Vol. 16(3), pp. 121-131, March 2022
DOI: 10.5897/AJMR2020.9434
Article Number: 61E378E68844
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

Full Length Research Paper

Assessment of antiplasmodial and immunomodulatory activities of endophytic fungal metabolites from *Azadirachta indica* A. Juss

Nonye T. Ujam[1*], Cyril C. Adonu[1], Thaddeus H. Gugu[2],Restus Onwusoba[2], Chibueze Ike[3], Raymond O. Offiah[4], Malachy C. Chigbo[5], Festus B. C. Okoye[6], Charles O. Esimone[7]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria.
[2]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka Enugu State, Nigeria.
[3]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.
[4]Department of Pharmacology and Therapeutics, Faculty of Clinical Medicine, College of Medicine, Enugu State University of Science and Technology, Enugu, Nigeria.
[5]Department of Applied Biochemistry, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.
[6]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.
[7]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria.

Received 9 October, 2020; Accepted 17 June, 2021

This study reported the antiplasmodial and immunomodulatory activities of extracts of endophytic fungi isolated from *Azadirachta indica*. The extracts were evaluated for potential *in-vivo* and *in-vitro* antiplasmodial activity using murine malaria models and microtechnique methods respectively. Immunomodulatory potentials of the extracts were assessed using cyclophosphamide-immunomyelosuppression in mice and hypersensitivity and hemagglutination reactions in rats, using sheep red blood cells (SRBC) as the antigen. The extracts inhibited the growth of *Plasmodium berghei in-vivo* and *Plasmodium falciparum in-vitro*. At 100 and 200 mg/kg oral doses, extracts of AIL1, AIL3, AIS1 and AIS2 recorded parasite inhibition of 95.62-97.87, 73.47-85.71, 83.11-98.63 and 94.31-100% respectively. *In vitro* inhibition of schizont maturation was concentration-dependent; extract of AIS2 at 1 mg/ml gave the highest activity (86.67%). A dose-related increase in the mean total white blood cell (WBC) and a significant $p < 0.001$ increase in neutrophil counts compared to the positive control was shown by the extracts at 100 and 200 mg/kg with a significant $p < 0.05$ increase in the hypersensitivity reaction to the SRBC antigen and an increase in the antibody titer value, to SRBC in rats. Thus, extracts of the isolated fungi exhibited immunomodulatory activity in both the innate and adaptive immune components of the immune system which correlated positively with the antiplasmodial activity.

Key words: *Azadirachta indica*, endophytes, antiplasmodial, immunomodulatory activity.

INTRODUCTION

Infectious diseases are a significant burden on the public health and economic stability of societies all over the world. They have for centuries been among the leading causes of death and disability and presented growing challenges to health security and human progress (Nii-Trebi, 2017). The emergence of new diseases, re-emergence of old diseases, development of resistant strains, side effects of some currently available drugs including toxicity and other undesirable effects in allergic patients are a few major problems that require immediate attention to combat these diseases with effective drugs of high therapeutic index (Nii-Trebi, 2017). Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. It is preventable and curable (WHO, 2001).

In 2019, the global tally of malaria cases was 229 million, an annual estimate that has remained virtually unchanged over the last 4 years and the disease claimed some 409 000 lives in 2019 (WHO, 2020). The World Health Organization (WHO) is calling on countries and global health partners to step up the fight against malaria, a preventable and treatable disease that continues to claim hundreds of thousands of lives each year (WHO, 2020).

Plants are still considered as one of the important sources of biologically active compounds in natural products research (Marcellano et al., 2017). Many plant species have been utilized globally in traditional healing and have been studied extensively for their pharmacological properties. Medicinal plants are also reported to host some fungi that are involved in the co-production of active metabolites (Alvin et al., 2014). Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate disease symptoms or overt negative effects (Bacon and White, 2000).

Endophyte biology is an emerging field. The backbone of the endophytes is plants. The microorganisms like bacteria and fungi live in the tissues, leaves and roots of plants for some reason like habitat, food and protection (Preethi et al., 2021). The importance of studying the endophytes is their secondary metabolite production. The secondary metabolites have been isolated, and many studies have been carried out (Okoye et al., 2015, Ujam et al., 2020, 2021). These are biologically active compounds which include alkaloids, flavonoids, steroids and phenols (Preethi et al., 2021). The production of selected bioactive secondary metabolites by medicinal plants and by the endophytes provided for countless drugs selected as important therapeutic options innumerable diseases. The endophytes still have wide potential to be explored what could expand even more the phenomenal contribution to health and well-being. Considering the multi-resistant pathogenic micro-organisms and the producing capacity of antimicrobial metabolites by endophytes, it is indispensable for the search of antibiotic substances with new mechanisms of action, less toxic effect, and/or medication enhancement through these inexhaustible bioactive metabolites source (Demain and Sanchez, 2009).

