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Immunomodulatory Potential of Alkaloid-Rich Fraction of *Abrus precatorius* seeds Methanol Extract

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ABSTRACT

BACKGROUND: Modulation of the immune system plays an essential role in managing health and disease conditions in humans. Immunomodulation has been reported to effectively treat some diseases, especially those involving immune responsiveness or adverse immune reaction. A critical current feature of medicinal plants is the modulation of immune functions by certain plants and plant-derived products. **OBJECTIVE**: The aim of this research was to ascertain the immunomodulatory properties of *Abrus precatorius* alkaloid-rich seed fraction. **METHODS:** This study employed the delayed-type hypersensitivity reaction in rats, titer of haemagglutinating antibodies (HA), and in-vivo leukocyte mobilization models. The experimental animals were split into five groups of four. Group 1 received the vehicle, while Groups 2, 3, and 4 received doses of seed fraction of 50, 100, and 200 mg/kg b.w., respectively, and Group 5 received 2.5 mg/kg b.w. of the reference drug levamisole. **RESULTS**: Alkaloid-rich seed fraction significantly ($p<0.05$) decreased rat paw volume (oedema) relative to the control in a graded manner. Intraperitoneal (i.p.) administration of alkaloid-rich seed fraction at doses of 100 and 200 mg/kg b.w. to experimental animals significantly ($p<0.05$) elevated both primary humoral antibody titer as well as the secondary in a dose-related manner. Also, the tested groups showed a substantial and dose-dependent rise in leukocyte mobility into the peritoneum, with the lowest tested dose being the most effective. **CONCLUSION:** The inhibition of DTH response (immunosuppression) and the stimulation of both the humoral immunity and in-vivo leukocyte mobilization (immunostimulation) establish the immunomodulatory properties of the alkaloid-rich seed fraction of *Abrus precatorius.*

Keywords: *Abrus precatorius,* Alkaloids, immunomodulation, hypersensitivity, humoral immunity .

INTRODUCTION

Exposure to various antigens activates both cell-mediated immunity and humoral immunity. Excessive immune system activity results in a hypersensitivity reaction, which may be harmful to the body or cause death. The immune system overreacts in delayed-type hypersensitivity (DTH) reaction, causing phagocytes to destroy local tissue. The DTH reaction can be generated in experiments by injecting low doses of sheep red blood cells (RBCs), which triggers the proliferation of TH1 cells (Ofokansi et al., 2018). TH1-type cytokines typically generate proinflammatory cytokines. However, excessive pro-inflammatory responses may result in uncontrolled tissue damage (Chen et al., 2017), so it is essential to keep them under control. Antigen exposure activates naive B lymphocytes, separated into antibody-producing plasma cells and memory cells in humoral immunity.

Antibodies will carry out their effector functions regardless of where they were produced. For example, the innate immune response of leukocyte mobilization involves recruiting non-specific leukocytes to the site of tissue damage or infection. These white blood cells, or leukocytes, are in charge of the organism's protection, forming the immune system. Immunomodulators are substances that can strengthen or weaken the immune system's component and could be classified as immunostimulants or suppressants. Immunostimulants help improve the body's resistance to infections, treat immunodeficiency disorders and cancers (Bascones-

Martinez et al., 2014). Immunosuppressants inhibit immune responses and may be used to treat pathological immune responses, including Crohn's disease, and prevent organ transplant rejection.

Abrus precatorius Linn belongs to the Fabaceae family of leguminous plants. Its seeds, roots, and leaves are commonly used in Africa and Asia for medicinal purposes (Nwodo, 1981; Okhale and Nwanosike, 2016). For example, in Nigeria, the Igbos use aqueous decoctions of the seeds to treat many conditions, including malaria, ulcer, infections, hypertension, diarrhoea, infarct, and Ogbanje (Nwodo and Alumanah, 1991; Okhale and Nwanosike, 2016). Thus, this research aimed to ascertain the immunomodulatory properties of *Abrus precatorius'* alkaloid-rich seed fraction.

2. MATERIALS AND METHODS

2.1 Plant material

Mr. A. Ozioko of Bioresource Conservation and Development Programme (BDCP) Nsukka, Enugu State Nigeria authenticated *Abrus precatorius* Linn seeds (Fabaceae) accessed from local regions of Enugu Ezike, Nsukka (Nigeria).

