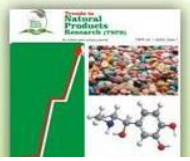
Trends in
Natural Products Research



Immunomodulatory activities of A*llium sativum* L and P*iper nigrum* L on albino mice

Charles Modilim[1], Stella Amarachi Ihim[2,4*], Vivian Chidimma Onyali[3], Moses Ikegbunam[1], Malachy Ugwu[1] and Chukwuemeka Sylvester Nworu[2]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria
[2]Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, Nigeria;
[3]Department of Medical Pharmacology and Physiology, University of Missouri, Columbia, USA
[4]Pharmacology and Physiology unit, Department of Science Laboratory Technology, University of Nigeria, Nsukka

Keywords: *Piper nigrum*, *Allium sativum*, immunomodulatory, complement, humoral, cyclophosphamide

Abstract: *Piper nigrum* and *Allium sativum* have been recognized as spices and a rich source of natural compounds which possess interesting and diverse pharmacological activities. The immunomodulatory activities of these reputed spices were evaluated using different experimental models, such as delayed type hypersensitivity test, carbon clearance test, cyclophosphamide induced myelosuppression, humoral antibody response and hemolytic complement fixation assay. Aqueous extracts of the spices of *Piper nigrum* (APN) and *Allium sativum* (AAS) were prepared from the commercially available ground spices and their immunomodulatory potentials evaluated. The oral treatment with APN or AAS (100, 200 or 400 mg/kg) evoked a significant ($p < 0.05$) increase in carbon clearance at all doses and potentiated the delayed type hypersensitivity response induced by sheep red blood cells (SRBC) compared to the untreated mice except AAS,100 mg/kg. Following short-term oral supplementation with APN or AAS (200 or 400 mg/kg), total lymphocytes, neutrophils and lymphocyte count increased significantly ($p < 0.05$) at all doses, indicating that mice were protected from cyclophosphamide induced myelosuppression. The hemolytic activity of the complement protein and sensitized SRBC were significantly ($p < 0.05$) inhibited at all doses of APN and AAS except at 62.5µg/ml. Furthermore, the extracts produced significant ($p < 0.05$) high titres of total ovalbumin-specific or tetanus-specific IgG1 and IgG2A compared with the untreated control.
These results suggest that the extracts of *Piper nigrum* (APN) and *Allium sativum* (AAS) may be useful as potential sources of natural immunomodulatory agents with immunostimulatory effects

*Corresponding author:
ihim.stella@unn.edu.ng;
+2348039576748
DOI: 10.48245/tnpr-2734391.2022.3.102
Page No.: 23-39
Volume: 3, Issue 1, 2022
Trends in Natural Products Research
Copy Right: NAPREG

INTRODUCTION

Piper nigrum (black pepper), which is also known as the king of spices, is one of the most widely used spices in the world. Aside from its remarkable role as a spice and flavouring agent in the human diet, it has also been used for its medicinal value as a preservative, biological pest control agent (Sharma *et al.*, 2018) and in the prescriptions of Ayurvedic medicine. *P. nigrum* has a spicy tang which is due to its most important and well-researched compound, piperine, a bioactive naturally occurring alkaloid. Most of the beneficial health and therapeutic effects of *P. nigrum* has been attributed to this alkaloid (Gorgani *et al*., 2017). Some of the major pharmacological effects of piperine that have been widely explored includes its effects on the neuromuscular system, sedative effect, anticarcinogenic, anti-asthmatic, stimulatory, hepatoprotective, anti-inflammatory, antimicrobial, antioxidant, antiulcer (Arcaro *et al*., 2014; Meghwal and Goswaini, 2013; Nurul *et al*., 2018). Other notable pharmacological activities exhibited by *P. nigrum* and its isolated constituents are insecticidal, larvicidal, antimicrobial, digestive properties, analgesic, antipyretic, anticancer, antidiarrheal, antihypertensive antithyroid, antidepressant (Bang *et al*., 2009; Ahmad *et al*., 2012; Dumanhouri and Ahmad, 2014; Vasavirama and Upender, 2014; Moreno *et al*., 2017; Wang *et al*., 2017; Dirgha *et al*., 2018; Sharma *et al*., 2018; Swathy *et al*., 2018). *Allium sativum* L. (garlic) is another commonly used spice. It belongs to the Alliaceae family and is the second most widely consumed bulb after onion (Kuete, 2017). *A. sativum* has a widespread distribution in temperate and tropical regions of the world, including central Asia and Africa (Tayebeh *et al*., 2018). It is characterized by a high production of organosulfur compounds that possess interesting biological and pharmacological properties (Rodrigo *et al*., 2015). Such pharmacological beneficial effects attributable to its organ sulphur contents include cardioprotective, anticarcinogenic, anti-inflammatory, anti-infectious (Mouna *et al*., 2018; Rodrigo *et al*., 2015), antifungal, anticancer, antiviral, antibacterial and immunomodulatory effects (Iciek *et al*., 2009; Chandrashekar and Venkatesh, 2016; Tayebeh *et al*., 2018).

It is now widely recognized that the modulation of the immune system by the suppression or stimulation of the immune responsiveness of an organism against invading antigens through induction, expression, amplification or inhibition of the response could provide an alternative to conventional therapy for diverse diseases (Nimrata *et al.,* 2016; Rama, 2018; Joo *et al*., 2017; Shah *et al.,*2022). The immune system is a remarkably sophisticated and major defence mechanism evolved in an organism to protect against foreign invaders and eliminate diseases (Shruthi *et al*., 2017). In phytomedicine, immunomodulation using medicinal plants is a novel approach which can enhance the host defence mechanism (Shruthi *et al*., 2017). Numerous medicinal plants and plant-based drugs have been used in the treatment of various diseases due to their rich bioactive component with immunomodulatory potentials, abundant availability, relatively cheaper cost and nontoxic nature compared to modern medicine (Njoku *et al*., 2017; Basavray and Milind,2018; Shi *et al.*, 2021). Culinary herbs and spices are plant products which are mostly used for seasoning and flavouring. They have also been shown to possess promising medicinal properties which are well documented (Aliyu *et al*., 2008; Otunola, 2022). Among such popular spices are *Allium sativum* and *Piper nigrum*. Not much scientific data is available on the immunomodulatory effects of these spices. Hence, this research was focused on the in vivo evaluation of the immunomodulatory activities of the crude aqueous extracts of the two spices in mice.

MATERIALS AND METHODS

Collection of spices

Allium sativum (Garlic) and *Piper nigrum* (Black pepper) ground spices were purchased from Eke-Awka market in Awka.

Aqueous extraction of spices

Aqueous extracts of both spices were prepared by maceration for 3 h with distilled water heated continuously to 40^0C and stirred at 30 mins intervals. The extracts were allowed to cool and filtered through Morcelain cloth and thereafter through Whatman No. 1 filter paper. The aqueous test extracts of *Allium sativum* (AAS) and *Piper nigrum* (APN) were then stored in aliquots at -20 ^{0}C for further use. Preliminary phytochemical tests were carried out using previously described standard procedures (Evans, 2009; Harborne, 1998).

Animals

This study was conducted using Adult Swiss albino mice (20-30g), procured from the laboratory Animal Facility at the Nnamdi Azikiwe University, Awka. The animals were housed in the institutional animal facility under standard conditions 25 ±2 ^{o}C, relative humidity of 50±5 %, and a 12 h light/dark cycle, and had access to standard pellets (Livestock Feed PLC, Lagos, Nigeria) and tap water *ad libitum*. All mice were acclimatized for 1w before the experiments. The use and care of laboratory animals in the study were in accordance with ethical guidelines contained in the European Union Directives for the Protection of Animals used for Experimental and other

Scientific Purposes (EU Directive: 2010/63/EU) of 2010.

Acute toxicity (LD_{50}) test

The acute toxicity (LD_{50}) of AAS and APN was estimated in mice by the oral route using the method of Lorke (1983). In the first phase, the toxic dose range was determined. The mice were placed in groups and AAS or APN (10, 100, or 1000 mg/kg) was administered by oral gavage. The treated mice were then monitored for 48 h for toxicity and mortality. In the second phase, four different doses of AAS or APN (1600, 2900, 3600 and 5000mg extract/kg body weight) were administered orally based on the earlier outcomes. The mice were then observed for lethality and signs of acute intoxication for 24 h.

Experimental protocol and design

The animals were randomized into eight (8) groups of six (6) animals each in the experiments for the assessment of immunomodulatory activities as follows;

Group 1: received commercial pellet diet and distilled water (negative control)
Group 2: received 100 mg/kg of Noni$^{(R)}$ (a known immune booster *Morinda citrifolia* Good 'N Natural, Ronkonkoma, NY) (positive control) p.o.
Group 3: received 100 mg/kg of the aqueous extract of *A. sativum* (AAS) p.o.
Group 4: received 200 mg/kg of the aqueous extract of *A. sativum* (AAS) p.o.
Group 5: received 400 mg/kg of the aqueous extract of *A. sativum* (AAS) p.o.
Group 6: received 100 mg/kg of the aqueous extract of *P. nigrum* (APN) p.o.
Group 7: received 200 mg/kg of the aqueous extract of *P. nigrum* (APN) p.o.
Group 8: received 400 mg/kg of the aqueous extract of *P. nigrum* (APN) p.o.