Focus has also been on the exploration of secondary metabolites of fungi to obtain new bioactive molecules with potential applications in the medical, pharmaceutical, industrial, agricultural, and environmental fields (Okoye et al., 2015; Ujam et al., 2020, 2021). The discovery of novel antimicrobial secondary metabolites and bioactive compounds from different types of endophytic microorganisms is an important alternative to overcome the increasing levels of drug resistance to various pathogenic microorganisms (Godstime et al., 2014).

Azadirachta indica has been reported to be a fast-growing evergreen popular tree found in India, Africa, and America (Panjak et al., 2011). Extracts of the fresh leaves of this plant is reported in folk medicine for their antimicrobial, antimalarial, anthelminthic, antiviral, antiulcer actions etcetera (Orwa et al., 2009; Panjak et al., 2011). Several reports in recent years show that the endophytic fungi from neem produce several bioactive compounds (Li et al., 2007; Wu et al., 2008). This study was carried out to determine the acute toxicity, antiplasmodial and immunomodulatory activities of endophytic fungi metabolites isolated from *A. indica.*

MATERIALS AND METHODS

Plant material

Fresh leaves and stem of *A. indica* were collected from Agulu, Awka, Anambra State, South-East Nigeria and authenticated by a taxonomist, Mrs. Anthonia U. Emezie of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. A voucher specimen (PCG474/A/046) was deposited in the herbarium of the Faculty.

Experimental animals

Albino rats and mice of both sexes were procured from the animal facility of the Faculty of Veterinary medicine, University of Nigeria, Nsukka, Nigeria. Animals were handled in compliance with the National Institute of Health Guidelines for the care and use of laboratory animals (Pub. No.85-23, revised 1985) as approved by the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Nigeria.

*Corresponding author. E-mail: nonyetreasure@yahoo.com. Tel: +2348064667066, +2348111821962.

Malaria parasite

Chloroquine-sensitive *Plasmodium berghei*, used in the study was obtained from the University of Nigeria, Nsukka, Enugu State Nigeria. Parasite's viability was maintained through a weekly passage in mice, by aseptic inoculation of a known amount of parasite into healthy mice every week.

Drugs

Cyclophosphamide (500 mg) was used as a standard immunosuppressant. Cycloxan® (Biochem–Pharmaceutical Industries Ltd., Mumbai). NONI® (10 mg/kg) and Artemether/ lumefantrine (120/80 mg) were used as standard immunostimulatory and antimalarial drugs respectively. Drug dilutions were made using sterile water for injection according to manufacturers' instruction.

Antigen

The antigen used in the work was fresh Sheep Red Blood Cell (SRBC) obtained from Animal Farm in Agulu, Awka, Anambra State. The SRBCs were washed three times in a large volume of pyrogen-free sterile normal saline by repeated centrifugation at 2500 rev/s for 10 min on each occasion. The washed SRBC was adjusted to a concentration of approximately 1×10^9 cells/ml and used for both immunization and challenge.

Endophytic fungal extracts

Information regarding the fungal endophyte extract samples (AIL1, AIL3, AIS1, and AIS2), isolation, identification, and extraction were previously described by Ujam et al. (2020).

Acute toxicity (LD_{50}) study

Acute toxicity study of the endophytic fungal extract was assessed in mice following Lorke's method (1983).

***In vivo* antiplasmodial assay**

The *in vivo* antiplasmodial activity of the extract against blood schizonts of *P. berghei* was evaluated following Peter and Reyley's curative test method (Peter and Anatoli, 1998). Donor albino mice were infected with chloroquine-sensitive *P. berghei* and rising parasitemia of 30% determined using thin blood film, the blood sample was collected using an EDTA bottle. The collected blood sample was diluted using phosphate-buffered saline (concentration of 137 mMNaCl, 10 mM Phosphate, 2.7 mMKCl, pH 7.4) such that 0.2 ml contained 10,000 infected red blood cells. To avoid variability in parasitemia, all the animals used were infected from the same source. Fifty adult albino mice were used to assess the antiplasmodial effect of the four endophytic fungi isolate (AIL1, AIL3, AIS1, and AIS2) extracts. Animals were inoculated with 10,000 *P. berghei* infected red blood cells and allowed for three days to establish infection. On day 3 the mice were randomized into Ten groups of 5 mice each such that the mean parasitemia levels of the groups are almost similar. Groups 1 and 10 served as the negative and positive controls and were given distilled water (10 ml/kg) and Artemether-lumefantrine respectively (0.3/0.2 mg/kg), while groups 2 and 3; 4 and 5; 6 and 7; 8 and 9 were treated with two doses (200 and 100 mg/kg) of AIL1, AIL3, AIS1, and AIS2 extracts respectively. Treatment was carried out once daily from day 1 to day 4. On day 4, blood was collected from the tail vein of the mice, and blood films made using a clean glass slide (Devi et al., 2000). The dry blood films were fixed with methanol and subsequently stained with 10% Giemsa for 10 min. They were washed with clean tap water and allowed to air dry. To ensure optimal film quality each film was duplicated. The slides were microscopically examined using x100 magnification in oil immersion (Model Olympus microscope) and the level of parasitemia was assessed. Treatment was continued from day 4 to day 7 and the above procedure was repeated (Dikasso et al., 2006). The percentage curative activity of parasitemia was calculated using the following formula:

$$\%\ \text{Cure for Parasitamia on day 3} = \frac{\text{Basal parasitamia count-Parasitemia count on day 3}}{\text{Basal parasitamia count}} \times \frac{100}{1} \quad (1)$$

$$\%\ \text{Cure for Parasitamia on day 7} = \frac{\text{Basal parasitamia count-Parasitemia count on day 7}}{\text{Basal parasitamia count}} \times \frac{100}{1} \quad (2)$$