2.2 Animals

Swiss albino male rats (100-250 g) were collected from the animal house of the Department of Veterinary Medicine, University of Nigeria, Nsukka. Under normal conditions of 12 h light/dark cycle and temperature of 25 ^{0}C, these animals were held in galvanized wire cages and fed standard pellets with a free supply of water. They acclimatized to conditions in the lab for two weeks. Experimental procedures were reviewed by the University's Animal Ethical Committee. The number of experimental rats used in the research was minimal, with optimal care to reduce animal suffering.

2.3 Antigen

Blood (10 ml) was drawn aseptically from a healthy male adult sheep's jugular vein and transferred to EDTA bottles under sterile conditions. Sheep erythrocytes (red blood cells) were centrifuged for 10 minutes at 3000 g per time after being washed 4–5 times with pyrogen-free sterilized regular saline, according to the method of Nworu et al. (2007) and Omeje et al. (2011). The prepared SRBC batch was used for sensitization and challenge after being adjusted with normal saline to a concentration of 109 cells/ml.

2.4 Processing of plant material

In a round bottom flask with a glass stopper, pulverized *Abrus precatorius* Linn (2 kg) seeds were soaked in five volumes of chloroform/methanol in a 2:1 ratio, forcefully shaken allowed to remain for 18 hours at a temperature of 25 ^{0}C. The filtrate was washed thoroughly using 20 percent distilled water in a separating funnel, leading to two different layers. Aqueous methanol component of the upper phase was drained, dried, and weighed.

2.5 Fractionation of the aqueous methanol plant extract

On a 1.5 x 25 cm column filled with Sephadex G-15 gel swollen in distilled water, aqueous methanol extract (2 g) dissolved in distilled water was layered and eluted with distilled water. In serially arranged test tubes, 50 aliquots of 3 ml each were collected. The ultraviolet absorption of each fraction was measured at 265 nm.

2.6 Thin-layer chromatography (TLC)

Aliquots of the fractions were spotted on 20 x 20 cm activated chromatoplates precoated with silica gel F254 (110 ^{o}C for one h in preheated oven). The plates were air-dried after being developed for 1 hour in a chromatographic tank pre-equilibrated with a solvent system of butanol, acetic acid, and water (65:13:22). Following that, Drangendorff's spray reagent was used to test several bands of the fractions. The alkaloid-rich fraction was made up of fractions that were violet on orange in color.

2.7 Delayed type hypersensitivity (DTH) response

Sharma et al. (1996) investigated the delayed-type hypersensitivity (DTH) response induced by SRBC in rats. In the experiment, there were five groups of four animals each. As a control, Group 1 received normal saline (0.2 ml). The fraction was given to groups 2, 3, and 4 at doses of 50, 100, and 200 mg/kg b.w. respectively, while Group 5 received a commonly used immunomodulatory drug Levamisole (2.5 mg/kgb.w.). The administration started 72 hours before the challenge and continued every day until it was exhausted. Animals were sensitized on day 0 of the study by injecting into the animals' right hind paw plantar region, 109 cells/ml SRBC (0.1 ml). On the 5th day, equal volume of antigen (SRBC) was injected into the foot plantar region of the left hind. The technique described by Fletcher et al. (2006) was used to assess oedema. The difference in the left and right plantar circumferences of the injected paw was measured with a thread at the metatarsal level 24 hours post-challenge and correlated to the values obtained for group that received the vehicle.

2.8 Humoral antibody response

Intraperitoneal inoculation of 109 cells/ml of SRBC (0.2ml) in normal saline was given to rats on day 0, and on the 7th day, the rats were challenged with the same volume of antigen (i.p). The alkaloid-rich leaf fraction of *Abrus precatorius* was orally given to the experimental animals in the respective test groups. The standard control group was given 2.5 mg/kg b.w. Levamisole 72 hours before immunization and a single daily dose for seven days after that. Every antigenically sensitized rat's blood drawn from retro-orbital plexus was centrifuged for 10 minutes at 3000 x g, and 25 ml serum was serially diluted two-fold in a 96-well microtitre

plate with standard saline. After being defied with 25 ml of 1% (v/v) SRBC, the plates were incubated for 24 hours at 37°C for 1 hour before being checked for haemagglutination (prepared in regular saline). On day 7 (before the challenge), the primary antibody titer was assessed, while the secondary antibody titer was determined on the 14th day using the standard haemagglutination test (Nelson and Mildenhall, 1967). The antibody titer was determined using the optimum dilution that resulted in evident haemagglutination. Antibody titers were rated from least (0.5) to most diluted (calculated as – Log2 of dilution factor). For statistical significance, the mean values of various treatment groups were compared.

