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# IMMUNO-MODULATORY AND MEMBRANE STABILISING ACTIVITIES OF ENDOPHYTIC FUNGI ISOLATED FROM MANGROVE PLANTS: *RHIZOPHORA RACEMOSA, AVICENNA ALBA* AND *ACROSTICHUMAUREUM*

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## ABSTRACT

**Objective**: To investigate the immuno-stimulatory and membrane stabilising activities of endophytic fungi isolated from mangrove plants: *Rhizophora racemosa*, *Avicenna alba* and *Acrostichum aureum.* **Method**: The pure endophytic fungal cultures were fermented using semi-solid rice substrate which produced the crude extract. The crude extract was then extracted using ethyl acetate and methanol and dried under controlled temperature of 37 ˚C and standard atmospheric pressure. Hypotonicity-induced and heat-induced haemolysis assays were used to evaluate the membrane stabilizing activity, and phagocytises stimulatory assay was used to evaluate the immuno-modulatory activity of the isolated endophytic fungi. **Results**: Eight endophytes coded as 1S2, 2L1, 2S2, 3L1M, 3L2-B, 3L2-amc, 3S1 and 3S2- amc were isolated from leaves and seeds of *R. racemosa, A. alba* and *A. aureum.* Ethyl acetate extracts of 1S2, 2L1, 2S2, 3L2-B and 3S2-amc were found to stabilize the membrane more than the standard drug. Their mean percentage inhibitions at 100 µg/ml are 95.41,

86.07, 88.35, 90.65 and 76.01% respectively when compared to the standard (48.98%) in a hypotonic environment. Only 1S2, 2S2, 3L2-B and 3S2-amc showed significant membrane stabilizing activity comparable to the standard in the heat induced environment. All the ethyl acetate extracts did not show any significant immuno-stimulatory activity ($p>0.05$). **Conclusion**: 1S2, 3L2-B and 3S2-amc have promising membrane stabilizing activity and can serve as leads in the development of novel bioactive compounds.

**KEYWORDS:** Immuno-stimulatory, membrane stabilising, endophytic fungi, *Rhizophora racemosa*, *Avicenna alba* and *Acrostichum aureum.*

## 1. INTRODUCTION

Endophytes are microorganisms (mostly bacteria, actinomycetes and fungi) dwelling in plant tissues in diverse symbiotic associations, without any immediate symptomatic overt negative impacts on the plant (Bacon and White, 2000; Petrini, 1991). The long term interaction that endophytes have with the defence mechanisms of the plant hosts is a potential selection pressure for the development of novel metabolic pathways in them - a potentially beneficial situation for bio-prospectors (Calhoun *et al*., 1992; Lu *et al*., 2000; Schulz *et al*., 1995; Tan and Zou, 2001; Wang *et al*., 2000; Weber *et al*., 2007). Therefore, most of endophytes are capable of producing active metabolites and some of these compounds are proven to have medical values (Santiago et al, 2012; Weber et al, 2007; Tan et al, 2001 and Zhaoet et al, 2009), serving as leads in drug discovery and development.

A number of mangroves and associates contain substances which show biological activities such as antiviral, antibacterial and antifungal properties (Bandaranayake, 2002; Bandaranayake, 1995 and Chandrasekaran et al, 2009). It has been found that Mangrove plants are a rich source of phytocompounds including steroids, triterpenes, saponins, flavonoids, alkaloids, tannins (Agoramoorthy et al, 2007; Bandaranayake, 2002 and Bandaranayake, 1995).

Therefore, some plant parts of *Rhizophora racemosa* ***Avicenna alba*** **and** ***Acrostichum aureum*** were studied with a view of isolating any endophytic fungi and subsequent evaluations of their immuno-modulatory and membrane stabilising activities.

## 2. MATERIALS AND METHODS

### 2.1 Equipment

Autoclave, laminar air flow hood, pH meter, BOD incubator, vacuum rotary evaporator, analytical balance, centrifuge, microscope, ultraviolet-visible light spectrophotometer, and analytical high performance liquid chromatography (HPLC), Dionex UltiMate-3400SD with an LPG-3400SD pump, coupled with photodiode array detector (DAD 300RS) were used.

### 2.2 Collection of Plant Materials

Nigeria mangrove plant species which include *Rhizophora racemosa, Avicenna alba* and *Acrostichum aureum* were collected from the mangrove forest at Degema, Rivers State, Nigeria on August 16, 2015. The whole-plant specimen was identified and authenticated by Dr. Ekeke Chimezie, Taxonomist, Department of Plant Science and Biotechnology, University of Port Harcourt, Port Harcourt, Nigeria. Voucher specimen of the sampled mangrove plants were deposited at the University of Port Harcourt Reference Herbarium for Research and Germplasm Conservation (UPH/RHRGC) with the herbarium number UPH/V/78.

