IN-VIVO IMMOUNOMODULATORY ACTIVITIES OF ISOLATED COMPOUNDS FROM THE LEAVES OF *AMARANTHUS SPINOSUS* AND *ACHYRANTHES ASPERA*

Abstract

The aim of the present study was to investigate the Immounomodulatory 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 IIA, IAS and Levamisole significantly increased the circulating antibody titre in indirect haemagglunation 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. From the above findings, 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 doseof (5 mg/kg).

Keywords: Isolated compound**,** *Achyranthes aspera* (IAA), *Amaranthus spinosus* (IAS) Immunomodulation; Neutrophil adhesion; Phagocytic response

Introduction

A global reliance on alternative system of medicine for chronic and acute ailments resulted in an intense area of research and discovery of a number of herbs with potential to curb diseases. Among them, ample number of herbs has been exploited for modulation of immune system from Ayurvedic formulation either alone or in combinations. Environmental pollutants and dietary habits cause disturbances in immune activities and diet containing micronutrients and antioxidants are known to prevent these alterations [1]. The use of herbs as Immounomodulatory in the indigenous system of medicines, indeed, can modulate the body's defense mechanism. The following active constituents of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported to modulate the

immune system in different experimental models [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.

In addition, it also has many vitamins and minerals including vitamin C, vitamin A, thiamine, riboflavin, niacin, calcium, and phosphorus [3]. Therefore, the chemical profile indicates *Achyranthes aspera*and *Amaranthus spinosus* as good sources of Immounomodulatory agents. Further, the various parts of these plant has been used for many disorders such as chronic diarrhea& dysentery and act as a tonic for the heart and brain. It is widely used as indigenous traditional medicine for variety of stress disorders including immunodeficiency. However, till date no scientific evaluations are conducted for confirming its role as immune-stimulant on isolated compounds. Thus, this study was designed to study the Immounomodulatory activity of isolated compound of *Achyranthes aspera*(IAA) and *Amaranthus spinosus*(IAS) in different experimental models of cellular and humoral immunity in animals.

Materials and methods

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. Bedding material was removed and replaced with fresh paddy husk as often as necessary to keep the animals clean and dry. 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 experimental protocol was approved by Institutional ethical committee (RKDFCP/IAEC/2020/32). The animals were subjected for quarantine (10 days) prior to experimentation

Procurement of plant material

Medicinal plants were selected by the help of local herbal healers and the fresh leaves of selected medicinal plants, were collected from Akshat Nursey karond Bhopal (M.P), during March to June, 2020. These were further identified by Dr. Saba Naaz, HOD Department of Botany, Saifia Science College Bhopal, and specimens have been submitted and preserved in the Department of Botany, The Specimen voucher no. (*Achyranthes aspera* is

196/Saif/Sci/Clg/Bpl) and (*Amaranthus spinosus* is 197/Saif/Sci/Clg/Bpl) dated 17/6/2020.

Chemicals and their sources

Leishmann's stain and gluteraldehyde were purchased from Merck (NS Scientific, Bhopal). Indian ink from HIMEDIA (NS Scientific, Bhopal). WBC diluting fluid and EDTA from (Gupta Pathology, Bhopal). Pasteurella multocida of bovine origin and its vaccine and Nylon fibers From (Bhopal Memorial Hospital and research Centre, Bhopal).

Antigen preparation

Fresh sheep blood was collected from the local slaughterhouse. 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 x 109cells/ml for immunization and challenge [4].

Preliminary phytochemical

Preliminary phytochemical analysis was carried out to check and identify the active constituents of the Hydro alcoholic extract of *Achyranthes aspera* and *Amaranthus spinosus* (Leaves)such as alkaloids, carbohydrates, flavonoids, terpenes and steroids, Saponins and tannins by using test methods of Dragendroffs and Mayer's test, Molisch's and Fehling's test, lead acetate and magnesium ribbon test, Liebermann–Burchard test, foam formation test, ferric chloride test and gelatin test, respectively.

Acute toxicity studies [5]

The acute toxicity study was carried out to select the dose, by using up and down or stair case method. Two mice were selected with a dose of 50 mg/kg orally and examined for a period of 24 h for mortality. The subsequent doses are then increased by 1.5 factors to attain maximum non-lethal and minimum lethal dose. The Isolated compounds was found to be safe at the dose of 5 g/kg p.o According to office of pollution prevention and toxics (OPPT) guidelines, 1/10th of the maximum safe dose (5 g/kg).

Experimental protocol

The drug solutions were prepared in distilled water for oral administration. Immounomodulatory activity was checked both at cellular and humoral levels. Cellular immunity was evaluated by neutrophil adhesion test and carbon clearance assay, whereas, humoral immunity was analyzed by mice lethality test and indirect haemagglutination assay. All the experimental models had four common groups consisting of six animals each. Group I, was served as control and received (vehicle 1 ml/100 g, p.o), group II, received the Levamisole (0.68 mg/kg, p.o) , whereas groups III and IV were administered IAA (5 mg/kg, oral) and IAS (5 mg/kg, oral) of *Achyranthes aspera* and *Amaranthus*

*spinosus*isolated compounds, respectively. However, in mice lethality test, an additional negative control group was also present.

Neutrophil adhesion test [6-7]

The rats were pre-treated orally with vehicle or isolated compounds for 14 days. At the end of treatment day 14, blood samples were collected from the retro-orbital plexus into heparinized vials and analyzed for differential leukocyte count (DLC). After the initial counts, blood samples were incubated with 80 mg nylon fibres/ml for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC, respectively to give neutrophil index of blood samples. The percent neutrophil adhesion was calculated as follows:

$$\text{Neutrophil adhesion } \% = \frac{\text{Nlu} - \text{Nlt}}{\text{NIu}} x100$$

Where NIu is the neutrophil index of untreated blood samples and NIt is the neutrophil index of treated blood samples.