Preparation of antigen

Sheep red blood cells (SRBC) were collected and washed three times in large volumes of pyrogen-free 0.9 % normal saline. The cells were adjusted to a concentration of 0.1ml containing 1×10^9 cells for immunization and challenge.

The effect of AAS or APN on delayed type hypersensitivity (DTH) response in mice

Delayed hypersensitivity reaction was induced in mice using SRBCs as antigen. The mice were treated according to the experimental schedule with either AAS or APS (100, 200, or 400 mg/kg) or Noni$^{(R)}$ (100 mg/kg) or distilled water daily for 5 d. On day 5, mice in all groups were primed (i.p.) with an injection of 0.1 ml of 1×10^9 cells of sheep red blood cell (SRBC; Animal house, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria). Treatments were continued for 14 d. The mice were sensitized with the same antigen at the end of day 14and treatments continued.7 days after the challenge, the thickness of the left hind footpad was measured using a standard vernier calliper and the mice received a booster immunization through an injection with 1 x 10^9cells SRBCs in the right hind footpad. The sensitized right footpad thickness was measured again after 24 hours of challenge. The difference between prior and post challenge footpad thickness was reported as DTH response and expressed as mean percent thickness/edema (Sneha *et al*., 2017).

The effect of oral AAS or APN supplementation on phagocytic activity

The *in-vivo* phagocytic activity of the reticuloendothelial system in mice using AAS or APN was determined using the carbon clearance test as previously described by Oumar *et al* (2017) with modification. Mice were treated according to the experimental schedule for 10 d. A single intravenous injection of colloidal carbon suspension (1:50 dilution of Indian ink; Hi-Media Laboratories Pvt. Ltd, Mumbai, India) at 5 ml/kg body weight was administered to all mice, 48 hours after the last dose via the tail vein. Blood samples were drawn from the retro-orbital venous plexus before injection (0 min) and 15 min after injection of the carbon clearance. Each blood sample (50 µl) was lysed with 4ml of 0.1 % sodium carbonate (Na_2CO_3) solution and the optical density (OD) of the lysed sample was measured spectrophotometrically at 660 nm in a UV-2102 PC_ spectrophotometer (Unico, Shanghai, China). The phagocytic index was then calculated as $k = ([\ln OD_{0\ min}] - [\ln OD_{15\ min}]) / [t_{15\ min} - t_{0\ min}]$ where $OD_{15\ min}$ and $OD_{0\ min}$ are the optical densities at time $t_{15\ min}$ and $t_{0\ min}$, respectively.

The effect of oral AAS or APN supplementation on cyclophosphamide-induced myelosuppression in mice

The mice received treatment as scheduled respectively for 10 d. On day 10, baseline total leukocytes, total neutrophils and total lymphocyte counts of the mice were determined by collecting blood through the retro orbital plexus of the mice. On days 11, 12, 13, a neutropenic dose of cyclophosphamide (30 mg/kg i.p.) was administered to compromise the immune system of the mice, one hour after treatment with the extracts, Noni$^{(R)}$ or vehicle. Blood samples were then collected by retro-orbital puncture on the 14th day. Haematological parameters were determined for total leucocyte count (TLC) and differential leucocyte count (DLC)

(Lymphocytes and neutrophils). Total and differential white blood cell values in the treated groups were compared with the control groups (Verma *et al*., 2012).

The effect of oral AAS or APN on antibody responses to tetanus toxoid and ovalbumin antigens

The effect of AAS and APN on humoral immune responses was determined in mice in two separate experiments using ovalbumin (OVA) or tetanus toxoid (TT) as the antigen in a homologous prime-boost strategy. Briefly, mice were randomized into groups as shown in the treatment design for use in these studies. All the mice were immunized and challenged respectively on day 5 and 20 with 100 µg OVA/mouse (50 µl/footpad) or 0.1 ml of tetanus toxoid/mouse injected into their hind footpads. All mice were bled by retro-orbital venous puncture on day 19 for primary immune response and also sampled on day 26 (7 d post-booster- secondary immune response). Sera samples recovered each time were used for the determination of host primary and secondary humoral responses using ELISA (see below).

Determination of antibody titre by ELISA

The antibody titres elicited against OVA in the treated and control groups were estimated by enzyme linked immunosorbent assay. Flat bottom polystyrene plates were coated with 2 µg of OVA or TT in 100 µl bicarbonate-coating buffer (pH 9.6) and incubated overnight at 4 ^{o}C Unbound OVA or TT was then washed off with ELISA wash buffer (PBS-T; containing 0.05% Tween-20 in 0.15 M phosphate-buffered saline [PBS, pH 7.2]). Non-specific binding was blocked by the addition of 100 µl of 5 % fat free milk in phosphate buffer saline (BSA; Sigma, St. Louis, MO) in PBS to each well and the plate was incubated for 1 h at room temperature (RT). The wells were then washed again with PBS-T before the serum (100 µl), diluted with 2 % fat free milk in phosphate buffer saline (1:20), and was added to dedicated wells. The plates were incubated 1 h at 37^{o}C before unbound serum proteins and other constituents were washed off using PBS-T. To assess the antibody titre, 100 µl horseradish peroxidase (HRP)-conjugated goat anti-mouse total IgG, IgG1, or IgG2a secondary antibodies (BD Bioscience, Heidelberg, Germany) were added (each at 1:1000 dilution) to dedicated wells in the plates and the plates were then incubated another 1 h at RT. Thereafter, the unbound conjugates were washed off with PBS-T and 100 µl/well of freshly prepared 2,2-azino-bis-3-ethylbenzothiazoline-6-sulpuriic acid solution (ABTS) substrate was added. The plate was incubated at RT for 30 min before the reaction in each well was stopped by the addition of 100 µl of peroxidase stop solution (Kirkegaard & Perry Laboratories, NC., KPL, Gaithersburg, MD). The color developed in each well was assessed at 540 nm using an automatic Thermomax® ELISA Plate Reader (Molecular Device, Sunnyvale, CA). The mean absorbance value of the sera samples for each treatment group (assayed in duplicate) was then calculated. Sera samples from naive mice that received neither extract nor immunized with any antigens were also included in the ELISA to monitor for non-specific background signals (Beck and Spiegelberg, 1989).

Haemolytic complement fixation assay

The effect of AAS or APN on the haemolytic activity of the complement system through the classical pathway was investigated in vitro by a modified micro titre assay method (Garbacki *et al*., 1999; Nworu *et al*., 2008).
Sheep red blood cell was obtained from a female sheep and was washed 3 times using normal saline by centrifugation. A 0.1ml of 1×10^9 cells solution of the washed SRBC was injected into the rat intraperitoneally (i.p) and it was noted as day zero. On the 14th day, the rat was given the second immunization. Seven days after the last immunization, blood samples were collected through retro-orbital vein and it was centrifuged to obtain the serum, the serum was used as antisheep antiserum.
A 1:10 dilution of sheep red blood cells (SRBC) and veronal buffer was incubated at 37°C for 30 min with an equal volume of rat antisheep antiserum that was obtained from the immunized rat (1:1000 dilution).1mg/ml of both extracts was prepared in distilled water and serially diluted two-fold in 0.5ml of Veronal buffer solution. Four different concentrations of each test sample were obtained in triplicate test tube. Thereafter, a 0.5ml of 1:20 dilution of fresh guinea pig pooled serum in veronal buffered saline was added to each tube. After pre-incubation at 37^{0}C for 30 min, a 0.5ml of the suspension of the sensitized SRBC (1×10^9) was added. Tubes were incubated at 37 °c for 1 hour and the reaction was stopped by centrifugation at 1500 × g for 5 min. A 0.5 ml aliquot was drawn from each tube and mixed with 2 ml of distilled water and the degree of haemolysis was measured spectrophotometrically at 541nm. Control in this assay consisted of similarly treated incubates in which samples (0 % stimulation) were omitted. The effect of the treatment on the hemolytic activity of complement was calculated.

Statistical analysis

All results were expressed as mean± standard error of mean (SEM). Data were analysed by one-way analysis of variance (ANOVA) using GraphPad

Prism Software (Version 7.0). Differences between mean observations were considered significant at *p* values < 0.05.

RESULTS

Acute toxicity test

The oral administration of APN caused no death or no signs of acute intoxication at doses up to 5000 mg kg-1 while the oral LD_{50} of AAS was calculated as 3633 mg/kg.

