the foreign matter[1].

There are various side effects are connected with the use of these drugs i.e. Pulmonary toxicity, Myelosuppression, Alopecia, Increased threat of infection, Hepatic fibrosis, Lymphoma (Epstein–Barr virus associated), Nephrotoxicity, neurotoxicity (tremor, headache, motor disturbances and seizures), GI complaints, hypertension, hyperkalemia, hyperglycemia, and diabetes, Renal dysfunction, tremor, hirsutism, hypertension, hyperlipidemia, gum hyperplasia, hyperuricemia, hyper cholesterolemia, Nephrotoxicity, hypertension, diabetogenic, Elevated LDL cholesterol etc[2].

Although many drugs are used in ethno-medical practices in India, most of them are not pharmacologically evaluated. There is an imperative need to settle on the true therapeutic value of medicinal drug. The herb appears to be a safe drug.

The objective of the present effort is to study the phytochemical and pharmacological investigation of *Alstonia scholaris* for immunomodulatory activity.

MATERIALS & METHODS

Glass wares: Borosil and ASGI make glass wares were used.

Chemicals: All chemicals used were of analytical grade. Acetic acid S.D. Fines Chemicals; Bombay; Coomassie blue-Sigma Chemical Company, USA, DAB- Sigma Chemical Company, USA, DMSO- Sigma Chemical Company, USA, Dichlomethane Qualigens, Ethanol-Merck/Bengal Chemical; Ethyl acetate-Qualigens; EDTA-Qualigens; Methanol-Merck/Qualigens, Pet. ether, chloroform, methanol, water, molisch reagents, fehling solutions, Dilute hydrochloride, ferric chloride, glacial acetic acid, mercuric chloride, dragendroff reagents, millons reagents, hagers reagents, Ninhydrine reagents, SRBC, Precoated plates, silica gel, Indian ink etc.

Animals: Wistar Albino of either sex (150 to 200 g) was purchased from the CPCSEA approved vendors for *in-vivo* immunomodulatory activity. They were maintained under standard laboratory conditions at 25 ± 2°C and normal 12-hour light-dark cycle were used for the experiment. Commercial pellet diet and water were provided *ad libitum* throughout the course of study. All the experimental trial was carried out in agreement with the CPCSEA guidelines. The study designs were permitted by the Institutional Animal Ethical Committee of Oriental College of Pharmacy and Research, Oriental University, Indore (MP), India.

METHODS

Collection and authentication of plant material

The stem bark of *Alstonia scholaris* were collected from outfield medicinal garden near to Gwalior (M.P.) that show the green color with rough surface. The stem bark was washed thoroughly in tap water, dried in shade, finely powdered and used for extraction. Plant was identified by the Dr. Anurag Titov, Professor, Department of Botany, Govt. Madhav Sciences, PG College, Dewas Road, Ujjain and herbarium specimen was submitted in Department of Botany for future references.

Extraction of plant material

The extraction was done by following continuous or extraction procedure. Powdered material (stem bark) was packed in soxhlet apparatus. The drug was defatted with petroleum ether (60-80°C) for about 30-35 complete cycles. Defatted material was subjected to further extraction process by chloroform, methanol and water. All the extracts were concentrated under vacuum. The yield values and other physical properties were observed[3].

Qualitative phytochemical screening

Preliminary phytochemical screening was performed for presence of fatty acids, steroids, terpenoids, alkaloids, flavonoids, phenolic compounds, glycosides etc in all the extracts [4, 5].

Immunomodulatory activity of plant extracts

Delayed-type hypersensitivity (DTH) response by SRBC

Animals were divided into ten groups each having 6 animals. Group 1-received Vehicle, Group-2 received pet ether extract of *Alstonia scholaris* (200mg/kg), Group-3 received pet ether extract of *Alstonia scholaris* (400mg/kg), Group-4 received chloroform extract of *Alstonia scholaris* (200mg/kg), Group-5 received chloroform extract of *Alstonia scholaris* (400mg/kg), Group-6 received methanolic extract of *Alstonia scholaris* (200mg/kg), Group-7 received methanolic extract of *Alstonia scholaris* (400mg/kg), Group-8 received water extract of *Alstonia scholaris* (200mg/kg), Group-9 received water extract of *Alstonia scholaris* (400mg/kg), Group-10 received Standard drug, extract of *Ocimum sanctum.* (100mg/kg),

The pet ether, chloroform, methanolic and water extracts of *Alstonia scholaris* were administered orally on day 0 and continued till day 7 of challenge [6]. One group of each extract contains 6 animals. *Ocimum sanctum* extract was used as a standard drug.

