



## ***In-vivo* Immunomodulatory Activities of Isolated Compounds from the Leaves of *Amaranthus spinosus* and *Achyranthes aspera***

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### **Authors' contributions**

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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### **ABSTRACT**

The purpose of this study was to look into the Immunomodulatory action of isolated compound of *Achyranthes aspera* and *Amaranthus spinosus* in experimental model of immunity. Cellular immunity was carried out by neutrophil adhesion test and carbon clearance assay, whereas, humoral immunity was analyzed by mice lethality test and indirect haemagglutination assay. The dose was selected after acute toxicity study of isolated compound of *Achyranthes aspera* and *Amaranthus spinosus* and administered at 5 mg/kg orally. The Levamisole (0.68mg/kg, p.o) was used as standard. Isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) at 5 mg/kg produced significant increases in adhesion of neutrophils and an increase in phagocytic index in carbon clearance assay. Both doses of IAA and IAS significantly prevented the mortality induced by bovine *Pasteurella multocida* in mice. Treatment of animals with IAA, IAS and Levamisole significantly increased the circulating antibody titre in indirect haemagglutination test. Among the different Isolates, (IAS) was more effective in cellular immunity models than the (IAA). However, Both the Isolates IAA and IAS exhibited similar protection in humoral immunity procedures. It is concluded that isolated compound of *Achyranthes aspera*(IAA) and *Amaranthus spinosus*(IAS) possesses potential for augmenting immune activity by cellular and humoral mediated mechanisms more at dose of (5 mg/kg).

**Keywords:** *Isolated compound; Achyranthes aspera (IAA); Amaranthus spinosus (IAS) immunomodulation; neutrophil adhesion; phagocytic response.*

## 1. INTRODUCTION

A worldwide dependence on alternative medicine for chronic and acute illnesses has resulted in extensive research and the discovery of a number of plants with disease-fighting properties. A large range of herbs have been used in Ayurvedic formulations to modulate the immune system, either alone or in combination. Environmental contaminants and dietary habits disrupt immune functions, but a diet rich in micronutrients and antioxidants has been shown to mitigate these changes [1]. In the indigenous system of medicine, the usage of herbs as immunomodulators can indeed affect the body's defence mechanisms. Polysaccharides, lectins, peptides, flavonoids, and tannins are active elements of plant derivatives that have been shown to regulate the immune system in various experimental paradigms [2]. The leaves of *Achyranthes aspera* and *Amaranthus spinosus* are reported to contain many functional and bioactive compounds such as carotenoids, phenolic, alkaloids, coumarins, flavonoids, Terpenoids, and other antioxidants.

Vitamin C, vitamin A, thiamine, riboflavin, niacin, calcium, and phosphorus are just a few of the vitamins and minerals found in it [3]. As a result of the chemical profile, *Achyranthes aspera* and *Amaranthus spinosus* are likely to be good sources of immunomodulatory drugs. Furthermore, the plant's many parts have been utilized to treat a variety of ailments, including chronic diarrhea and dysentery, as well as work as a heart and brain tonic. It's frequently utilized as an indigenous traditional medicine for a number of stress-related conditions, including immunodeficiency. However, no scientific studies on isolated chemicals have been carried out to far to confirm its significance as an immunological stimulant. As a result, the goal of this research was to investigate the immunomodulatory activity of isolated compounds from *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) in several cellular and humoral immune models.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Animals

Laboratory bred Wistar albino rats (180–200 g) and albino mice (20–25 g) of either sex were housed at 25° ± 5 °C in a well-ventilated animal house under 12/12 h light/dark cycle. The mice were procured from College of Veterinary

Science and Animal Husbandry Mhow, Indore M.P, (India). The animals had free access to standard food pellets (Bird House Bhopal, India) containing (% w/w) protein 22.10, oil 4.13, fiber 3.15, ash 5.15, sand (silica) 1.12, and water ad libitum. To keep the animals clean and dry, bedding material was removed and replaced as needed with fresh rice husk. The animals were maintained under standard conditions in an animal house approved by Committee for the purpose of control and supervision on experiments on animals (CPCSEA). The animals were subjected for quarantine (10 days) prior to experimentation [4].

### 2.2 Procurement of Plant Material

Medicinal plants were chosen with the assistance of local herbal healers, and fresh leaves of selected medicinal plants were collected from Akshat Nursery karond near Bhopal (MP) between March and June 2020. Dr. Saba Naaz, HOD Department of Botany, Saifia Science College Bhopal, identified them further, and specimens were submitted and stored in the Department of Botany, Saifia Science College Bhopal. The specimen voucher numbers are 196/Saif/Sci/Clg/Bpl for *Achyranthes aspera* and 197/Saif/Sci/Clg/Bpl for *Amaranthus spinosus*, both dated 17/6/2020.