### 2.3 Cultivation of endophytic fungi

Plant parts (leaves and seeds) were washed under running distilled water and surface-sterilized twice by immersion in 70% ethanol for 1 min, sodium hypochlorite solution (3% available chlorine) for 5 mins and distilled water for 1 min. The surface-sterilized plant tissues were cut into small pieces. The slices were further sterilized as stated above.

### 2.4 Isolation of endophytic fungi

Fungal isolation was performed under sterilised conditions from the inner tissues of *R. racemosa, A. alba* and *A. aureum* following an isolation protocol described previously (Kjer *et al,* 2010). The fungus was cultured on solid rice medium and identified based on the morphology of the fungal culture, the mechanism of spore production and characteristics of the spore. A voucher specimen of the fungus was deposited at our mycology laboratory.

### 2.5 Extraction of endophytic fungi secondary metabolites

The fermentation was performed on solid rice for four (4) weeks. The culture was diced and extracted with ethyl acetate, and the crude extract dried *in vacuo* using a rotating evaporator. The dried extract was weighed and stored in a refrigerator at less than 4 ºC, before used for bioassays.

**2.6 Membrane stabilising effect assay**

**2.6.1** Heat-induced hemolysis: Fresh whole human blood (5 ml) was collected and transferred to an ethylene diamine tetraacetate (EDTA) centrifuge tube. The tube was centrifuged at 2000 rpm for 5 min, and washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as a 40 % v/v suspension with isotonic phosphate buffer solution (pH 7.4). The composition of the buffer solution (g/l) was NaCl (4.4 g), NaH2PO4 (1.6 g), and Na2HPO4 (7.6 g). The isotonic buffer solutions (5 ml) containing 20, 50, or 100 $\mu$g/ml of the secondary metabolites were each put in sets of four (4) centrifuge tubes per concentration. Control tubes contained 5 ml of vehicle, 5 ml of 50 $\mu$g/ml indomethacin, or 5 ml of 100 $\mu$g/ml prednisolone, or 5 ml of 100 $\mu$g/ml diclofenac. Erythrocyte suspension (0.005 ml) was added to each tube and gently mixed. A pair of the tubes from each set was incubated at 54 °C for 20 min in a regulated water bath. The other pair was maintained at 0 – 4 °C in a freezer for 20 min. At the end of the incubation, the reaction mixture was centrifuged at 1000 rpm for 3 min and the absorbance (OD) of the supernatant measured spectrophotometrically at 540 nm. The percent inhibition of haemolysis (Shinde *et al*, 1999), was calculated using the following formula:

Inhibition of haemolysis (%) = {1 – [(OD2 – OD3)/(OD3 – OD1)]} * 100

Where OD1 = absorbance of test sample unheated, OD2 = absorbance of test sample heated, and OD3 = absorbance of control sample heated.

**2.6.2 Hypotonicity-induced hemolysis**

This was done by a slight modification of the method reported by Mounnissamy *et al*, 2007. Two per cent (2 % w/v) dextrose, 0.8 % w/v, sodium citrate, 0.05 % w/v citric acid and 0.42 % w/v sodium chloride was dissolved in distilled water and sterilised to obtain sterilised Alsever solution. Blood was collected from median cubital vein of healthy volunteers, placed in ethylene diamine tetraacetic acid (EDTA) centrifuge tubes and swirled for 30 seconds. A portion of the collected blood was mixed with equal volume of sterilized Alsever solution, centrifuged at 3000 rpm for 10 minutes and the packed cells were washed trice with sterilised isosaline (0.9 % NaCl) solution in distilled water), and a suspension of compact packed blood cells in 10 % (v/v) sterile isosaline was made. A 1 ml aliquot of filtered solutions of varying concentrations (20 – 100 $\mu$g/ml) of the *F. equiseti* secondary metabolites in phosphate buffer (pH 7.4) was mixed with 2 ml hyposaline (0.3 % NaCl solution in distilled water) in a centrifuge tube, followed by addition of 0.5 ml of the human red blood cell (HRBC) suspension. The assay mixture was incubated at 37 ºC for 30 minutes and centrifuged at 3000

rpm for 10 minutes. Haemoglobin content in the supernatant solution was estimated using ultraviolet/visible light spectrophotometry at 560 nm. Diclofenac sodium (100 $\mu$g/ml) was used as the reference drug and positive control. For the negative control, the 2 ml of hyposaline was replaced with an equivalent volume of sterile distilled water. The per cent inhibition of haemolysis was calculated by assuming the haemolysis inhibition produced in the presence of distilled water was 0 %. The percentage of HRBC membrane stabilization or protection was calculated using this equation:

Percentage inhibition of Haemolysis = [(OD1 – OD2)/OD1] * 100

Where OD1and OD2, are the optical absorbance of the negative control and test sample respectively.