2.9 In vivo leukocyte migration

Applying the method of Ribeiro et al. (1991), alkaloid-rich seed fraction on *in vivo* leukocyte mobilization in rats was examined. Animals were given alkaloid-rich seed fraction (50, 100, and 200 mg/kgb.w) orally and intraperitoneally administered 1 ml of a 3 percent (w/v) agar suspension in sterile saline. After four hours, the animals were sacrificed and phosphate buffered saline (PBS) containing 10 percent EDTA (0.5 ml) was used to wash the peritoneal cavities. On the perfusates recovered in the peritoneal wash, total leukocyte count (TLC) and differential leukocyte counts (DLC) were done. The strength of inhibition (percent) of neutrophil and lymphocyte migration was determined using the relationship:

$$\text{Inhibition of leukocyte mobilization (\%)} = \frac{\text{TLC (control)} - \text{TLC (test)}}{\text{TLC (control)}} \times 100$$

2.10 Estimation of total leukocyte count (TLC)

The perfusates in the tubes were drawn up to the 0.02 ml calibration of the white blood cell dilution capillary and mixed with 0.38 ml of leukocyte diluting fluid (3 percent acetic acid). This was left to stand for 10 minutes to lyze the erythrocytes. The diluted sample was then placed in the Neubauer counting chamber, and the setup was microscopically examined under 100 magnifications in oil immersion. The total leukocyte count (TLC) was calculated at the end by counting the number of white cells in each of the four broad squares:

TLC = n × 20 × 2.5.

The dilution factor is 1:20, and the counting chamber volume factor is 1/0.4, where n is the number of cells counted.

2.11 Estimation of differential leukocyte count (DLC)

The Leishman staining technique was used by Dacie et al. (2006). Using an applicator, a drop of the fluid was applied on one end of the glass slide. Applying the push wedge method, another glass slide was used to produce a spread of the liquid on the glass. After applying the stain to the film, it was allowed to sit for two minutes before being removed. The thin film was then flooded with twice as much distilled water as a dye. The rig was gently rocked for 2 minutes before rinsing the stain and standing for 15 minutes. After drying, the slide was observed under a microscope at X 100 magnification with an oil immersion objective lens. Using a tally clock, the cells were counted and morphologically distinguished.

2.12 Statistical analysis

The Duncan multiple test range was used to compare means in this study's findings, which were statistically analyzed using one-way ANOVA. The data were presented as a mean with a standard deviation (SD). The IBM SPSS statistical program version 20 was used to carry out the study.

3.0 RESULTS

3.1 Effect of alkaloid-rich seed fraction on DTH response in rats

As determined by a rise in rat paw volume, the mean DTH response (0.625±0.047 mm), in experimental animals treated with sheep red blood cells (RBCs) formulated in normal saline was relatively high. Treatment with Levamisole, a reference drug, inhibited this response by 24.00 %, as shown in Table 1. Furthermore, when compared to the control, the alkaloid-rich seed fraction inhibited the DTH response significantly ($p<0.05$), with a decline from 72.00 percent to 48.00 percent as the dose increased from 50 to 200 mg/kg. Alkaloid-rich seed fraction of *Abrus precatorius* inhibited DTH response by 72 percent and 56 percent at 50 and 100 mg/kg b.w., respectively, relative to standard control, Levamisole.

Table 1: The inhibitory effect of alkaloid-rich seed fraction of *Abrus precatorius* treatment on DTH in rats

Treatment	Dose mg/kg, p.o	DTH response Oedema (mm) (Mean ± SD)	Inhibition (%)
Alkaloid-rich seed fraction	50	0.175 ± 0.075	72.00*
	100	0.275 ± 0.063	56.00*
	200	0.325 ± 0.095	48.00*
Levamisol	2.5 mg/kg	0.475 ± 0.025	24.00*
Control (NS)#	0.2 ml	0.625 ± 0.047	0

Values represent the mean ± SD of 4 animals in each group. * indicates signifiance at $p<0.05$. NS#: Normal saline

