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Original Research Article

Susceptibility Studies of Vancomycin Resistant *Staphylococcus aureus* (VRSA), Extended *β*-lactamase Producing *Escherichia coli* and *P. falciparum* to Ethylacetate Extract of Endophytic Fungal Metabolites from *Annona senegalensis*

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ABSTRACT

Search for novel compounds can impact positively in the development of action plans in the control of existing and emerging antimicrobial drug-resistance (ADR). This study, aimed at assessing the susceptibility of vancomycin resistant *Staphylococcus aureus* (VRSA), extended *β*-lactamase (*ESβL)* producing *Escherichia coli (E. coli)* and *Plasmodium falciparum (P. falciparum)* to ethyl acetate extract of secondary metabolites of endophytic fungi isolated from the leaves and root of *Annona senegalensis* Pers. Seven endophytic fungi were isolated from cultured, freshly collected root and leaves of *A. senegalensis*. Six (Root(RT)1, Root (RT)2, Root(RT)3, Leaf-Blade(LB)1, Mid-Rib(MR)1 and Mid-Rib(MR)2) out of the seven isolates whose toxicity profiles had been ascertained in our previous study were selected for antibacterial and antimalarial studies. The antibacterial activities of the endophytic fungal extracts were tested using the agar well diffusion technique. The activities were tested against VRSA and ESβL *E. coli* and ciprofloxacin- (5 µg) was used as the positive controls. *In vitro* anti-malarial activity was also carried out with the extracts. The result showed that out of the six isolates, RT1 metabolite had marked activities against ESβL *E. coli* and *P. falciparum*. Similarly, RT2, had an impressive activity against VRSA, ESβL *E. coli* and *P. falciparum*. The highest concentration of RT2 gave 58 % inhibition of the parasite whereas that of the control was 66 %. This impressive susceptibility of VRSA, ESβL *E. coli and P. falciparum* to these fungal metabolites calls for further studies on these metabolites to purify and elucidate the bioactive component of each compound.

***Keywords*:** Multidrug-resistant, Antimicrobial resistance, ESβL *E. coli*, Endophyte, VRSA, *P. falciparum*

Introduction

Emergence of multidrug-resistance organisms have adversely affected public health worldwide and often result in increased healthcare cost, limited treatment options, uncertainties in therapeutic outcome as well as high mortality.[1-3] It has been reported that multidrug-resistant bacteria, fungi, viruses, and parasites have developed in clinical setting and are now frequently identified in community settings, suggestive of the fact that numerous antibiotic-resistant organisms exist outside the hospital.[4] Similarly, microbial resistance to antibiotic has been projected to bring about approximately 300 million untimely deaths by 2050, with a cost of about $100 trillion (£64 trillion) to the world economy.[5]

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In addition, it has been reported that, *Staphylococcus aureus* isolates with complete resistance to vancomycin have emerged.[1] *S. aureus* had been implicated in many severe infections especially when entering the bloodstream or internal tissues and it remains primarily human pathogen that causes a multiplicity of clinical conditions going from relatively mild skin and soft tissue infections to generalized life threatening disease conditions.[6] Vancomycin has often been used as drug of choice in the treatment of methicillin resistant *Staphylococcus aureus* in clinical setting. Resistance of *S. aureus* to vancomycin is mediated by a van A gene cluster, which is transferred from vancomycin resistant enterococcus. Whereas, some proteins encoded by the vanA gene cluster, VanS, VanR, VanH, VanA and VanX are essential for vancomycin resistance.[1,7] In the same way, the incidence of extended-spectrum *β*-lactamase (ESBL)-producing enterobacteriaceae resistant to penicillins and third generation cephalosporins has been growing considerably since the turn of the millennium[8-12] Worthy of mention among the enterobacteriaceae-producing ESBL is *Escherichia coli*, a common cause of urinary tract and wound infections. Its ubiquitous nature in various habitats and its capability to acquire resistance genes via mobile genetic elements assists *E. coli* to rapidly spread antimicrobial resistance (AMR) among people, animals, and the environment.[8] In Africa, various plants and plant materials have been useful traditionally in the prevention, treatment and management of various disease conditions affecting man. One of such plants of high ethno medicinal potential is *Annona*