***In vitro* antiplasmodial assay**

The antiplasmodial assay was carried out based on the *in-vitro* microtechnique method by Rieckmann et al. (1978) with little modification. The blood samples were collected from the malaria-infected subject by a certified medical laboratory scientist on the consent of the patient. The blood samples were screened for *Plasmodium falciparum* infections. One to two drops of the blood samples obtained were used to prepare thick and thin smears on clean slides. Prepared slides were stained for 10 min with 10 % Giemsa solution prepared in phosphate buffer of pH 7.3 and examined microscopically for parasites (Molta et al., 1992). The patient who had mono-infection of *P. falciparum* was included in the *in vitro* drug susceptibility test (WHO, 2001).

The fresh blood samples were centrifuged at 2000 rpm for 10 min, the blood plasma was removed and the blood pellets were suspended and washed thrice in a sterile Roswell Park Memorial Institute (RPMI) medium before use for parasite cultivation (Flyg et al., 1997). Artemether - Lumefantrine was used as the standard drug.The ethyl acetate extracts were first dissolved in DMSO and two-fold dilutions were carried out to prepare the following concentrations of 1.0, 0.5, 0.25, 0.125 mg/ml. A 1 ml volume of the extracts at various concentrations was first distributed into the plates after which 1 ml of culture medium was added into the well plates. The plates were incubated in a 5% CO_2 incubator (Thermo Fisher Scientific, Inc. Corporate Office is located in Waltham, MA,

USA) at 37°C for 24-30 h. After incubation, the contents of the plates were harvested and the red cells were transferred to a clean microscopic slide to form a series of thick films. The films were stained for 10 min in 10% Giemsa solution of pH 7.3. Schizont growth inhibition per 200 asexual parasites was counted in 10 microscopic fields. The control parasite culture freed from extracts was considered 100% growth. The percentage inhibition per concentration was calculated using the formula:

$$\% \text{ Inhibitio of schizont growth} = \frac{(\% \text{ parasitaemia in control wells} - \% \text{ parasitaemia of test wells})}{(\% \text{ parasitaemia of the control})} \times 100 \quad (3)$$

(WHO, 2001; Ngemenya et al., 2006)

Immunomodulatory assay

Preliminary evaluation of the immunomodulatory potential of the endophytic fungi extracts was carried out using the cyclophosphamide-induced immunosuppression method as previously reported by Ujam et al. (2021).

Delayed type hypersensitivity response (DTHR)

Hypersensitivity reaction to SRBC was induced in rats following the method reported by Gabhe et al. (2006) with modifications. SRBCs collected in Elsevier's solution, were washed three times with pyrogen-free sterile normal saline and adjusted to a concentration of 1×10^8 cells/ml for sensitization and challenge. Animals were divided into five groups of five animals each, one group served as control while the remaining served as experimental groups for the treatment with AIL1, AIL3, AIS1, AIS2 extracts. Animals were sensitized by injecting 0.1 ml suspension of 10% freshly prepared SRBCs (1×10^8 cells /ml) on days one and six. The experimental groups received 200 mg/kg of test extracts for eight days whereas the control group was administered with equal volume distilled water. On day 8, 2 h after giving the extracts, animals were challenged by injecting 0.1 ml of SRBC intradermally in the left hind footpad. The thickness of the footpads was measured using a micrometer screw gauge before the challenge and at 24 h after the challenge. The difference between 0 and 24 h values of footpad thickness was taken as a measure of DTH reaction and the mean percent edema was determined using the formula:

$$\% \text{ Edema} = \frac{\text{Mean final reading-Mean initial reading}}{\text{Mean initial reading}} \times 100 \quad (4)$$

Haemagglutination inhibition activity

The crude extracts of AIL1, AIL3, AIS1, AIS2 at 200 mg/kg were administered to the animals (test groups) orally for eight days and the vehicle (normal saline) was administered to the control animals. Each group consists of five rats and was immunized intraperitoneally by injecting 0.1 ml suspension of freshly obtained Sheep Red Blood Cells (SRBCs) (1×10^8 cells/ ml) on days 0 and 6. Blood samples were collected by a retro-orbital puncture on day 8 after the immunization. Antibody levels were determined by the haemagglutination technique. 5 µl of 10% SRBC suspension was added to 5 µl of two-fold diluted serum samples in a glass test tube. After 18 h of incubation, the highest dilution giving haemagglutination was considered as the antibody titer. The antibody titer was expressed in a graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance.

$$\% \text{ Inhibition} = 100 - (a/b \times 100/1) \quad (5)$$

Where a = mean rank of the control group and b = mean rank of the treated group.