Effect of oral AAS or APN supplementation on delayed-type hypersensitivity response in mice

The potential efficacy of the APN extract on cell-mediated immunity was evaluated by DTHT response to SRBC. Pre-treatment of the APN showed a significant dose related increase in footpad thickness ($p < 0.05$) at all doses when compared to the DTHT response of the untreated negative control group. Percentage increase in footpad oedema in mice treated with APN 200 and 400 mg/kg doses were 60.13 and 64.00 % respectively compared to the corresponding untreated negative control group, 37.39 %. The increases in footpad thickness in the treatment groups at all doses were statistically significant ($p < 0.05$) and comparable to the group that received the standard drug, Noni (100 mg/kg), 61.34 %. The DTH response to SRBCs, showed a dose-dependent increase due to the treatment with AAS. With doses of 200 and 400 mg/kg/day of AAS, the DTH response was 0.23±0.01123 and 0.24±0.01871, respectively, in comparison with corresponding value of 0.13±0.00860 for the untreated negative control group. The differences in DTH response were statistically significant ($p < 0.05$). The results of DTH responses are shown in Figure 1.

Effect of AAS or APN on phagocytic index of reticulo-endothelial system using carbon clearance Assay

The rate of carbon clearance in mice was used as an index of the phagocytic ability of macrophages to evaluate the effect of APN and AAS on phagocytic activity as shown in Figure 2.

Test animals treated with AAS extract showed a significant ($P < 0.05$) dose-dependent increase in the phagocytic index when compared to the negative control. Elevations were observed in the phagocytic index at all doses of AAS extract (100, 200 and 400 mg/kg) as 0.00850±0.00038, 0.00939±0.00020 and 0.01996±0.00089 respectively.

The phagocytic index for APN extract was significantly high ($p < 0.05$) at 100 mg/kg (0.00899±0.00011), 200 mg/kg (0.00957±0.00017) and 400 mg/kg (0.02157±0.00120) dose levels as compared to negative control group. The standard drug, Noni (100 mg/kg) showed a higher phagocytic index of 0.01701±0.00093 which was statistically significant ($p < 0.05$) compared to the negative control group.

The effect of AGL or MGL supplementation on differential white blood cell counts using cyclophosphamide induced leukopenia test

Pre-treatment with APN and AAS before cyclophosphamide administration showed a significant protection against cyclophosphamide-induced neutropenia when compared with the control group. The results are given in the figure 3. Administration of the extract AAS (100, 200 and 400 mg/kg) or APN (100, 200 and 400 mg/kg) Significantly ($p < 0.05$) ameliorated the cyclophosphamide induced immunosuppression of Total leucocyte count (TLC) in mice by as much as 61.60 %, 43.98 %, 20.55 % and 59.79 %, 43.58 %, 19.28% respectively. These results were comparable with the amelioration in mice administered the standard drug, Noni, 36.12 %.

In the same vein, treatment with APN and AAS remarkably attenuated the cyclophosphamide induced immunosuppression of lymphocyte and neutrophils levels as comparable with the positive control group that received the standard drug. Treatment with AAS (400 mg/kg) and APN (400 mg/kg) caused significant elevations in neutrophil and lymphocyte levels by as much as 33.58 %, 29.9 % (neutrophils) and 11.04 % and 16.39 % (lymphocytes) as compared to the negative control group of mice that received only cyclophosphamide.

Effect of AAS and APN oral supplementation on humoral antibody induced by ovalbumin or tetanus toxoid

Total IgG and IgG1 levels varied amongst test animal group and control animals. Administration of AAS and APN showed a significant increase in the serum immunoglobulin levels when compared with the control. Short term daily oral administration of AAS (100, 200 and 400 mg/kg body weight) followed by a homologous prime boost immunization with ovalbumin caused significantly ($p < 0.05$) higher titres of OVA-specific IgG1 (Figure 4a) and IgG2a (Figure 4b) in the sera of immunized mice compared to the negative control mice. The secondary antibody levels developed against OVA-specific antigen in immunized animals that received daily oral supplementation of AAS (100 and 400 mg/kg) or APN (400 mg/kg) was more than two-fold higher. The increases in antibody responses were similar to that elicited by the group that received daily oral administration of the standard drug, Noni$^{(R)}$. Similarly, daily oral supplementation with AAS or APN caused a remarkable increase in TT-specific IgG1 (Figure 4c)

and IgG2a (Figure 4d) in the sera of mice also immunized with TT in a homologous prime boost schedule. Administration of 400 mg/kg AAS increased TT-specific IgG1 from 0.644 (± 0.0041) to 1.430 (± 0.0188) and OVA-specific IgG2a from 0.672 (±0.0109) to 1.411 (±0.0053) respectively. Also, administration of 400 mg/kg APN increased TT-specific IgG1 from 0.627 (±0.0023) to 1.361 (±0.0143) and OVA-specific IgG2a from0.655 (±0.0048) to1.388 (±0.0141) respectively. The results at all doses were statistically significant ($p < 0.05$) compared to the negative control.

Haemolytic complement fixation assay with AAS and APN

As shown in Figure 5, the inhibitory effects of each spice extracts on complement activation through the classical pathway were examined. The control showed a relatively high optical density when the hemolytic activity of complement proteins was measured. AAS and APN significantly ($p < 0.05$) decreased the optical density of the medium as the concentration increased relative to the negative control and hence inhibited greatly the hemolytic activity of the complement proteins on the sensitized sheep red blood cells.

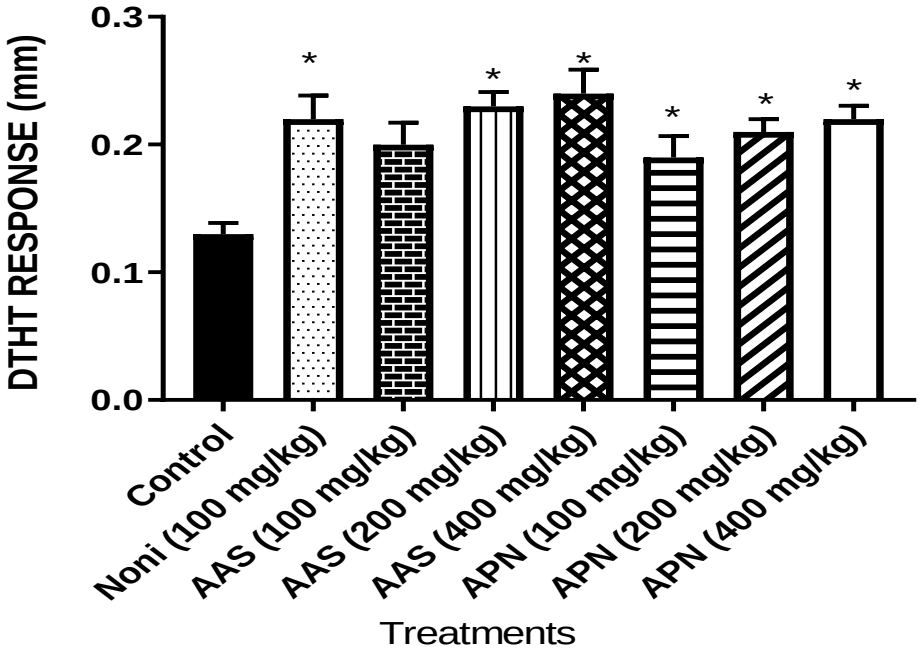


Figure 1: Effect of the extracts on Delayed type hypersensitivity response in mice.
*$p < 0.05$ versus untreated control. AAS—*Allium sativum*, APN---*Piper nigrum*,