**2.7 Phagocytises stimulatory assay**

**2.7.1** *Candida albicans* culture was incubated in Sabouraud dextrose broth (SDB) overnight and centrifuged to form a cell button at the bottom of the test tube. The supernatant was discarded and the cell button washed four (4) times with sterile phosphate buffer saline (PBS) and centrifuged. The washed cell button was re-suspended in equivolume of a mixture of PBS and serum in the ratio 4:1.

**2.7.2** Slides were appropriately labelled for the various concentrations of the secondary metabolites, ascorbic acid, and control (water). One drop of human blood was smeared on each slide and the slides were incubated at 37$^{o}$C for 20 minutes to allow for clotting. The slides were drained slowly with sterile normal saline, taking care not to wash off the adhered neutrophils. The slides consisting of polymophornuclear neutrophils (PMNs) were flooded with 5 ml of solution of predetermined concentrations of the various test samples, respectively, and incubated at 37 $^{o}$C for 15 minutes. After which, the PMNs on the slide was covered with 1 ml of the *Candida albicans* suspension and incubated at 37 $^{o}$C for 1 hour. The slides were drained with normal saline, fixed with methanol and stained with Giemsa stain.

**2.7.3** The slides were viewed under the microscope and the number of *Candida albicans* cells phagocytosed by PMNs on the slides was determined by a count of the dark blue spots on gray background. This number was regarded as the phagocytic index (PI) and was compared with the PI of the control treatment as described in the equation below.[11]

$$\text{Stimulation of phagocytosis} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI control}} \times \frac{100}{1}$$

### 2.8 Statistical Analysis

Where applicable, results were expressed as mean ± SEM. Means were compared for statistical significant difference by one-tailed *t*-Test and one-tailed analysis of variance (ANOVA) using Duncan post-hoc test, as programmed in Graphical Prism® statistical software version 7.0. Effects were considered significant at p = 0.05 in all cases.

## 3. RESULTS

**3.1 Table 1 Endophytic fungi isolated showing the plant and plant parts they were isolated from.**

| Fungal Code No. | Plant Part | Plant | Family |
|---|---|---|---|
| 1S2 | Seed | *Rhizophora racemosa* | Rhizophoraceae |
| 2L1 | Leaf | *Avicenna alba* Blume | Avicenniaceae |
| 2S2 | Seed | *Avicenna alba* Blume | Avicenniaceae |
| 3L1M | Leaf | *Acrostichum aureum* L | Pteridaceae |
| 3L2-B | Leaf | *Acrostichum aureum* L | Pteridaceae |
| 3L2-amc | Leaf | *Acrostichum aureum* L | Pteridaceae |
| 3S1 | Leaf | *Acrostichum aureum* L | Pteridaceae |
| 3S2-amc | Leaf | *Acrostichum aureum* L | Pteridaceae |