Statistical analysis

Results of the study were presented as mean ± Standard error of the mean (SEM) of sample replicate, n=5. Raw data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Turkey's test and independent students't-test. Bivariate correlation was used to assess the relationship between the antiplasmodial and immunomodulatory activity. The analysis was done using the statistical package for Social Sciences (SPSS) version 20 for windows. Statistical significance was established when $p<0.05$. The graphical illustration was carried out using Microsoft Excel, 2010.

RESULTS

The extracts of the endophytic fungi coded AIL1, AIL3, AIS1, and AIS2 isolated from the leaves and stems of *A. indica* were examined in this study. The acute toxicity result showed the extract of AIL1 did not produce any mortality or obvious signs of toxicity at the first stage doses (10-1000 mg/kg) but was toxic at the second dosage levels (2000-5000 mg/kg) causing mortality of the animals (mice). However, the extracts of the fungi AIL3, AIS1, AIS2 showed no mortality of the mice even at 5000 mg/kg dose (Table 1).

The results of the curative *in vivo* anti-plasmodial study showed that at 100 and 200 mg/kg dose level, the extracts of AIL1, AIL3, AIS1, and AIS2 recorded percentage parasite inhibition of 95.62-97.87; 73.47-85.71; 83.11-98.63 and 94.31-100% respectively (Table 2). The fungal extracts significantly ($p<0.001$) inhibited the growth of the plasmodium parasite after 3 days of treatment. On the 7th day, of the curative experiment, the parasites were further inhibited and total clearance of the parasites was displayed by AIS2 extract. The result of the *in-vitro* antiplasmodial assay against *P. falciparum* is shown in Figure 1. The parasite growth decreases as the concentration of the extracts increases, the negative

Table 1. Acute toxicity/oral median lethal dose (LD_{50}) study of the fungal extracts.

S/N	Extract codes	D_0 (mg/kg)	D_{100} (mg/kg)	The oral median lethal dose (LD_{50}) in mice (mg/kg)
1	AIL1	1000	2000	1414
2	AIL3	10	5000	>5000
3	AIS1	10	5000	>5000
4	AIS2	10	5000	>5000

Do = Highest dose that gave no mortality, D_{100} = Lowest dose that produced mortality.

Table 2. *In-vivo* antiplasmodial activity of crude extracts of endophytic fungal isolates against *Plasmodium berghei.*

Animal groups	Endophytic fungal extracts/Doses (mg/kg)		Mean parasitemia count			% cure	
			B	D_3	D_7	D_3	D_7
1	AIL1	200	44.67 ± 0.33	1.00 ± 0.58[a]	1.00 ± 0.58	97.87	97.76
2		100	45.67 ± 2.03	2.00 ± 0.58[a]	2.00 ± 0.58	95.62	95.62
3	AIL3	200	49.00 ± 0.00	7.00 ± 3.46[a]	3.50 ± 0.00[*]	85.71	92.86
4		100	47.67 ± 2.03	13.50 ± 4.33[a]	8.50 ± 0.29[*]	73.47	84.27
5	AIS1	200	49.00 ± 0.58	1.56 ± 1.00[a]	0.67 ± 0.88[*]	95.74	98.63
6		100	45.00 ± 1.16	7.60 ± 0.88[a]	2.00 ± 1.00[*]	83.11	95.56
7	AIS2	200	47.00 ± 1.73	1.50 ± 0.29[a]	0.00 ± 0.00	94.98	100.00
8		100	47.00 ± 0.58	2.67 ± 0.67[a]	0.00 ± 0.00	94.31	100.00
9	AL	9.8	49.00 ± 1.16	4.50 ± 0.87[a]	3.50 ± 0.87	90.81	92.86
10	DW	10 ml/kg	43.00 ± 2.00	51.00 ± 1.16	59.00 ±3.84	-	-

Values are expressed as mean ± sem, n = 5, * indicates significant difference, *** = a ($p < 0.001$), ** = ($p<0.01$), * = ($p<0.05$) b = basal, d3 = day 3 and d7 = day 7 after inoculation. Dw =distilled water (negative control), AL = artemether-lumefantrine/20mg-120mg (positive control), - = no activity.