Preparation of antigen

Fresh blood was collected from sheep sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in large volumes of Pyrogen free 0.9% normal saline and adjusted to a concentration of 0.5×109 cells/ml for immunization and challenge.

Delayed-type hypersensitivity (DTH) response

The rats were challenged by injection of 0.5×109 cells SRBCs in right hind foot pad. Foot thickness was measured after +24 and +48 h 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. The pet ether, chloroform, methanolic and water extracts of *Alstonia scholaris* was administered orally on day 0 and continued till day 7 of challenge [7]. One group of each extract contains 6 animals. *Ocimum sanctum* extract was used as a standard drug.

Phagocytic response of Different Extracts

Dose and Treatment

Animals were divided into ten groups each having 6 animals.

Group 1-Received Vehicle

Group-2 Received pet ether extract of *Alstonia scholaris* (200mg/kg)

Group-3 Received pet ether extract of *Alstonia scholaris* (400mg/kg)

Group-4 Received chloroform extract of *Alstonia scholaris* (200mg/kg)

Group-5 Received chloroform extract of *Alstonia scholaris* (400mg/kg)

Group-6 Received Methanolic extract of *Alstonia scholaris* (200mg/kg)

Group-7 Received Methanolic extract of *Alstonia scholaris* (400mg/kg)

Group-8 Received water extract of *Alstonia scholaris* (200mg/kg)

Group-9 Received water extract of *Alstonia scholaris* (400mg/kg)

Group-10 Received Standard drug, extract of *Ocimum sanctum.* (100mg/kg),

The animals were treated from day 0 today 7 with the vehicle and pet ether, chloroform, methanolic and water extracts of *Alstonia scholaris* (200 & 400 mg/kg). *Ocimum sanctum* (100mg/kg) extract was used as a standard drug. On day 7, all the animals of the entire groups received the treatment of an intravenous injection of (0.3 ml per 30 g) Indian ink dispersion (pre-warmed at 37°C).

The method was described by Cheng [8]. Rate of carbon clearance (*K*) and phagocytic index (α) were calculated by using following formula:

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

Where OD2 is the log absorbance of blood at 2min; OD10 is log absorbance of blood at 10 min; T2 is the last time point of blood collection; T1 is the first time point of blood collection. Rate of carbon clearance and phagocytic index of treated group animals were compared with the control group animals.

Cyclophosphamide-induced myelosupression

The control group received normal saline solution. Group II was administered with only cyclophosphamide at the dose of 30 mg/kg, i.p. while groups III, IV,V, VI and VII rats received cyclophosphamide with chloroform, methanolic extract (200 & 400 mg/kg) and *Ocimum sanctum* extracts (100 mg/kg) for 10 days.

Group 1: Served as control, received 10 ml/kg normal saline

Group 2: Received cyclophosphamide at the dose of 30 mg/kg, i.p

Group 3: Cyclophosphamide at the dose of 30 mg/kg, i.p+chloroform treated (200 mg/kg)

Group 4: Cyclophosphamide at the dose of 30 mg/kg, i.p+chloroform treated (400 mg/kg)

Group 5: Cyclophosphamide at the dose of 30 mg/kg, i.p+Methanolic treated (200 mg/kg)

Group 6: Cyclophosphamide at the dose of 30 mg/kg, i.p+Methanolic treated (400 mg/kg)

Group 7: Cyclophosphamide at the dose of 30 mg/kg, i.p+*Ocimum sanctum* extracts (100 mg/kg)

Ziauddin *et al*., (1996) method was employed for cyclophosphamide-induced myelosupression. On day 11, blood samples were collected from the retro-orbital plexus of individual animals and analyzed for hematological parameters[9].

Estimation of Hematological Parameters

Red Blood Corpuscles[10]

Reagents

Red blood cell diluted fluid (Hayem's fluid) – 5g of sodium sulphate, 1g of sodium chloride, 0.5g of mercuric chloride were dissolved in 200ml of distilled water.

Procedure:

Blood was sucked exactly up to the 20µl mark in the RBC pipette and the diluting fiuid was drawn immediately up to the mark and the blood mixed thoroughly with the diluting fluid. It was left for 2-3 min for proper mixing. The Neubauer counting chamber was placed along with the cover glass in position. The capillary stem of the pipette was emptied which contains only the diluting fluid. This was done by discarding first 3-5 drops.

Charging of the Counting Chamber

One drop of diluted blood was released into the groove of the Neubauer counting chamber. It was left for cells to settle for 2 – 3 minutes the counting chamber was put under the microscope and the ruled area was located. Erythrocytes were counted in the 5 squares of the counting area of 1 mm square. The number of cells in the 4-corner square was counted.