### 2.3 Chemicals and where they come from

The Leishmann's stain and gluteraldehyde (NS Scientific, Bhopal). HIMEDIA ink from India (NS Scientific, Bhopal). EDTA and WBC diluting fluid from (Gupta Pathology, Bhopal). *Pasteurella multocida* from cattle and its vaccine, as well as nylon fibres originating (Bhopal Memorial Hospital and research Centre, Bhopal).

### 2.4 Antigen Preparation

The local slaughterhouse provided fresh sheep blood for the experiment. For immunization and challenge, sheep red blood cells (SRBCs) were washed three times in large volumes of pyrogen-free 0.9 percent normal saline and adjusted to a concentration of 0.5 x 10<sup>9</sup>cells/ml [5].

### 2.5 Phytochemical Results

Preliminary phytochemical analysis was performed to check and identify active constituents of the hydro alcoholic extract of *Achyranthes aspera* and *Amaranthus spinosus* (Leaves), such as alkaloids, carbohydrates, flavonoids, terpenoids and steroids, Saponins

and tannins, using test methods such as Dragendorff's and Mayer's test, Molisch's and Fehling's test, lead acetate and magnesium ribbon test, Liebermann–B.

## 2.6 Acute Toxicity Studies [6]

To choose the dose, an acute toxicity study was conducted utilizing the up and down or stair case method. Two mice were chosen and given a dose of 50 mg/kg orally for 24 hours before being evaluated for death. To achieve the maximum non-fatal and minimum lethal dosages, the successive doses are increased by 1.5 times. According to the Office of Pollution Prevention and Toxics (OPPT) recommendations, the isolated chemicals were found to be safe at a dose of 5 g/kg p.o, which is 1/10th of the maximum acceptable dose (5 g/kg).

## 2.7 Experimental Protocol

For oral administration, the drug solutions were prepared in distilled water. Both cellular and humoral immunomodulatory activity were examined. The neutrophil adhesion test and carbon clearance assay were used to assess cellular immunity, while the mice lethality test and indirect haemagglutination assay were used to assess humoral immunity. There were four common groups of six animals in each of the experimental models. Group I was given vehicle (1 ml/100 g, p.o.), group II was given Levamisole (0.68 mg/kg, p.o. ), and groups III and IV were given IAA (5 mg/kg, oral) and IAS (5 mg/kg, oral) of *Achyranthes aspera* and *Amaranthus spinosus* isolated compounds, respectively. However, in the mouse lethality test, there was also a negative control group.

## 2.8 Neutrophil Adhesion Test [7,8]

The rats were given a 14-day oral pre-treatment with vehicle or isolated substances. Blood samples from the retro-orbital plexus were collected into heparinized vials at the end of treatment day 14 and tested for differential leukocyte count (DLC). Blood samples were incubated with 80 mg nylon fibres/ml for 15 minutes at 37 °C after the initial counts. To calculate the neutrophil index of blood samples, the incubated blood samples were examined for TLC and DLC again. The following formula was used to compute the % neutrophil adhesion:

$$\text{Neutrophil adhesion \%} = \frac{Nlu - Nlt}{Nlu} \times 100$$

Where Nlu is the neutrophil index of untreated blood samples and Nlt is the neutrophil index of treated blood samples.

## 2.9 Carbon Clearance Test [9,10]

Isolated compounds of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS), vehicle, and Levamisole therapy were given orally to Swiss albino mice for 10 days in their respective groups. Animals in all groups received an intravenous injection of (0.3 ml per 30 g) Indian ink (colloidal carbon) into the tail vein 48 hours after the previous dosage of the medication. After the ink injection, blood samples were taken from each animal via the retro-orbital plexus at intervals of 0 and 15 minutes. The absorbance of a 50-ll blood sample was measured at 660 nm after it was combined with 4 ml of 0.1 percent sodium carbonate solution. The phagocytic index K was calculated using the following formula:

$$K = \frac{(\text{Loge OD1} - \text{Loge OD2})}{15}$$

where OD1 and OD2 are the optical densities at 0 and 15 min, respectively.

## 2.10 Mice Lethality Test [11]

In each group, Swiss albino mice were given an isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) as well as Levamisole orally for 21 days. The animals were subcutaneously inoculated with haemorrhagic septicaemic vaccine (HS vaccine) on the 7th and 17th days of therapy. The animals were given 0.2 ml of fatal dosage (25x LD50) *Pasteurella multocida* (bovine origin) comprising 107 cells per ml subcutaneously on the 21st day. The animals were monitored for 72 hours before the fatality percentage was calculated.

## 2.11 Indirect Haemagglutination Test [7]

The medications were given to rats in separate groups for 14 days before they were inoculated intraperitoneally with 0.5x10<sup>9</sup> sheep red blood cells (SRBCs). The first day of vaccination was called day 0. The medication therapy was maintained for another 14 days, after which blood samples were obtained from each rat and the titre value was evaluated by titrating serum dilutions (50–100 l) with SRBC (0.025 x10<sup>9</sup> cells) in microtitre plates. The plates were incubated for 2 hours at room temperature before being visually checked for agglutination.