3.2 Effect of alkaloid-rich seed fraction of *Abrus precatorius* on SRBC-induced humoral antibody titers in rats

The control group had relatively low antibody titer values after being immunized and then challenged with SRBCs prepared in normal saline, with mean antibody titer values of 0.753±0.15 (primary) and 1.505±0.30 (secondary). As shown in Table 2, groups treated with reference drug levamisole showed significantly higher levels of antibodies in primary (2.408±0.00) as well as secondary (5.418±1.52) antibody responses as compared to the control group ($p<0.05$). The alkaloid-rich seed fraction, at all the tested doses, increased both primary and secondary humoral antibody titers significantly ($p<0.05$) and dose-dependently at i.p doses of 100 and 200 mg/kg b.w. Surprisingly, on the 14th day, the significant ($p<0.05$) stimulatory effect produced by alkaloid-rich fraction on secondary antibody production at 50 mg/kg was similar to the standard immunostimulant drug (levamisole). In addition, at the highest tested dose of 200 mg/kg, alkaloid-rich seed fraction of *Abrus precatorius* produced a maximum secondary antibody titer value of 10.080±3.03, which was found to be significant when compared to the effect of levamisole.

Table 2: Effect of alkaloid-rich seed fraction of *Abrus precatorius* on SRBC-induced humoral antibody titres in rats

Treatment	Dose	Haemagglutination antibody titre	
		Primary	Secondary
Alkaloid-rich seed fraction	50 mg/kg	1.505 ± 0.30 (99.87)	5.418 ± 1.52 (260)*
	100 mg/kg	2.107 ± 0.30 (179.81)*	6.020 ± 1.20 (300)*
	200 mg/kg	3.010 ± 0.60 (299.73)*	10.080±3.03 (570)*
Levamisol	2.5 mg/kg	2.408 ± 0.00 (219.79)*	5.418 ± 1.52 (260)*
Control (NS)#	0.2 ml	0.753± 0.15	1.505 ± 0.30

***** indicates significance at $p<0.05$; n = 4, NS#: Normal saline; Percentage of humoral stimulation is shown in parenthesis.

3.3 Effect of alkaloid-rich seed fraction of *Abrus precatorius* on *in vivo* leukocytes mobilization

The control group had a lower mean total leukocyte count (TLC) of 3366.75±138.94 mm^3 in the evaluation of *in vivo* leukocyte mobilization induced by intraperitoneal injection of agar suspension in normal saline. Levamisol, the standard treatment, increased the mean total leukocyte count by 112 percent (7137.50±1252.06 mm^3). Administration of the alkaloid-rich seed fraction to the respective groups of animals increased leukocyte recruitment into the peritoneum in a dose-dependent manner. Maximum activity of up to 145.41 percent was observed at the lowest tested dose of 50 mg/kg b.w. Though not statistically significant or dose-related, there were higher proportions of neutrophils in the perfusates compared to lymphocytes in all groups given the alkaloid-rich seed fraction (Table 3).

Table 3: Effect of alkaloid-rich seed fraction of *Abrus precatorius* on *in vivo* leukocytes mobilization

Test sample	Dose	TLC (cells/mm^3)	Leukocyte mobilization (%)	Differential leukocyte Mobilization (%)	
				Neutrophils	Lymphocytes
Alkaloid-rich fraction	50 mg/kg	8262.50 ± 772.54	145.41*	71.75 ± 3.52	26.25 ± 2.72
	100 mg/kg	7875.00 ± 914.35	133.91*	71.75 ± 2.84	26.25 ± 3.57
	200 mg/kg	7662.50 ± 154.94	127.60*	73.00 ± 2.38	22.75 ± 2.14
Levamisol	2.5 mg/kg	7137.50 ± 125.06	112.00*	72.25 ± 3.33	26.25 ± 2.87
Control (NS)#	0.2 ml	3366.75 ± 138.94	-----	69.25 ± 4.29	26.00 ± 3.34

TLC= total leukocyte count; n = 4; * indicates significance at $p<0.05$; NS#: Normal saline