Calculation

The total number of cells found in 5 groups of 16 squares is multiplied by 10,000 to give the number of cells in millions/mm of blood.

White Blood Corpuscles[11]

WBC diluting fluid or Turk's fluid was used as the diluents which can destroy RBC'S.

Reagents

WBC diluting fluid was prepared by mixing Glacial acetic acid, Gentian violet 1% and Water 95 ml.

Procedure

The method of counting is similar to RBC counting except that the count is made in 4 large (1 mm) cover squares of the Neubauer counting chamber.

Calculation

The total number of cells in 4 squares is multiplied by a factor of 2500 to give the count/mm of blood.

Hemoglobin

Principle

Haemoglobin is converted into acid haematin by the action of HCl. The acid haematin solution is further diluted with distilled water until its colour matches with exactly that of permanent standard of comparator block. The Hb concentration is read directly from the calibration tube.

Requirements

HCl solution, sahli's Haemoglobinometer, pipette, distilled water.

Procedure

0.1 N HCl was added in the Haemoglobinometer upto the lowest marking. 20µl of blood was drawn up to 20µl in the Sahli's pipette. Adjusted the blood column carefully without bubbles.Wiped the excess of blood on the sides of the pipette by using a dry piece of cotton.Blown the blood into the acid solution in the graduated tube, rinsed the pipette well. Mixed the reaction and allow the mixture to stand at room temperature for 10 minutes. Diluted the solution with distilled water by adding few drops of water carefully and by mixing the reaction mixture until the colour matches the colour in the comparator. The lower meniscus of the fluid was noted and reading was noted in g/100ml.

Statistical Analysis

Data were expressed as the mean standard error of mean (S.E.M.) of the means and statistical analysis was carried out employing one-way ANOVA. Differences between the data were considered significant at $P < 0.05$.

RESULT

Qualitative Phytochemical Screening

The preliminary phytochemical analysis revealed that different active constituent present in different extracts such as carbohydrates, proteins, amino acids, fat, oils, steroids, terpenoids, glycosides, alkaloids, tannins and other phenolics compounds.

Immunomodulatory Activity of Different Extracts of *Alstonia scholaris*

Delayed-type hypersensitivity (DTH) response by SRBC

The cell-mediated immune response of different extracts of *Alstonia scholaris* were assessed by DTH reaction, i.e. foot pad reaction. As shown in Table, the chloroform and methanolic extract of *Alstonia scholaris* produced a significant, dose-related increase in DTH reactivity in rats. Increase in DTH reaction in rats in response to cell

dependent antigen revealed the stimulatory effect of methanolic extract on T cells (Table).

Table 1: Effect of different extracts on DTH response using SRBCs

S No.	Groups	Treatments	Dose	DTH Response (mm) 24 Hrs	DTH Response (mm) 48 Hrs
1	Group I	Control (10 ml/kg vehicle control)	-------	0.21±0.05	0.14±0.03
2	Group II	Pet ether extract of *Alstonia scholaris*	200 mg/kg	0.37±0.05*	0.31±0.03*
	Group III	Pet ether extract of *Alstonia scholaris*	400 mg/kg	0.39±0.07*	0.33±0.07*
	Group IV	Chloroform extract of *Alstonia scholaris*	200 mg/kg	0.77±0.02***	0.71±0.04***
	Group V	Chloroform extract of *Alstonia scholaris*	400 mg/kg	0.79±0.05***	0.73±0.07***
	Group VI	Methanolic extract of *Alstonia scholaris*	200 mg/kg	0.66±0.05**	0.61±0.08**
	Group VII	Methanolic extract of *Alstonia scholaris*	400 mg/kg	0.71±0.05***	0.62±0.09***
	Group VIII	Water extract of *Alstonia scholaris*	200 mg/kg	0.32±0.02*	0.28±0.04*
	Group IX	Water extract of *Alstonia scholaris*	400 mg/kg	0.35±0.03*	0.31±0.07*
4	Group X	*Ocimum sanctum* Extract	100 mg/kg	0.84±0.04***	0.72±0.07***

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

Phagocytic response of Different Extracts

Oral administration of different extracts in 200 & 400 mg/kg for 7 days, and 10 min prior to carbon injection exhibited a dose-related increase in the clearance rate of carbon by the cells of the RES. Chloroform and methanolic extracts (400 mg/kg) showed highest phagocytic index 6.44±0.57 & 6.67±0.48 respectively. The phagocytic index of control (group I) was 3.78±0.22 (Table No 2).