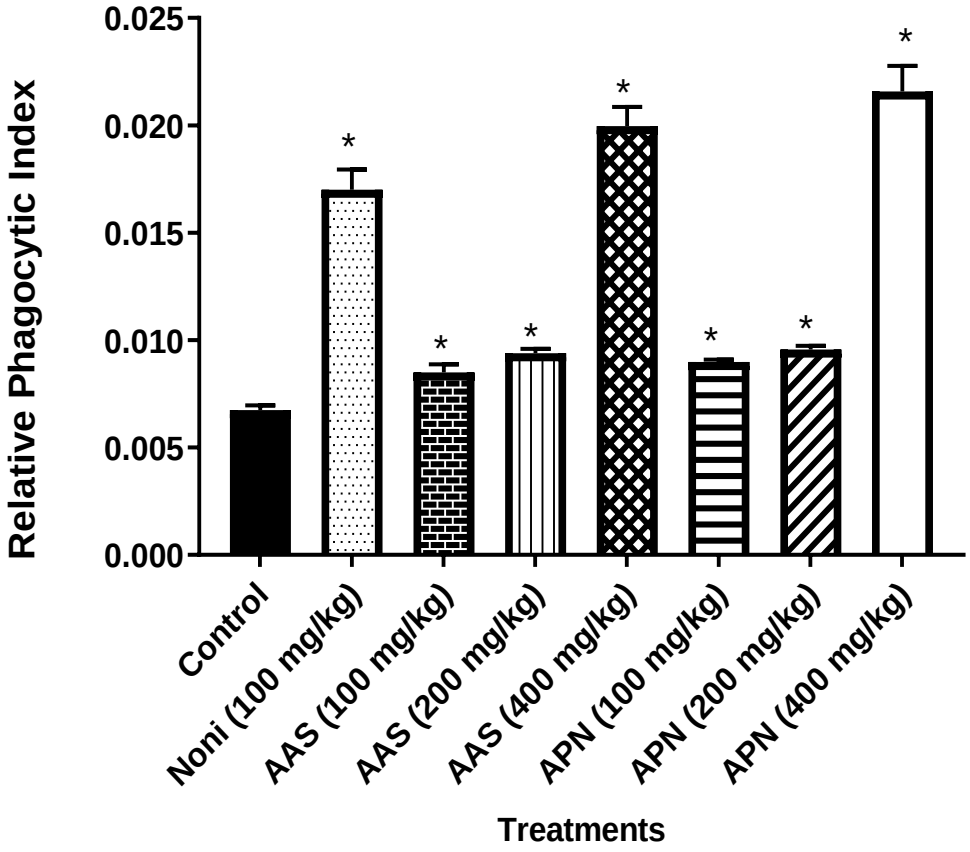


Figure 2: The effect of extracts treatment on phagocytic index. *p < 0.05 versus untreated control. AAS—*Allium sativum*, APN---*Piper nigrum*,

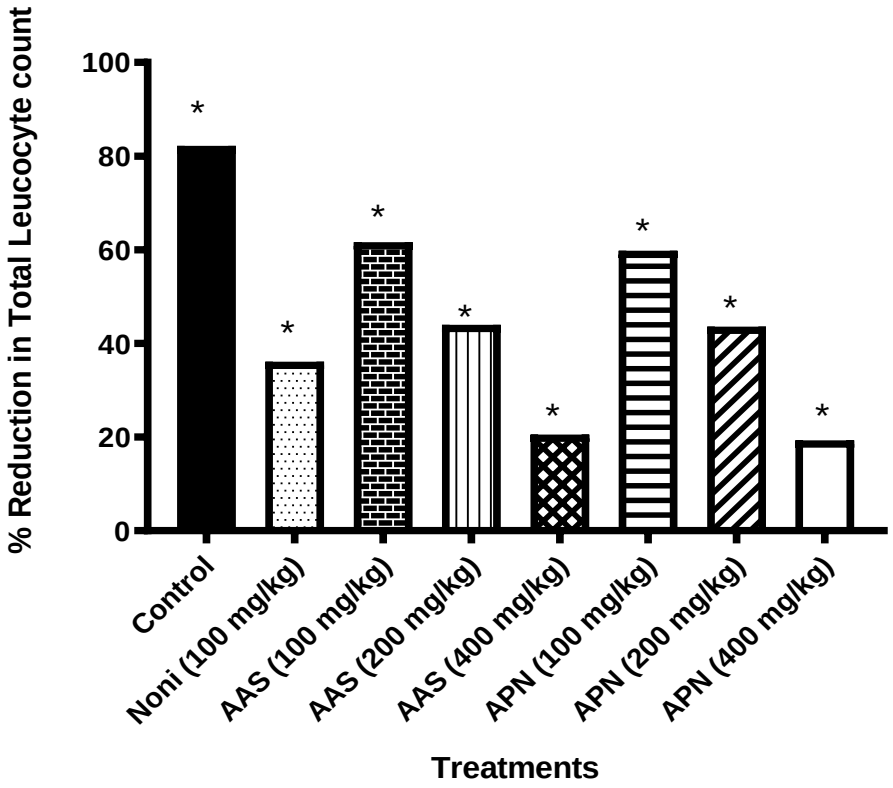


Figure 3a: Effect of cyclophosphamide induced myelosuppression on Total leucocyte count in mice treated with both aqueous extract *Allium sativum* (AAS) or *Piper nigrum* (APN). *$p < 0.05$ versus negative control.

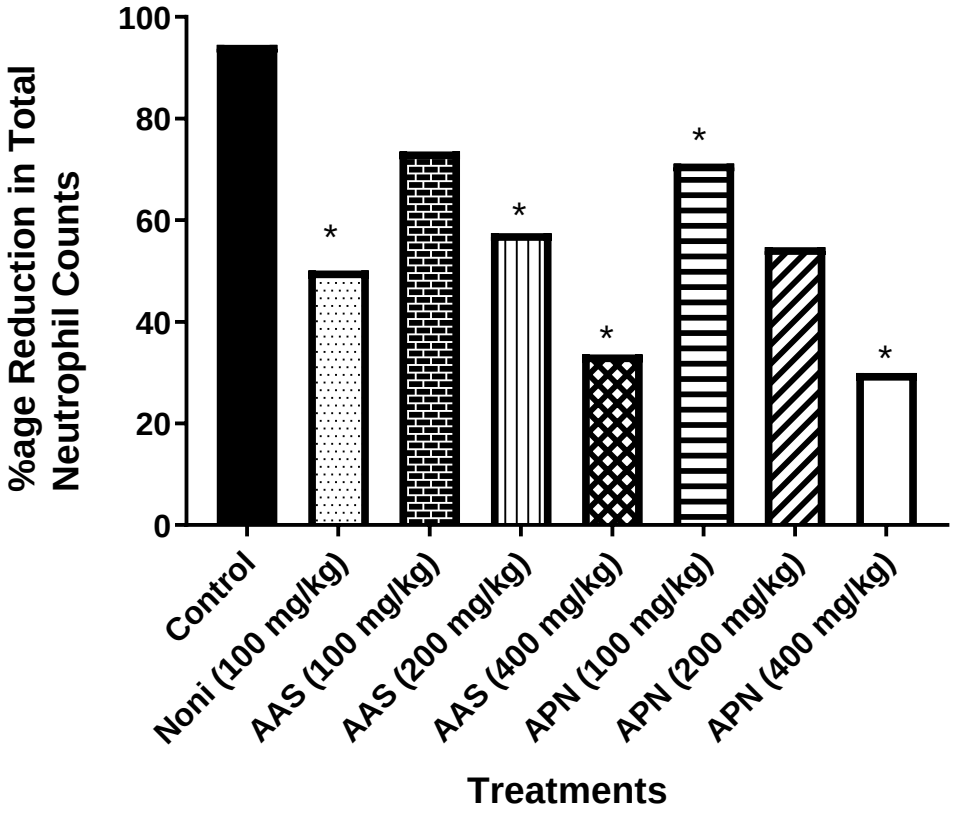


Figure 3b: Effect of cyclophosphamide induced myelosuppression on Total neutrophil count in mice treated with both aqueous extract of *Allium sativum* (AAS) or *Piper nigrum* (APN). *$p < 0.05$ versus control.