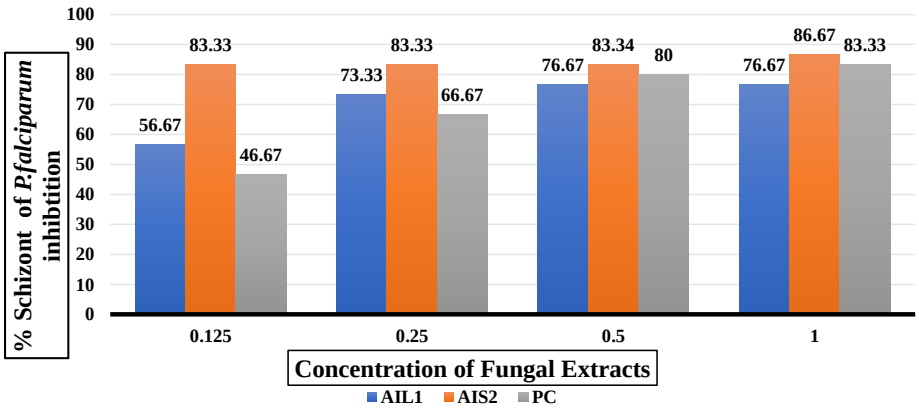

Figure 1. *In-vitro* antiplasmodial activity of the extracts of three endophytic fungal isolates against *P. falciparum.* Positive control= artemether-lumefantrine.

control (distilled water) had 100% parasite growth. Percentage inhibition of schizont maturation was a concentration-dependent extract of AIS2 at 1 mg/ml gave the highest activity (86.67%) which was comparable to

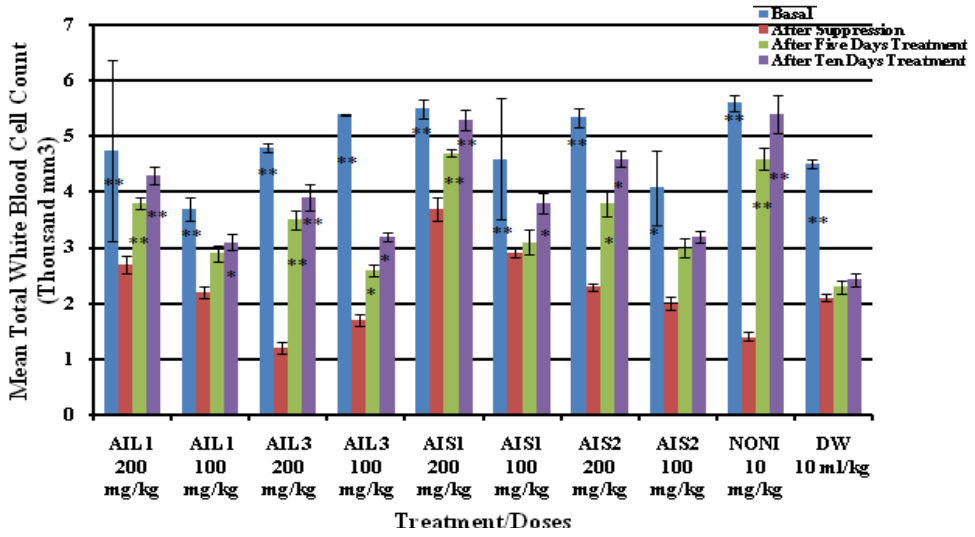

Figure 2. Mean total white blood cell counts (TWBC) for the curative experiment *p<0.05, *p<0.01 significantly different from WBC level at suppression.

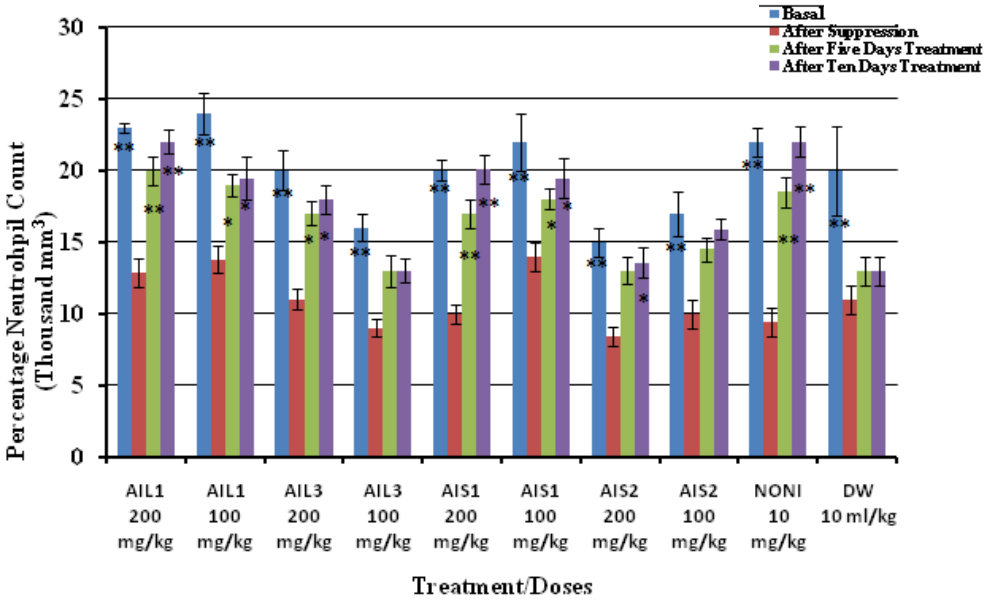

Figure 3. Mean differential white blood cell (Neutrophil) count for the curative experiment. *p<0.05, and **p<0.01 significantly different from the neutrophil level at suppression.

the activity of the positive control (artemether-lumefantrine) 83.33%.