**Table 1. Effect of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole on neutrophil adhesion test**

Treatment	TLC (103/mm <sup>3</sup> ) (A)		Neutrophil% (B)		Neutrophil index (A x B)		Neutrophil adhesion (%)
	UB	NFTB	UB	NFTB	UB	NFTB	
Control	5.6 ± 0.16	5.5 ± 0.16	23.3 ± 0.80	22.5 ± 0.8	158.58 ± 5.4	151.5 ± 4.5	3.4 ± 0.6
Levamisole(0.68 mg/kg)	6.6 ± 0.18	5.8 ± 0.15	26.6 ± 1.08	18.6 ± 0.4	207.23 ± 6.4	133.0 ± 4.2	33.4 ± 0.6***
IAS mg/kg	6.7 ± 0.12	5.8 ± 0.13	27.0 ± 1.33	16.6 ± 1.0	218.13 ± 4.7	119.3 ± 8.5	44.1 ± 1.2***
IAA 5mg/kg	6.3 ± 0.86	5.9 ± 0.49	24.5 ± 1.23	17.6 ± 1.2	185.88 ± 8.8	128.9 ± 9.1	28.7 ± 2.0***

All values are expressed as mean ± SEM of six observations; UB, untreated blood; NFTB, nylon fiber treated blood; \*\*\* P < 0.001 when compared to control.

**Table 2. Effect of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole on phagocytic index and HA titre**

Treatment	Phagocytic index in carbon clearance assay	Haemagglutination (HA) titre (µl)
Control	0.0174 ± 0.0019	0.0874 ± 0.2562
Levamisole (0.68 mg/kg,po)	0.0482 ± 0.002***	0.0018 ± 0.0003***
IAS mg/kg,po	0.0422 ± 0.0027***	0.0018 ± 0.0003***
IAA 5mg/kg,po	0.0415 ± 0.0016***	0.0043 ± 0.0008***

All values are expressed as mean ± SEM of six observations. . \*\*\* P < 0.001 when compared to control.

**Table 3. Effect of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole on mice lethality test**

Treatment dose	Mortality first day	Second day	Third day	Mortality percentage
No drug, no vaccination	2	4	-	100
No drug, vaccination	1	3	1	83.33
Levamisole(0.68 mg/kg,po)+ vaccination	-	1	3	66.66
IAS mg/kg,po + vaccination	-	2	2	66.66
IAA 5mg/kg,po + vaccination	-	3	1	66.66

Haemagglutination (HA) titre was defined as the minimal volume of serum that showed haemagglutination.

### 2.12 Statistical Analysis

One-way analysis of variance (ANOVA) and Bonferroni's comparison test were used to determine statistical significance. The values were expressed as mean  $\pm$  SEM and  $P < 0.05$  was considered significant.

## 3. RESULTS AND DISCUSSION

### 3.1 Neutrophil Adhesion Test

Due to neutrophil adherence to the fibres, incubation of blood with nylon fibres (NF) resulted in a drop in neutrophil numbers. When compared to control, both dosages of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole significantly increased neutrophil adherence. It was discovered that the 5mg/kg dose of (IAS) was more efficacious than the 5mg/kg dose of (IAA). The neutrophil count increased in all treatment groups' untreated blood (Table 1).

### 3.2 Carbon Clearance Test

When compared to control, both dosages of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole exhibited a substantial rise in the phagocytic index, indicating that colloidal carbon clearance from the blood increased following administration of these medications. However, a 5mg/kg dosage of (IAS) plus Levamisole provided the greatest clearance (Table 2).

### 3.3 Mice Lethality Test

When *Pasteurella multocida* was given to the control group, 100 percent of them died within 72 hours. Without any prior medication therapy, the vaccinated group had an 83.33 percent mortality rate. The 5mg/kg doses of IAA and IAS as well as Levamisole reduced the mortality percentage to 66.66% (Table 3).

### 3.4 Indirect Haemagglutination Test

The haemagglutination antibody (HA) titre value was significantly increased in animals that received vaccination along with 5mg/kg dose of IAA and IAS or Levamisole compared to animals that received vaccination alone (Table 2).

## 4. CONCLUSION

We discovered that an isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) had immunomodulatory effects in cellular and humoral immunity models in this work. The research was conducted using four distinct ways, each of which offers information on the influence on various immune system components. Plant products can either stimulate or depress immunological responses, and may be used as a supportive treatment with conventional medications in immune-compromised patients [12].

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

The experimental protocol was approved by Institutional ethical committee (RKDFCP/IAEC/2020/32).

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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