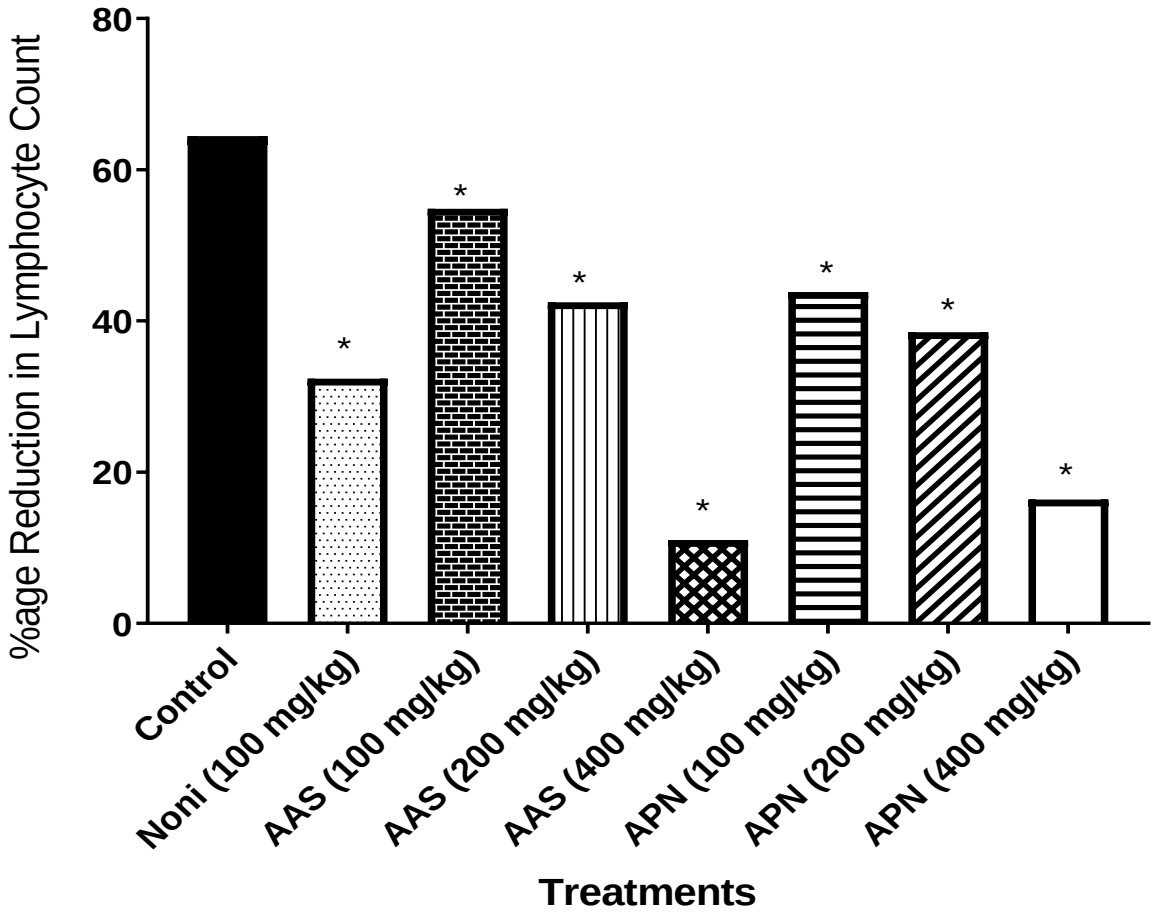


Figure 3c: Effect of cyclophosphamide induced myelosuppression on Total lymphocyte count in mice treated with both aqueous extract of *Allium sativum* (AAS) or *Piper nigrum* (APN). *p < 0.05 versus control

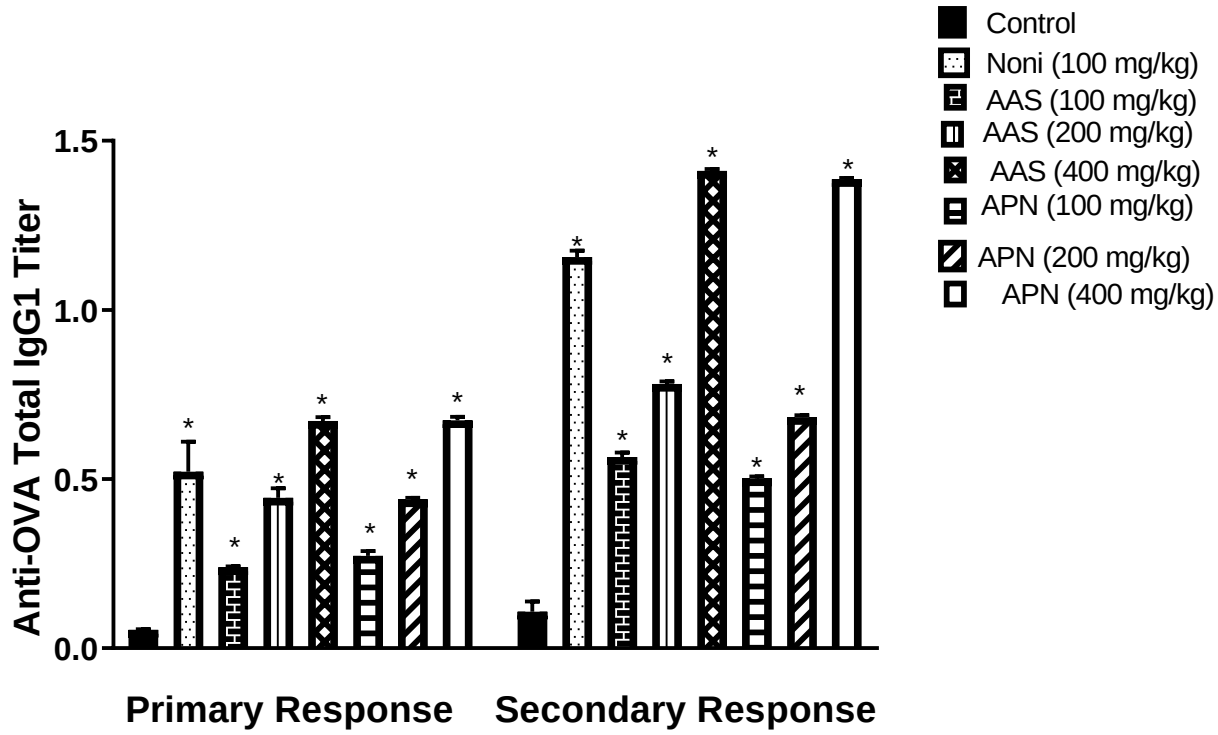


Figure 4a: Effect of the extracts on Ovalbumin specific IgG1 responses in mice. * $p < 0.05$ versus control.

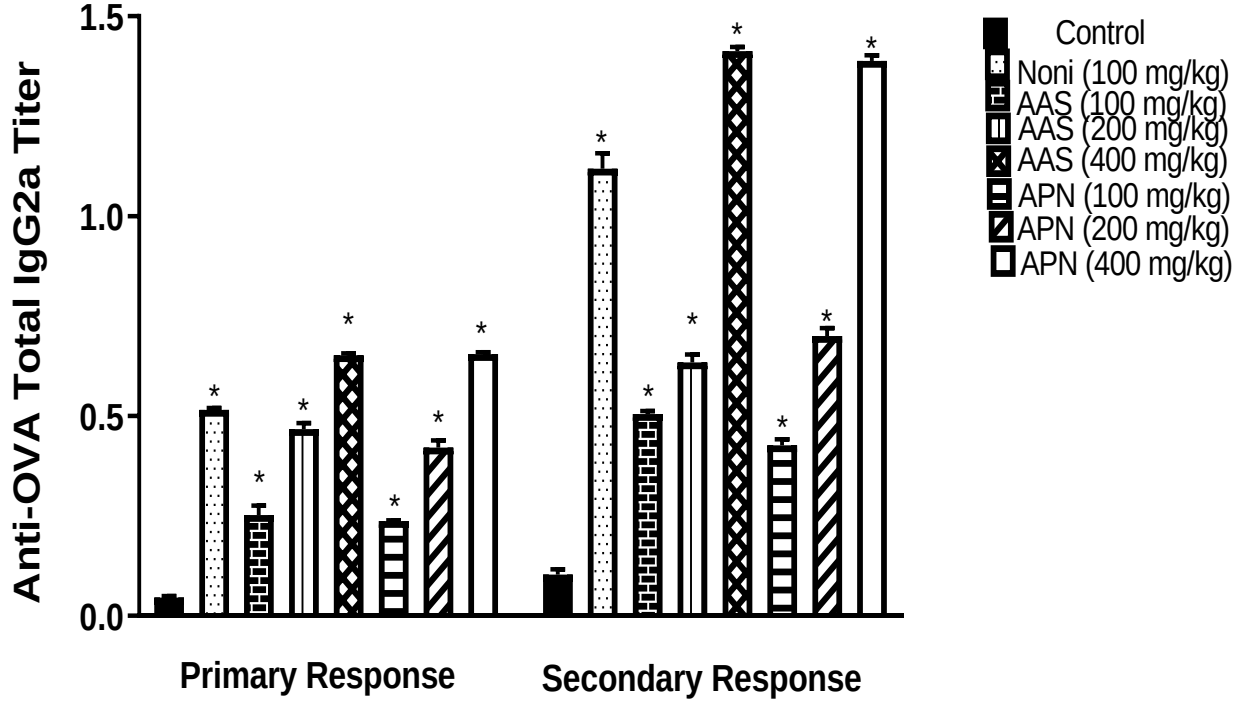


Figure 4b: Effect of the extracts on Ovalbumin specific IgG2a responses in mice. * $p < 0.05$ versus control