The result of the curative immunomodulatory study of the AIL1, AIL3, AIS1, and AIS2 extracts is shown in Figures 2 and 3. The mean basal total white blood cell (TWBC) count of the mice ranged from 4.08- 5.60 × 10^3/ mm^3. Following induction of cyclophosphamide, the TWBC count significantly $p < 0.01$ decreased compared to the basal white blood cell count, ranging from 1.20 to 2.90 × 10^3 / mm^3 (Figure 2). After five days of treatment, extracts of AIL1, AIL3, AIS1, and AIS2 raised the TWBC count of the mice, mean TWBC count ranged from 2.30 to 4.70 × 10^3 /mm^3. Further increase of the total WBC counts was exhibited by the extracts after ten days of treatment, mean TWBC count was from 2.90 to 5.40 ×10^3/mm^3 (Figure 2).

The mean basal neutrophil count of all the groups in the experiment ranged from 14.90 to 24.00 × 10^3/mm^3 (Figure 3). After administration of cyclophosphamide the mean neutrophil count of the mice significantly decreased

Table 3. Mean total white blood cells counts (thousand mm^3) for the prophylactic experiment.

Treatment groups	Doses (mg/kg)	Basal blood sample	Blood sample after 10 days treatment	Blood sample after the suppression
AIL1	200	5.50 ± 0.15	10.60 ± 0.14^{aaa}	9.40 ± 0.14
AIL3	200	6.60 ± 0.15	8.60 ± 0.10^{aaa}	6.80 ± 0.10 ^{b}
AIS1	200	5.40 ± 0.10	7.80 ± 0.06^{aaa}	7.20 ± 0.04
AIS2	200	4.50 ± 0.16	9.79 ± 0.12^{aaa}	6.50 ± 0.06 ^{bb}
Distilled water	10 (ml/kg)	8.30 ± 0.13	10.30 ± 0.18^{aaa}	5.42 ± 0.16^{bbb}

Values were presented as mean ± Standard error of the mean of five (5) replicates (n=5). $^{a}P<0.05$ and $^{aa}P<0.01$ significantly different from basal WBC level. $^{b}P<0.05$ and $^{bb}P<0.01$ significantly different from WBC level on day 10. Distilled water = Negative control.

Table 4. Mean Percentage Neutrophils Counts for the Prophylactic Experiment.

Treatment groups	Doses (mg/kg)	Basal blood sample	Blood sample after 10 days administration of extracts	After suppression blood sample
AIL1	200	19.00 ± 0.71	25.00 ± 0.71^{aa}	24.00 ± 0.63
AIL3	200	17.00 ± 0.95	20.00 ± 1.22^{a}	17.00 ± 1.14
AIS1	200	22.00 ± 0.63	26.00 ± 1.22^{a}	24.00 ± 1.22
AIS2	200	16.00 ± 1.30	19.00 ± 1.05^{a}	15.00 ± 0.20 ^{b}
Distilled water	10 ml/kg	21.00 ± 1.22	22.60 ± 0.84	12.00 ± 1.14^{bbb}

Values were presented as mean ± Standard error of the mean (SEM) of five (5) replicates (n=5). $^{a}p<0.05$, and $^{aa}p<0.01$: significantly different from basal neutrophil level. $^{b}P<0.05$ and $^{bb}P<0.01$: significantly different from the basal neutrophil level on day 10.

compared to the basal blood sample, $p< 0.01$, ranging from 7.00 to 14.20 $\times10^3/mm^3$.Comparison of the neutrophil count of mice after 5 days with the counts after suppression showed that AIS1, AIL1, and AIL3 extracts and NONI (positive control) significantly ($p < 0.01$) increased the neutrophil count while AIS2 extracts showed an increase at ($p<0.05$). The Negative Control (DW) exhibited no significant increase ($p > 0.05$) (Table 3). After 10 days of treatment, AILI, AIL3, AIS1 extracts, and NONI further increased the neutrophil count of the mice ($p<0.005$). At 200 mg/kg the fungal endophyte extracts showed a higher increase compared to the 100 mg/kg dose level (Figures 2 and 3).

In the prophylactic immunomodulatory study, the mice were pre-treated with the extract for ten days and there was a significant increase $p< 0.05$ in the TWBC (Table 3). Extracts of AIL1, AIL3, AIS1, and ASI2 significantly raised the leucocytes of the mice after the ten days administration and inhibited the suppressive effect of cyclophosphamide on the TWBC (Table 3) and Neutrophil count (Table 4) of the mice. Percentage inhibition of cyclophosphamide effect by AIS2, AIL3, AIL1, AIS1 extracts were 32, 63, 75 and 87% respectively (Figure 4).

Oral administration of 200 mg/kg of AIL1, AIL3, AIS1, and AIS2 extracts caused a significant inhibition of Delayed-Type Hypersensitivity Reaction (DTHR) induced by SRBC in rats. Percentage inhibition of DTHR ranged from 66, 53, 76, 69 and 53% for AIL1, AIL3, AIS1 and AIS2 extracts respectively (Table 5). The Mean HI antibody titers in the sera of all the groups obtained after vaccination were expressed as HI titer (log^2) and presented in (Table 6). A positive correlation ($r = 0.893$) was established between the immunomodulatory and antiplasmodial activity and was also statistically significant at $p < 0.05$.