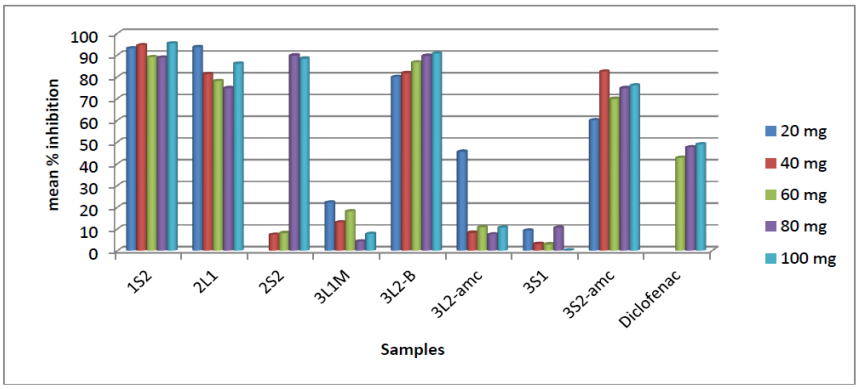


**Fig 1: Membrane stabilising effect of test drugs and the standard in a hypotonic environment.**

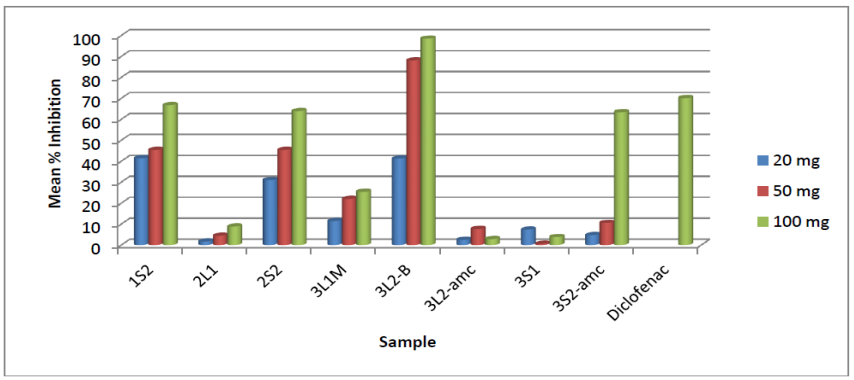


**Fig. 2: Heat-Induced Haemolysis: Membrane stabilizing effect of test drugs and standard in a heatenvironment.**

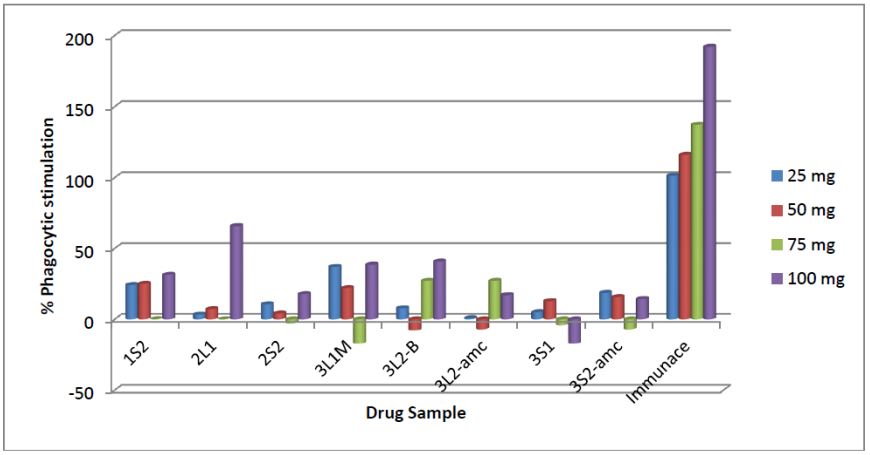


**Fig. 3: Percentage phagocytotic stimulation (% PS) of test and standard drugs.**

## 4. DISCUSSION

In this study, eight endophytic fungi designated as 1S2; 2L1, 2S2, 3L1M, 3L2-B, 3L2-amc, 3S1 and 3S2-amc have been isolated from the ethyl acetate extraction of three mangrove plants (Table 1). These endophytes were studied for *in vitro* membrane stabilizing activity and immune stimulatory activity. The extracts (1S2, 2L1, 3L2-B and 3S2-amc) showed significant membrane stabilizing property in an hypotonic environment, higher than the control used (diclofenac). The human red blood cell membrane is similar to lysosomal membrane. The stabilization of membrane is important in reducing inflammatory response caused by the release of pro-inflammatory enzymes from the lysosome of active neutrophils such as bactericidal enzymes and protease (Azzem et al., 2010; Chippada, 2011). Hypotonicity-induced haemolysis may have been inhibited by the extracts mentioned above by fortifying the human red blood cells (HRBC) membrane and thereby preventing the lysing of the cell. The mechanism is not yet known. The stability in heat-induced condition was also studied. The result shows that the extracts (1S2, 2S2 and 3L2-B) were able to stabilize the membrane more than the standard. The stability of membrane under heat is likely due to protection against protein denaturation. Denaturation of protein under elevated temperature is a well documented source of inflammation. Membrane stabilization under heat condition could possibly be due to enhanced membrane protein by the extracts. The extracts (1S2, 2S2 and 3L2-B) and the standard drug show similar cell membrane stabilizing effect under heat.

The *in vitro* immune-stimulatory assay revealed some immune boasting effects of the extracts: 1S2, 3L1M, 3L2-B and 3S2-amc, indicated by increased phagocytic activity of polymorphonuclear neutrophils (PMN). However, this activity was not significant compared to the standard.

## 5. CONCLUSION

Our showed that the plants used: *Rhizophora racemosa, Avicenna alba* and *Acrostichum aureum* contain endophytic fungi. The isolates (1S2, 2L1, 3L2-B and 3S2-amc) showed significant membrane stabilizing property in hypotonic environment while 1S2, 2S2 and 3L2-B provided membrane stability when heamolysis is heat-induced. There was no significant immuno-stimulatory effects observed in all the isolates.

## 6. Conflict of interests

The Authors declare no conflict of interests.

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