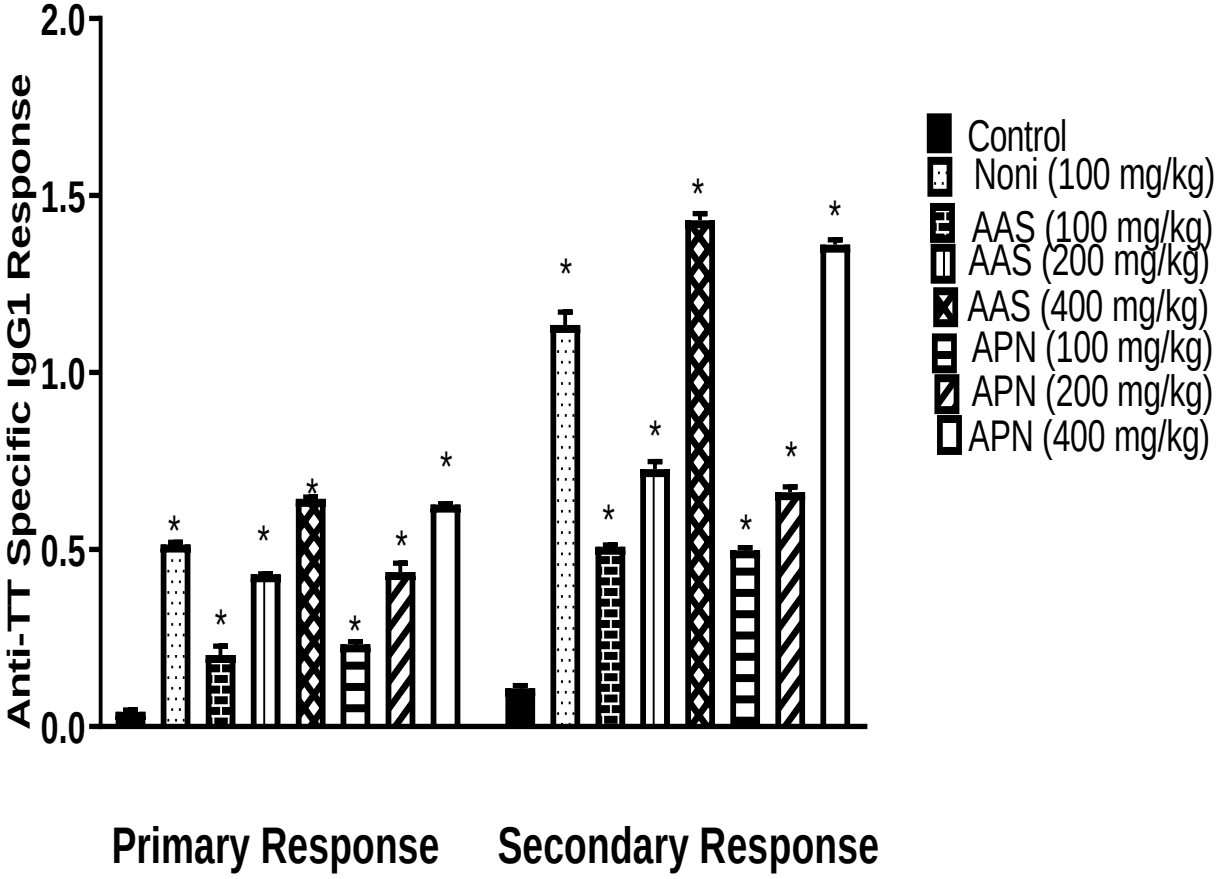


Figure 4c: Effect of the extracts on TT specific IgG1 responses in mice. * $p < 0.05$ versus control.

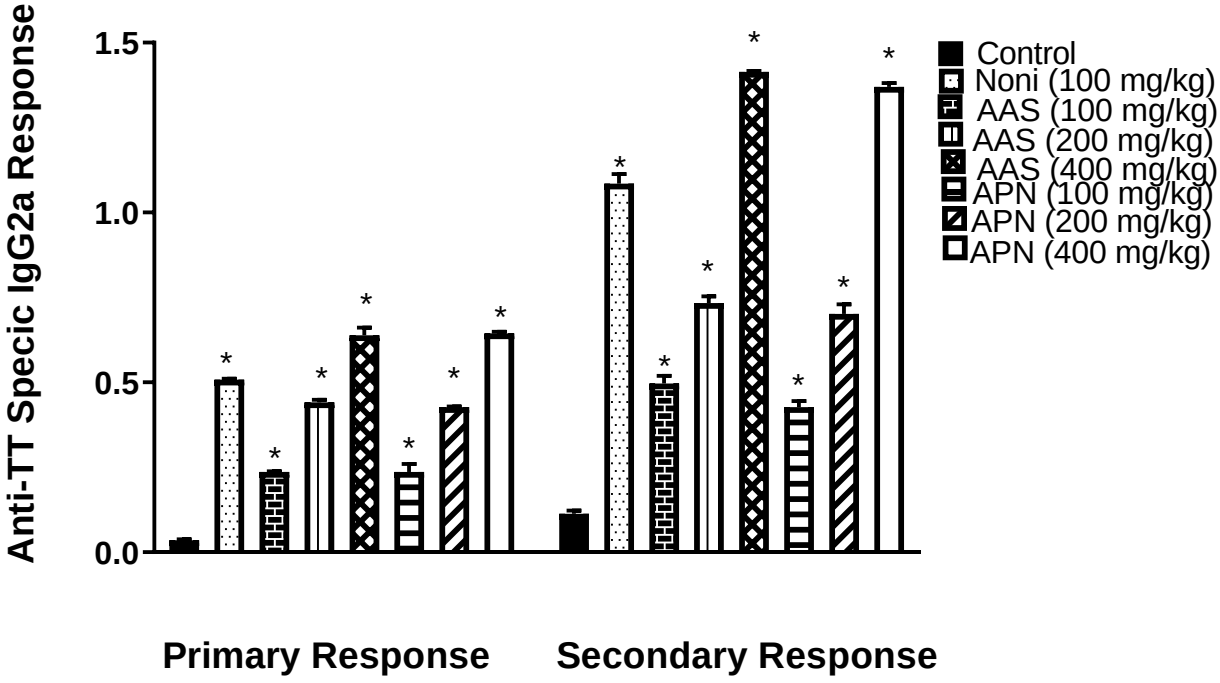


Figure 4d: Effect of the extracts on TT specific IgG2a responses in mice. * $p < 0.05$ versus control.

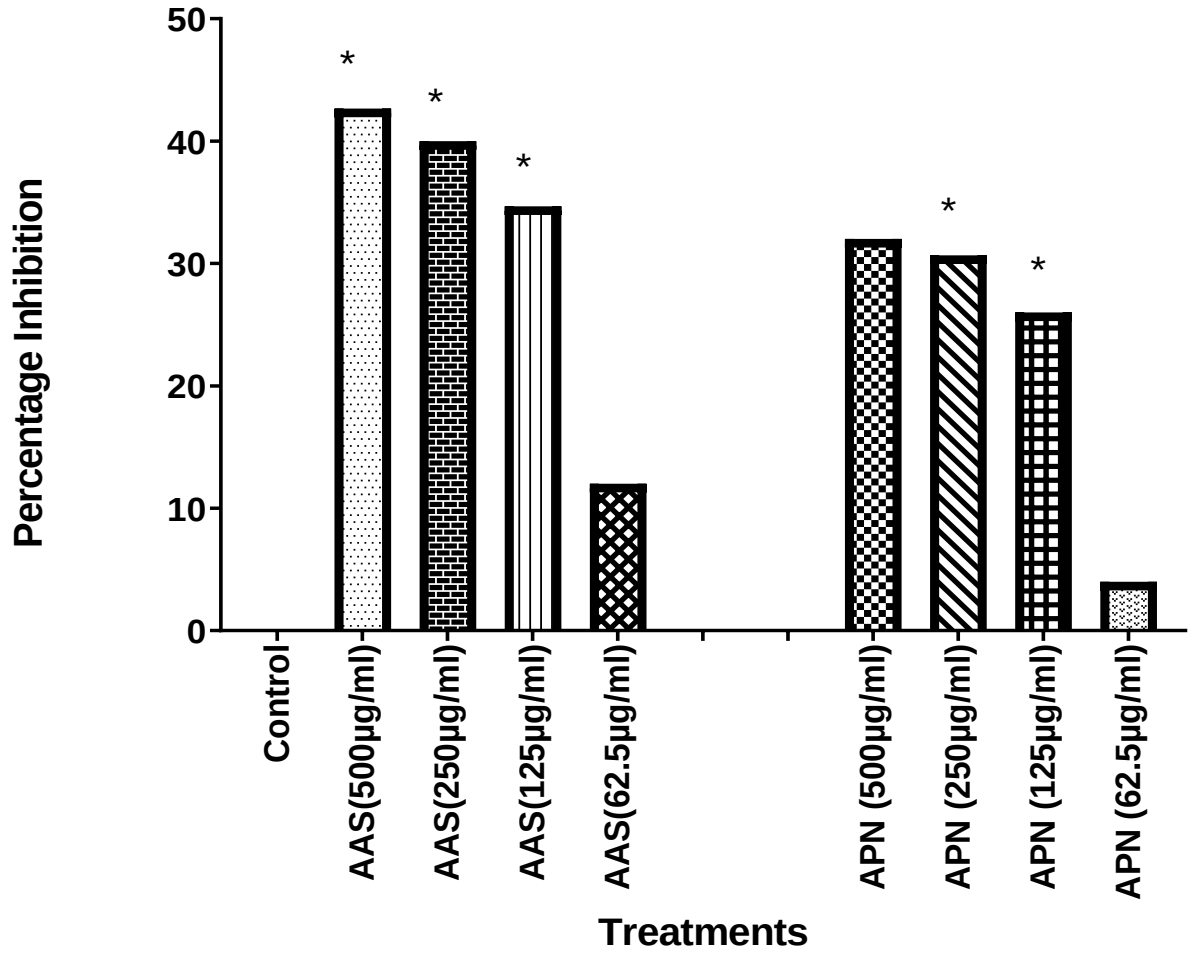


Figure 5: Effect of the aqueous extracts of *Allium sativum* (AAS) and *Piper nigrum* (APN) on hemolytic complement fixation. *$p < 0.05$ versus control

DISCUSSION

In the present study, the immunomodulatory effects of AAS and APN were evaluated using animal models to determine its effect on various immunological responses. Results showed that the crude aqueous extracts of the spices showed immunostimulant activity. It prevented the mortality of mice challenged with cyclophosphamide, increased the phagocytic index in the carbon clearance test and also increased serum immunoglobulin levels, suggesting the immunostimulatory effects of AAS and APN on both cell-mediated and humoral immunity.