DISCUSSION

A. indica is found throughout the geographical area of southeast Nigeria is a medicinal plant used in folk medicine. In this present study, the antiplasmodial and immunomodulatory properties of four endophytic fungi (AIL1, AIL3, AIS1, and AIS2) isolated from *A. indica* were studied in swiss albino rat and mice.

All (100%) the endophytic fungi (AIL1, AIL3, AIS1, and AIS2) extracts at 10 to 1000 mg/kg (phase 1) produced no physical signs of toxicity in the mice 24 h after administration and became increasingly pronounced as the dose increased towards 5000 mg/kg body weight. However, AILI extract showed mortality at Phase II of the experiment. An acute toxicity test gives clues on the range of doses that could be toxic to the animal. It could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics (Rang et al., 2001).

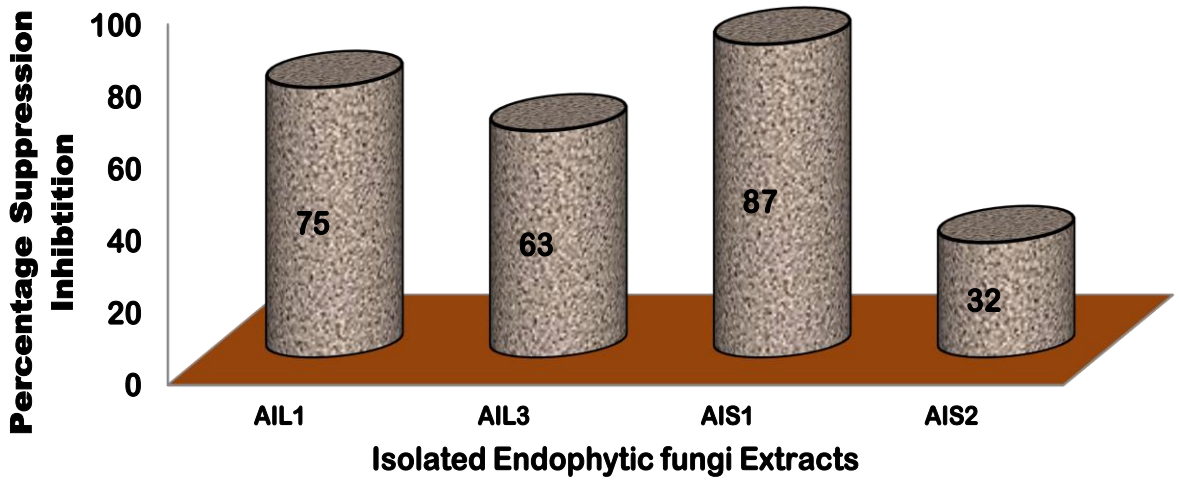

Figure 4. Percentage inhibition of the suppression by cyclophosphamide by the endophytic fungi extracts (prophylactic effect).

Table 5. Delayed type hypersensitivity response (DTHR) of the extracts in rats.

Treatment group	Dose (mg/kg)	DTHR edema (cm^3)	Inhibition (%)
AIL1	200	0.32 ± 0.03	65.59
AIL3	200	0.43 ± 0.10	53.76
AIS1	200	0.29 ± 0.04	68.81
AIS2	200	0.44 ± 0.12	52.68
Distilled water	10 ml/kg	0.93 ± 0.05	-

Values are expressed as mean ± Standard error of mean, n = 5 per group. DTHR: Delayed-Type Hypersensitivity Reaction, - = No inhibition.

Table 6. Antibody titre value of rats against different extracts.

Fungal extracts/ control	Doses (mg/kg)	Antibody titer value after seven days	Antibody titer	Haemagglutination inhibition (%)
AIL1	200	6 log2 ± 0.58	1.806	66.67
AIL3	200	4.5 log2 ± 0.29	1.355	55.56
AIS1	200	5.5 log2 ± 0.87	1.656	63.64
AIS2	200	5 log2 ± 0.58	1.505	60.00
Distilled water	10 ml/kg	2 log2 ± 0.00	0.602	-

Values are expressed as mean ± Standard error of the mean, n = 5 per group.

The mice were treated orally with endophytic fungi extracts and the route was chosen because of its sensitivity and rapid results. LD_{50} greater than 5000 mg/kg is thought to be safe (Erhirhie et al., 2018).

The present study demonstrates the antiplasmodial potentials of the fungal endophyte extracts against *P. berghei in vivo*. *P. berghei* has been used in studying the activity of potential antimalarials in mice (Kifle and Atnafie, 2020) and in rats (Pedroni et al., 2006). It produces diseases similar to those of human plasmodium infection (Peter and Anatoli, 1998; Shimada et al., 2019). Average percentage parasitemia decreased in the groups treated with the extracts comparable to the effect of the positive control (artemether-lumefantrine) while a daily increase of parasitemia was in the negative control group. *In vivo* models are usually employed in anti-malarial studies because they take into account the possible prodrug effect and probable involvement of the

immune system in the eradication of the pathogen (Waako et al., 2005; Mulisa et al., 2018).