Carbon clearance test[8-9]

Swiss albino mice were administered isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS), vehicle and Levamisole treatment orally for 10 days in their respective groups. Forty- eight hours after the last dose of the drug, animals of all the groups received intravenous injection of (0.3 ml per 30 g) Indian ink (colloidal carbon) via the tail vein. Blood samples were withdrawn from each animal by retro-orbital plexus at an interval of 0 and 15 min after the ink injection. A 50-ll blood sample was mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution was determined at 660 nm. The phagocytic index K was calculated using the following formula:

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

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

Mice lethality test [10]

Swiss albino mice were pretreated with isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisoleorally for 21 days in their respective groups. On the 7th and 17th day of the treatment, the animals were immunized with haemorrhagic septicaemic vaccine (HS vaccine) through subcutaneous route. On the 21st day, the animals were challenged subcutane-ously with 0.2 ml of lethal dose (25x LD50) of *Pasteurella multocida* (bovine origin)

containing 107 cells per ml. The animals were observed for a period of 72 h and the mortality percentage was determined.

Indirect haemagglutination test [6]

Rats of various groups were pretreated with the drugs for 14 days and all rats of entire groups were immunized with 0.5x109 sheep red blood cells (SRBCs) intraperitoneally. The day of immunization was referred to as day 0. The drug treatment was continued for 14 more days and blood samples were collected from each rat at the end of the drug treatment and the titre value was determined by titrating serum dilutions (50–100 µl) with SRBC (0.025 x109 cells) in microtitre plates. The plates were incubated at room temperature for 2 h and examined visually for agglutination. The minimum volume of serum showing heamagglutination was expressed as heamagglutination (HA) titre.

Statistical analysis

The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Bonferroni's comparison test. The values were expressed as mean ± SEM and $P < 0.05$ was considered significant.

Results and Discussion

Neutrophil adhesion test

Incubation of blood with nylon fibers (NF) produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibers. Both doses of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole showed significant increase in the neutrophil adhesion when compared to control. The 5mg/kg dose of (IAS) was found to be more effective than 5mg/kg dose of (IAA). There was also rise in neutrophil count in untreated blood of all treatment groups (Table 1).

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

Treatment	TLC (103/mm3) (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.

Carbon clearance test

Both doses of isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus* (IAS) and Levamisole showed significant increase in the phagocytic index when compared to control indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these drugs. However, the clearance was best with 5mg/kg dose of (IAS) and Levamisole (Table 2).

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.

Mice lethality test

Mortality was found to be 100% within 72 h in control group upon administration of *Pasteurella multocida*. There was 83.33% mortality in vaccinated group without any prior treatment of drug. The 5mg/kg doses of IAA and IAS as well as Levamisole reduced the mortality percentage to 66.66% (Table 3).

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

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).

Conclusion

In this study we found that isolated compound of *Achyranthes aspera* (IAA) and *Amaranthus spinosus*(IAS) possesses Immounomodulatory activity in experimental models of cellular and humoral immunity. The study was carried out using four different methods, each of which provides information about effect on different components of the immune system. The variety of plant products can modulate immune reaction either by stimulation or suppression and may assist as a supportive therapy along with conventional drugs in immune compromised patients (Wagner, 1984)[11].

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

References

1. Bafna, A. and Mishra, S., 2010. Antioxidant and immunomodulatory activity of the alkaloidal fraction of Cissampelo spareira linn. *Scientia pharmaceutica*, *78*(1), pp.21-32.
2. Shivaprasad, H.N., Kharya, M.D., Rana, A.C. and Mohan, S., 2006. Preliminary Immunomodulatory Activities of the Aqueous Extract of *Terminalia chebula*. *Pharmaceutical Biology*, *44*(1), pp.32-34.
3. Das B, Das RD. Medicinal properties and chemical constit- uents of *Aegle marmelos* Correa. Indian Drugs 1995; 32, 93–99.
4. Thomas, L., Asad, M., Hrishikeshavan, H.J. and Chandrakala, G.K., 2007. Effect of centchroman on cellular and humoral immunity. *Indian J Physiol Pharmacol*, *51*(4), pp.387-394.
5. Ghosh, M.N., 1984. Fundamentals of Experimental Pharmacology, second ed. Scientific Book Agency, Kolkata (India), pp. 156–157.

6. Fulzele, S.V., Satturwar, P.M., Joshi, S.B. and Dorle, A.K., 2003. Study of the immunomodulatory activity of *Haridra dighrita* in rats. *Indian journal of Pharmacology*, *35*(1), p.51.
7. Shinde, U.A., Phadke, A.S., Nair, A.M., Mungantiwar, A.A., Dikshit, V.J., Saraf, M.N., 1999. Preliminary studies on the immunomod- ulatory activity of *Cedrus deodara* wood oil. Fitoterapia 70, 333– 339.
8. Jayathirtha, M.G. and Mishra, S.H., 2004. Preliminary immunomodulatory activities of methanol extracts of Ecliptaalba and *Centella asiatica*. *Phytomedicine*, *11*(4), pp.361-365.
9. Gokhale, A.B., Damre, A.S. and Saraf, M.N., 2003. Investigations into the immunomodulatory activity of *Argyreia speciosa*. *Journal of ethnopharmacology*, *84*(1), pp.109-114.
10. Ramanatha, K.R., Lakshminaryana, R. and Gopal, T., 1995. Potency test of Duck Pasteurella vaccine in mice. *Mysore J Agri Sci*, *29*, pp.155-7.
11. Wagner, H., 1984. In Economic and Medicinal Plant Research (Hiroshi Hikino, NR, Farnsworth, Eds.), vol. I.