Natural products are often mistakenly considered as safe because they are natural. However, some may contain Phyto therapeutic and bioactive compounds that are likely to cause adverse effects hence the need for all-natural products used in therapeutics to be subjected beforehand to safety tests (Tang *et al*., 2017). In the acute oral toxicity study, no adverse reactions or mortality were observed when the extract of APN was administered up to a dose of 5000 mg/kg. This strongly suggests that APN is relatively safe as substances with LD_{50} value of 1000 mg/kg by the oral route are considered to be of low toxicity and safe (OECD, 2001; Obici *et al*., 2008).

Thus, oral LD_{50} of AAS in the present is an indication that the extract is not completely safe, especially when administered in high concentrations. The component of the plant extract responsible for the toxic manifestations after a higher oral dose are not known, but may be due to one or more of the phytochemicals present in the crude aqueous extract of APN.

DTHT response is a cell mediated immune response classified as type IV DTH (Ul-Taq *et al*., 2016; Hasson *et al*., 2019) and is critical in defending against infectious diseases (Vanita and Diptesh, 2018). DTHT is an antigen specific reaction, mediated by activated T cells and lymphocytes and characterized by large influxes of non-specific inflammatory cells, macrophage accumulation, increased vascular permeability, vasodilatation, release of cytokines and subsequently inflammation (Sneha *et al*., 2017). With time, there is a marked increase in the concentration of lyric enzymes for more effective killing. The dose dependent increase in DTHT response suggests the stimulatory effects of both APN and AAS extracts, denoting an increase in the cell mediated immunity and lymphocytes that are essential for the process of inflammation (Pereira and Akbar, 2016).

The carbon clearance assay was performed to assess the impact of APN and AAS on the reticuloendothelial system. Overall, the extracts enhanced phagocytosis and increased the clearance of colloidal particles, which showed stimulatory effects on the phagocytic activity of macrophages (Oumar *et al*., 2017). The process of phagocytosis involves certain cells known as phagocytes which ingest and remove microorganisms, malignant cells, inorganic particles and tissue debris (Stanilova *et al*., 2000). Phagocytosis constitutes the body's primary line of defence (Vanita and Diptesh, 2018). Phagocytes are important immune cells whose immune function is to ingest pathogens and abnormal cells and are considered to be the first line of defence of the body's immune response (Wong *et al*., 2011; Li *et al*., 2018). Monocytes/macrophages and granulocytes are the most important type of phagocytes and one of the crucial constituents of nonspecific immunity as they play a significant role in nonspecific immune defence mechanisms (Sunil *et al*., 2017).

The effect of cyclophosphamide induced myelosuppression resulting in neutropenia in mice was studied to determine the effect of the extracts APN and AAS on the haemopoietic system. Specifically, total leucocyte count and differential counts before and after cyclophosphamide were determined to show the effects of the extracts on haemopoiesis. Cyclophosphamide is a cytotoxic chemotherapeutic drug for tumor therapy and is known to cause myelosuppression and immunosuppression leading to neutropenia as a major side effect. In the present study, AAS and APN at all doses exhibited significant protection against cyclophosphamide induced neutropenia suggesting it may have an effect on the haemopoietic system (Annapurna *et al*., 2017; Kenneth and Oduor, 2017) as well as protection against immunosuppression and leukopenia.

Immunoglobulins, which are also known as antibodies, are proteins produced by B-lymphocytes and they fight against antigens. Antibodies are the effectors of humoral response against antigens. IgG is a major immunoglobulin in humoral immune response and plays an important role in complement activation, opsonization and toxin neutralization (Zhi *et al*., 2019). The estimation of serum immunoglobulin is a direct measure of the competence of the humoral immunity. Humoral immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation to antibody secreting plasma cells. In this study, SRBC was used as a specific antigen to trigger the production of specific antibodies which were estimated in the serum samples of different treated groups. Specifically, immunoglobulin IgG1 and IgG2a values were measured. The mean antibody titre values of APN and AAS treated groups and Noni treated animals were significantly higher compared to the negative control animals, confirming their effect on humoral immunity.

The haemolysis of the sensitized SRBCs significantly increased in a concentration-dependent manner, suggesting the immune stimulatory potentials of the extracts. The complement cascade is one of the major natural defence mechanisms towards parasitic, fungal, viral and bacterial infections. The activation of complement, via either the classical or alternative pathways, leads to the production of several soluble components involved in chemiotaxis, opsonization, phagocytosis and pathogen destruction (Ibrahim *et al*., 2015).

CONCLUSION

The results of this study suggest that the aqueous extracts of *Allium sativum* (AAS) and *Piper nigrum* (APN) have the potential to stimulate the cell-mediated immunity and the humoral immune system, and may be potential therapeutic candidates in several immunosuppressed and immunocompromised clinical conditions

Acknowledgement

The authors wish to thank all staff members of the Faculty of Pharmaceutical Science, Nnamdi Azikiwe University, Awka.

Conflict of Interest

The authors have no conflict of interest to declare.

REFERENCES

Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M, Khan MA (2012). Biological role of *Piper nigrum* L. (Black pepper): A review. *Asian Pacific Journal of Tropical Biomedicine* 2(3): S1945-53.

Aliyu AB, Musa AM, Ibrahim MA, Oyewale AO, Amupitan JO (2008). Potential of Nigerian Medicinal Plants as Antioxidants: A Review. *Journal of Chemical Education* 5:1-10.

Annapurna A, Chandi V, Gummalla P (2017). Immunomodulatory activity of nutraceutical formulation and its potentiation by self-fortification and cow urine distillate fortification methods. *International Journal of Pharmacy and Pharmaceutical Sciences* 9(8): 15-19.

Arcaro CA, Gutierres VO, Assis RP, Moreira TF, Costa PI, Baviera AM, Brunetti IL (2014). Piperine, a natural bioenhancer, nullifies the antidiabetic and antioxidant activities of curcumin in streptozotocin-diabetic rats. *PLoS One* 9(12): 1-21.

Bang JS, Oh DH, Choi HM, Sur BJ, Lim SJ, Kim JY, Yang HI, Yoo MC, Hahm DH, Kim KS (2009). Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models. *Arthritis Research and Therapy*11:R49.

Basavraj N, Milind D (2018). Natural Immunomodulators. *Journal of Immunology and Microbiology* 2(1) :1-2.

Beck L, Spiegelberg HL (1989). The Polyclonal and antigen-specific IgG and IgE subclass response of mice injected with ovalbumin in alum or complete Freund's adjuvant. *Cellular Immunology* 123: 1-8.

Chandrashekara PM, Venkatesh YP (2016). Immunostimulatory properties of fructans derived from raw garlic (Allium sativum L.*). Bioactive Carbohydrates and Dietary Fibre* 8(2):65-70.

Damanhouri Z, Ahmad A (2014). A Review on Therapeutic Potential of *Piper nigrum* L. (Black Pepper): The King of Spices. *Medicinal and Aromatic Plants* 3(3):161

Dirgha RJ, Abinash CS, Nisha A (2017). A review on diversified use of the king of spices: *Piper nigrum* (Black pepper*). International Journal of Pharmaceutical Research* 0975-8232.

Evans WC (2009). Trease and Evans Pharmacognosy, 16th ed. Edinburgh: WB Saunders.

Garbacki N, Gloaguen V, Damas J, Bodart P, Tits M, Angenot L (1999). Anti- inflammatory and immunological effects of cyanus flower-heads. *Journal of Ethnopharmacology* 68: 235–241

Gorgani L, Mohammadi M, Najafpour GD, Nikzad M (2017). Piperine-the bioactive compound of black pepper: From isolation to medicinal formulations. *Comprehensive Reviews in Food Science and Food Safety*16(1): 124-140.

Harborne JB (1998). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, 3rd ed. London: Chapman & Hall.

Hasson SS, AL-Manthari AA, Idris MA, Al-Busaidi JZ, Al-Balushi MS, Aleemallah GM, Rashan L, Hakkim FL, Al-Jabri AA (2019). Immunomodulatory Potential of Combining Some Traditional Medicinal Plants In Vivo. *Annals of Microbiology and Immunology* 2(1): 1014.