4.0 DISCUSSION

Fractionation of the alkaloid-rich seed fraction in butanol-acetic acid-water (63:13:22) yielded several bands. The least mobile of them (Rf = 0.10) was positive for alkaloid testing with Drangendorff's spray reagent, indicating a violet spot on orange color. This substantiates earlier finding on the methanol extract, reported by Nwodo and Botting (1983), to contain the violet-colored stimulatory substance. The effect of the Sephadex G15 separated alkaloid-rich seed fraction of *Abrus precatorius* on delayed-type hypersensitivity (DTH) response revealed that all groups of rats treated with the fraction showed a significant ($p<0.05$), dose-dependent decrease in the mean difference of immunological paw oedema over a 24-hour period when compared to the control group. The dose-dependent suppression of cellular immunity, as demonstrated by inhibitions of 72 % (50 mg/kg b.w), 56 % (100 mg/kg b.w), and 48 % (200 mg/kg b.w), indicates that the best effect was obtained at doses less than 50 mg/kg b.w. The immunosuppression caused by the 50 and 100 mg/kg b.w fractions was significant ($p<0.05$) when compared to the suppression caused by the immunostimulant drug levamisol, which was used as standard control. SRBC stimulates a T lymphocyte cell-dependent immune response that is solely mediated by CD4 T lymphocyte cells. A low-dose injection of SRBCs causes the initiation of TH1 cells (a subset of CD4 T lymphocyte cells), which then leads to DTH (Stamm et al., 2013). According to an earlier report by Lele (2001) that TH1 lymphocyte pathway regulates cell-mediated immunity, the significant ($p<0.05$) immunosuppression observed could be due to repression of the TH1 T-lymphocyte pathway cellular immune response, which prevents the synthesis and secretion of Interleukin-12, a cytokine which promotes TH1 T-lymphocyte-mediated immunity. As a result, recruitment of both specific and non-specific effector cells is inhibited, resulting in dermal inflammation.

Earlier report has shown that immune cells are involved in the mediation of inflammatory responses (Deng et al. 2017), therefore the immunosuppression elicited by alkaloid-rich seed fraction of *Abrus precatorius* indicates anti-inflammatory activity. Furthermore, previous research on the membrane stabilization properties of *Abrus precatorius* seed spasmolytic substance (BN) in

limiting the ratio of membrane surface area to cell volume (Nwodo et al., 2008) suggests that immunosuppression of cell-mediated immunity by alkaloid-rich fraction may be immunoprotective. Given the importance of cell-mediated immunity responses in the defense against infectious organisms, tumor immunity, and delayed hypersensitivity reactions, this fraction may be useful in the treatment of these conditions.

The titer of haemagglutinating antibodies is an important parameter to consider when studying humoral responses, which include antigen and antibody responses. In other words, antibody-mediated primary immune responses as well as the secondary immune rresponses to antigens are thus referred to as humoral immunity. Primary responses are elicited when naive B cells are activated, whereas secondary responses are elicited when memory B cells are stimulated. When compared to the control group, administration of alkaloid-rich seed fraction (50, 100, and 200 mg/kg b. w) elicited a dose-dependent elevation of humoral antibody titer in response to SRBC, with significant ($p<0.05$) increases observed at doses of 100 mg/kg and 200 mg/kg in both humoral immune responses, indicating an immunostimulatory activity. Improved function of T-, B-cells and macrophage subsets involved in synthesis of antibodies, as demonstrated by increased antibody responsiveness to SRBC, reflects an improved function of the humoral response (Rajesh et al., 2011). Because TH2 helper cells assist B cells and are required for antibody-mediated immunity, alkaloid-rich seed fraction of *Abrus precatorius* is likely to influence the TH2 helper cell pathway and facilitate the formation and proliferation of interleukin 4. (IL-4). Interleukin 4 (IL-4) promotes IgE antibody synthesis in B cells and acts as a positive feedback device, directing more pre-TH cells into the TH2 pathway. The formation and release of interleukin 4 (IL-4) results in increased antibody formation (Heeb et al. 2020). The potent immunostimulation of humoral immunity caused by the alkaloid-rich seed fraction of *Abrus precatorius* may possibly induce life-long immunity following infection due to the high level of protection conferred by the increased secondary antibody titer produced by memory cells. Following treatment with the alkaloid-rich seed fraction of *Abrus precatorius*, there was significant ($p<0.05$) and dose-related raise in agar-induced leukocyte recruitment into the peritoneum of rats, indicating immunostimulation. Because complement fragments (particularly C3a, C4a, and C5a) and chemokines primarily function as chemoattractants for leukocytes from the blood to the sites of infection, the immunostimulatory activity of the fraction on leukocyte chemotactic movement, as indicated by the increased number of leukocytes in the perfusate, suggests that the fraction may be acting as a chemoattractant for leukocytes. This is in accordance with the findings of Ganachari et al (2004) that the observed rise in the amount of neutrophils at the site of infection, as shown by the higher percentage of neutrophils relative to lymphocytes, correlates to the first and most critical step of phagocytosis, which is chemotactic migration of neutrophils towards the foreign substance.

CONCLUSION

The immunomodulatory properties of the alkaloid-rich seed fraction of *Abrus precatorius* are established possibly by the inhibition of DTH response (immunosuppression) and stimulation of both humoral immunity and in-vivo leukocyte mobilization (immunostimulation).

CONFLICT OF INTEREST:

Authors declare no conflict of interest

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