Table 2: Effect of different extracts of *Alstonia scholaris* on Phagocytic Index

S No.	Groups	Treatments	Dose	Phagocytic Index
1	Group I	Control (10 ml/kg vehicle control)	-------	3.78±0.22
2	Group II	Pet ether extract of *Alstonia scholaris*	200 mg/kg	3.98±0.33*
	Group III	Pet ether extract of *Alstonia scholaris*	400 mg/kg	4.10±0.26*
	Group IV	Chloroform extract of *Alstonia scholaris*	200 mg/kg	4.56±0.31*
	Group V	Chloroform extract of *Alstonia scholaris*	400 mg/kg	6.44±0.57***
	Group VI	Methanolic extract of *Alstonia scholaris*	200 mg/kg	4.77±0.59*
	Group VII	Methanolic extract of *Alstonia scholaris*	400 mg/kg	6.67±0.48***
	Group VIII	Water extract of *Alstonia scholaris*	200 mg/kg	4.13±0.55*
	Group IX	Water extract of *Alstonia scholaris*	400 mg/kg	4.16±0.28*
3	Group X	*Ocimum sanctum* Extract	100 mg/kg	7.78±0.38***

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

Cyclophosphamide-induced myelosupression

Cyclophosphamide at the dose of 30 mg/kg, i.p. caused a significant reduction in the haemoglobin, RBCs, WBCs and differential leukocyte count. In case of cyclophosphamide induced myelosupression, there was decrease in the WBCs count in the control group. In treatment groups, the RBCs, HB and WBC count was found to be increased with p<0 .01and p< 0.001 at 200 & 400 mg/kg dose respectively on 11th day.

Table 3: Effect of chloroform & Methanolic extracts on RBCs, WBCs and HB

S No.	Treatments	RBCs	WBCs	HB
		11th Day	**11th Day**	**11th Day**
1	Control	8.35±0.12	3.44±0.16	11.61±1.43
2	Cyclophosphamide	3.15±0.34**	0.98±0.22***	6.52±0.33**
3	Chloroform Extract of *Alstonia scholaris* (200 mg/kg)	6.14±0.55**	1.92±0.57**	8.91±0.66*
4	Chloroform Extract of *Alstonia scholaris* (400 mg/kg)	7.34±0.67**	2.95±0.78**	9.94±0.46*
5	Methanolic Extract of *Alstonia scholaris* (200 mg/kg)	6.91±0.73**	2.11±0.43***	9.33±0.21**
6	Methanolic Extract of *Alstonia scholaris* (400 mg/kg)	7.98±0.64**	2.99±0.76***	10.39±0.88**
7	*Ocimum sanctum* Extract (100 mg/kg)	8.18±0.42**	3.38±0.89***	10.78±0.59***

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.

DISCUSSION

The present aim of our study was to evaluate the pharmacological activity of different extracts of *Alstonia scholaris.*

In the preliminary phytochemical screening of extracts showed the presence of terpenoids, steroids, alkaloids, flavonoids, phenolic compounds, glycosides and sugar compounds.

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 (Smith and Kroes, 2000). Delayed type hypersensitivity (DTH) is antigen specific and cause erythema induction at the site of antigen infection in immunized animals. The histology of DTH can be different for different species but the general characteristics are influx of immune cells at the site of injection, macrophages and basophiles in rat's induction become apparent within 24-72 hrs[12].

In our study, chloroform extract and methanolic extract of *Alstonia scholaris* showed maximum activity in DTH model.

The carbon clearance assay was used to evaluate the effect on reticuloendothelial cell mediated phagocytosis[13,14]. When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. Rate of clearance of ink from blood is known as phagocytic index. The extract produced an increased in phagocytic index suggesting its effect on reticuloendothelial system.

In our study, chloroform and methanolic extract of stem bark of *Alstonia scholaris* showed maximum activity against phagocytic index.

Cyclophosphamide suppresses humoral, cellular, non-specific and specific cellular immune response. When animal was treated with cyclophosphamide then haemoglobin (Hb), RBC counts, WBC count, Lymphocyte% and Platelet count all are reduced significantly[13,14]. 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 chloroform and methanolic extracts 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.

CONCLUSION

On the basis of the results obtained in the present study it can be concluded that stem bark of *Alstonia scholaris* had 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.

Further detailed studies are required to isolate the active compounds from chloroform and methanolic extracts which may be responsible for immunomodulatory activity. The present findings are significant for the development of alternative, inexpensive and perhaps safer strategies for the treatment of diseases.

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