Ibrahim J, Waqas A, Syed NAB (2015). Plant derived immunomodulators: an insight on their preclinical evaluation and clinical trials. *Frontiers in Plant Science* 6:655

Iciek M, Kwiecień I, Włodek L (2009). Biological properties of garlic and garlic-derived organosulfur compounds. *Environmental and Molecular Mutagenesis*50(3): 247-65.

Joo WK, Jae-Suk C, Du JS, Jai JC, Sae KK (2018). Immunomodulatory effects of kuseonwangdogo-based mixed herbal formula extracts on a cyclophosphamide-induced immunosuppression mouse model. *Evidence Based Complementary and Alternative Medicine* ID 6017412.

Kenneth OO, Oduor MA (2017). Protection of cyclophosphamide induced myelosuppression by extracts of *asparagus setaceous kunth* and *caesalpiniavol kensiiharm* in albino rats. *International Journal of Pharmaceutics and Pharmacology* 1(3): 114

Kuete V (2017). Allium sativum medicinal spices and vegetables from Africa. *Science Direct.* 363-377.

Li C, Yundong W, Shimei Z, Mengliang Z, Xiaoting Y, Xiangyun G, Yutong G, Anwen Z, Ying G (2018). Antitumor and immunomodulatory activities of total flavonoids extract from persimmon leaves in H22 liver tumor-bearing mice. *Scientific Report* 8:10523.

Lorke D (1983). A new approach to practical acute

toxicity testing. *Archives of Toxicology*54:275–87.
Meghwal M, Goswami TK (2013). *Piper nigrum* and piperine: An update. *Phytotherapy Researc*h 27(8): 1121-1130.

Moreno IR, Guerrero IN, Escareno N, Soto MEF, Gertsch J, Paredes JMV (2017). An Endocannabinoid uptake inhibitor from black pepper exerts pronounced anti-inflammatory effects in mice). *Journal of Agricultural and Food Chemistry* 65 (43): 9435-9442.

Mouna M, Norddine H, Abdallah B (2018). In vitro and in vivo immunomodulatory activities of *allium sativum* L. *Evidence based complementary and alternative medicine* ID 4984659

Nimrata S, Mukul T, Meht SC (2016). A review on herbal plants as immunomodulators. *International Journal of Pharmaceutical Research* 7(9): 3602-3610.

Njoku UO, Nwodo OF, Robert I (2017). Effects of Alkaloid Rich-Fraction (ARF) of Methanol Extract of *Ricinus Communis* (RC) Seeds on Immune Responses, Inflammatory Reactions and Liver Functions. *Journal of Applied Science* 17: 384-39.1

Nurul SA, Noor-soffalina S, Wan AW (2018). Functional properties of Oleoresin extracted from white pepper (*Piper nigrum* L.) retting waste water. *Sains Malaysiana* 47(9): 2009–2015.

Nworu C, Akah P, Okoli C, Esimone C, Okoye F (2008). Immunomodulatory Activities of Kolaviron, a Mixture of Three Related Biflavonoids of Garcinia kola Heckel. *Immunopharmacology and Immunotoxicology* 30(2):317–332.

Rama BP (2018). A review on immunomodulatory effects of plant extracts. *Virology and Immunology Journal* 2(6):000167.

Rodrigo A, Saray Q, Rocío IL, Enrique OF, Juan PR, Lucrecia C, Daniel OT (2015). Immunomodulation and anti-inflammatory effects of garlic compounds. *Journal of Immunology Research*. 401630.

Obici S, Otobone FJ, Da silva SVR, Ishida K, Da silva JC, Nakamura CV, Garcia CDA, Audi EA (2008). Preliminary toxicity study of dichloromethane extract of Kielmeyeracoriacea stems in mice and rats. *Journal of Ethnopharmacology* 115:131-139.

Organization for Economic Co-operation and Development. OECD Guidelines for testing of chemicals. Revised Draft Guidelines 425: Acute Oral Toxicity-Up and Down Method 2001.

Otunola GA (2022). Culinary Spices in Food and Medicine: An Overview of *Syzygium aromaticum* (L.) Merr. and L. M. Perry (Myrtaceae). *Frontiers in Pharmacology*. 17(12):793200

Oumar M, Tume C, Kamanyi A (2017). Immunostimulation by Stem Barks of *Pseudocedrela Kotschyi* (Family: Meliaceae): Stimulation of Phagocytosis Activities of Macrophages and Proliferative Response of Lymphocytes by Aqueous, Methanol and Hexane Extracts. *International Journal of Scientific and Engineering Research* 8(11):1-26.

Pereira BI, Akbar AN (2016). Convergence of innate and adaptive immunity during human aging. *Frontiers in Immunology* 7:445.

Shah VK, Firmal P, Alam A, Ganguly D and Chattopadhyay S (2020) Overview of Immune Response During SARS-CoV-2 Infection: Lessons From the Past. *Frontiers in Immunology*. 11:1949.

Sharma L, Vidhi G, Puneeta A, Maria A (2018). Protective role of *Piper nigrum* in health and healing of diseases. *International Journal of Food Science and Nutrition* 3(2): 84-88.

Shi J, Weng J, Mitchison TJ (2021). Immunomodulatory drug discovery from herbal medicines: Insights from organ-specific activity and xenobiotic defences. *Biochemistry and Chemical Biology elife* 10: e73673.

Shruthi **S**, Vijayalaxmi KK, Bhaskar SS (2018). Immunomodulatory effects of gallic acid against cyclophosphamide-and cisplatin-induced immunosuppression in albino mice *Indian Journal of Pharmaceutical Science* 80(1):150-160.

Sneha JA, Spoorthisudha P, Swetha M, Ganga RM (2017). Invitro and invivo screening of *Clitoria ternatea* (Linn.) for Immunomodulatory activity. *International Journal of ChemTech Research* 10(1): 116-125.

Stanilova S, Zhelev Z, Dobreva Z (2000). Preliminary studies on the immunomodulatory effect of the C3 binding glycoprotein isolated from *Cuscuta europea*. *International Journal of Immunopharmacology* 22:15-24

Sunil K, Gaurav S, Amit K (2017). Immunomodulatory Activity of Bioactive Fraction (PBC) from *Phyllostachys bambusoides. Pharmacognosy Journal* 9(6):786-791.

Swathy JS, Mishra P, Thomas J, Mukherjee A,

Chandrasekaran N (2018). Antimicrobial potency of high-energy emulsified black pepper oil nano emulsion against aquaculture pathogen. *Aquacultur*e 2018, 491: 210-220.

Tang R, Tiana RH, Caib JZ, Wua JH, Shena XL, Hua YJ (2017). Acute and sub-chronic toxicity of *Cajanus cajan* leaf extracts. *Pharmaceutical Biology* 55:1740-1746.

Tayebeh R, Zahra HY, ToobaGh, Zahra N, Marzieh F (2018). The immunoregulatory effects of four A*llium* species on macrophages and lymphocytes viability. *Immunoregulation*1(3):143-152.

Ul-Haq Z, Naz S, Mesaik MA (2016). Interleukin-4 receptor signalling and its binding mechanism: A therapeutic insight from inhibitors tool box. Cytokine Growth Factor Rev32:3-15.

Vanita K, Diptesh TP (2018). Evaluation of *in vivo* immunomodulatory activity of aqueous and ethanolic extract of E*ulophia nuda*l. *Asian Journal of Pharmaceutical and Clinical Research*11(12): 374-379

Vasavirama K, Upender M (2014). Piperine: A valuable alkaloid from piper species. *International Journal of Pharmacy and Pharmaceutical Sciences* 6(4):34-38.

Verma S, Shaban KA, Purohit R, Chimata LM, Rai G, Verma O (2012). Immunomodulatory activity of Withaniasomnifera (L.). *Journal of Chemical and Pharmaceutical Research* 4(1): 559-561.

Wang B, Zhang Y, Huang J, Dong L, Li T, Fu X (2017). Anti-inflammatory activity and chemical composition of dichloromethane extract from *Piper nigrum* and *longum* on permanent focal cerebral ischemia injury in rats. *RevistaBrasileira de Farmacognosia* 27: 369-374.

Wong KH, Lai CKM, Cheung PCK (2011). Immunomodulatory activities of mushroom sclerotial polysaccharides. *Food Hydrocolloids* 25: 150–158.

Zhi XL, Guan DZ, Wei X, Ke GL, Qiu SM, Wai SC, Hua Y, Yitao W (2019). Immunomodulatory effects of new whole ingredients extract from Astragalus: a combined evaluation on chemistry and pharmacology. *Chinese Medicine* 14:12

This paper is published under Creative Common Licence BY 4.0

CITATION: Modilim C, Ihim SA, Onyali VC, Ikegbunam M, Ugwu M, Nworu CS (2022). Immunomodulatory activities of A*llium sativum* L and P*iper nigrum* L on albino mice. Trend Nat Prod Res 3(1). 23-39. DOI: 10.48245/tnpr-2734391.2022.3.102