The findings of this study agree with other studies carried out on the plant extracts of *A. indica* but the results from this fungal endophyte study showed a higher percentage of parasite inhibition. The *A. indica* stem bark and leaf extracts have been documented to have recorded about 56-87% and 51-80% parasitemia inhibition respectively (Akin-Osanaiye et al., 2013). Also, other researchers have reported earlier the antimalarial activity of different parts of *A. indica* tree (leaf, stem bark, and seed) against *P. berghei* and *P. falciparum* responsible for causing mammalian malaria (Deshpande et al., 2014).

In our *in vitro* experiment, AIL1 and AIS2 extracts inhibited the maturation of the schizont stage of the *P. falciparum* parasite, with percentage inhibition comparable to that of the positive control (Arthemether-lufamentrine) used in the study. Interestingly, AIS2 extract cleared the plasmodium parasites from the mice blood at treatment. The *A. indica* leaf extract has also been reported by Udeinya et al. (2008) to have both schizonticidal and gametocytocidal activities.

Basic research on natural substances with immunomodulating properties is performed by stimulating cells of the immune system including neutrophils, macrophages, T and B cells, NK cells (Yuandani et al., 2021). The present study demonstrates, for the first time, the immunostimulatory potential of the endophytic fungi of *A. indica*.

Cyclophosphamide administration induces acute and transient myelosuppression, primarily through damage to rapidly proliferating hematopoietic progenitors and their mature progeny leading to a decline in the number of peripheral blood cells (Sheeja and Kuttan, 2006). Cyclophosphamide induced immune-suppressive mice model was used because the dynamic and complex nature of the immune system in which a drug elicits its effect can be detected more reliably after immune challenge (Ahlmann and Hempel, 2016).

From our findings, injection of cyclophosphamide caused a significant drop of total white blood cells (TWBC) and the differential white blood cells (neutrophil) in mice. Nevertheless, treatment of the mice with the fungal endophyte extracts enhanced the proliferation of the TWBC, and neutrophil count was observed in groups of mice treated with the extracts compared to the group given the distilled water (negative control) group. The increase in the TWBCs count observed may have resulted from stimulation of leucocytosis by the extracts and enhanced production in the bone marrow (Okokon et al., 2004). The extracts worked in a dose-dependent manner. The present study demonstrated, for the first time, the immunostimulatory property of the extracts of endophytic fungi isolated from *A. indica.*

Pre-treatment of the mice with the fungal endophyte extracts increased its TWBC and neutrophil count and thus, protected them against cyclophosphamide-induced leucopenia. This indicates that the extracts possess the ability to prevent the occurrence of infection. Extracts of the endophytic fungi inhibited the delayed-type hypersensitivity reaction evoked by SRBCs in the rat. Hence, the extracts can modulate the cell-mediated adaptive immune response in rats as shown by the inhibition of DTHR. This indicated the stimulatory effect of the fungal endophyte on chemotaxis-dependent leucocyte migration. In the early hypersensitivity reaction, the antigen-antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema, and infiltration of PMN leucocytes. Similar results have been obtained by other researchers (Dhasarathan et al., 2010; Eze et al., 2014).

Furthermore, the humoral immune response (HIR) to sheep red blood cells was measured by the haemagglutination (HI) test. The fungal endophyte extracts demonstrated an increase in the hemagglutination titer in mice. Antibody molecules that are secreted by plasma cells mediate the humoral immune response. This augmentation of the humoral response to SRBC indicated enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis (Gabhe et al., 2006, Kaur et al., 2014). The immunoregulatory properties of the antibody have been recognized since the earliest passive immunization experiments, and the potential to modulate the immune response by deliberate immunization with antigen bound by antibody has been demonstrated in numerous instances over the decades (Brady et al., 2000; Alber et al., 2001; Antoniou and Watts, 2002; Rafiq and Clynes, 2002). The extracts of the endophyte fungi were found to have a significant immunostimulant activity on both the specific and non-specific immune mechanisms. From the positive significant correlation between antiplasmodial and immunostimulatory activities displayed by the fungal endophyte extract, it could be inferred that the activities shown by these fungal extracts were influenced by immunomodulatory property.

Conclusion

The extracts of the isolated fungal endophytes showed antiplasmodial and immunomodulatory activities. They modulated both cellular and humoral immunity. There was a positive correlation between antiplasmodial and immunostimulatory activities displayed by the fungal endophyte extract. Consequently, plant immunomodulators demonstrating therapeutic and immunomodulatory mechanisms of action possibly will be a perfect target for drug development.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors appreciate the laboratory workers in different labs where this study was carried out and are also grateful to the taxonomist, Mrs. Anthonia U. Emezie who authenticated the plant materials used in the study.

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