senegalensis.[13] It has been variously documented that extracts from *Annona senegalensis* possess antimicrobial, antioxidant, anticancer, anti-inflammatory, anticonvulsant, antimalarial, trypanocidal, anti-snake venom and anthelmintic properties.[13-18] In a study, an extract of recipe comprising six plants with *Annona senegalensis* had substantial antibacterial activity with MIC of 62.5 µg/ml against *S. aureus* and 250 µg/ml against *Candida albicans*.[19] Similarly, the antimicrobial potential of *Annona senegalensis* when evaluated, were established to be good against pathogenic bacteria strains like *Staphylococcus aureus, Shigella flexneri, Salmonella paratyphi* and *Pseudomonas aeruginosa*. It was detected that the ethanolic and aqueous leaf extracts showed good zones of inhibition though methanol extract possessed substantial antimicrobial activities against clinical isolates of *Salmonella enteritidis, Shigella dysenteriae* and *E. coli*.[20, 21] Furthermore, the various solvent extracts of leaf of *Annona senegalensis* displayed wide-ranging antimicrobial activity using Agar-well diffusion method against pathogenic microbes.[20] An extract obtained from the root of the plant equally possessed activity against *Plasmodium falciparum* that was not susceptible to chloroquine [22]. Despite avalanche of its reported medicinal property, the microbial diversity of *Annona senegalensis* has been under- researched vis- a-vis its endophytic fungi. It is therefore pertinent to assess the susceptibility of vancomycin resistant *Staphylococcus aureus* (VRSA), extended spectrum *β*-lactamase *Escherichia coli* and *P. falciparum* to ethyl acetate extract of its endophytic fungal metabolites.

Materials and Methods

Sample collection and authentication

The various plant parts of *Annona senegalensis* used in this study (root, stem and leaves) were collected from a non-diseased, mature plant at Mbu-Akpoti, in Isi-Uzo Local Government Area of Enugu State Nigeria in September 2017. The freshly collected plant samples were duly identified by a taxonomist, Mr. Alfred Ozioko, a retired staff of Botany Department, University of Nigeria Nsukka. Further authentication of the samples was done by a botanist Mr. Onyeukwu Chijioke John of Plant Science and Biotechnology (former Botany) Department, University of Nigeria, Nsukka. The samples were assigned a voucher number UNH NO 9a and deposited at the herbarium collection center of Plant Science and Biotechnology Department (Botany), University of Nigeria. The ethical clearance for this study with reference number DOR/UNN/19/00029 was obtained from the Research Ethics Committee of the University of Nigeria, Nsukka.

Cultivation and isolation of endophytic fungi

In the whole process of cultivation, isolation and purification, the methods of Okezie *et al.*[23] were adopted with little modifications because of preliminary studies. Here, all the freshly harvested samples (roots, stems and leaves) were washed carefully beneath running tap water, followed by sterile double distilled water, before processing. To remove epiphytic microorganisms, all the samples were subjected to four step surface sterilization which involved washing under running tap water-ethanol-sodium hypochlorite-distilled water. The samples were washed in running tap water, after which they were further immersed in 70 % ethanol for 3 min and washed twice with distilled water. In addition, the already washed samples were again immersed in sodium hypochlorite solution (4 %) for 5 min and washed meticulously, thrice in distilled water and then rinsed in 70 % ethanol for 3 min, prior to a final rinse in sterilized double distilled water. The washed samples were dried in the laminar flow on a sterile filter paper. Sterile knife was used to cut the samples to roughly 1 cm in length. Segments (a total of 30 at three to six segments per Petri dish) of samples were inoculated on previously sterilized media (malt extract agar) incorporated with chloramphenicol, 500 mg/L. The Petri dishes were appropriately sealed with Parafilm®, then incubated at 25^{o}C and the plates were checked on alternate days. After seven days, hyphal tips of actively growing fungi from the plant tissues were sub-cultured to other sterile malt extract agar (MEA) plates and incubated for five to seven days and the purity of the cultures was checked periodically. Sub culturing was done at an interval of two weeks to maintain the pure cultures. For studying the cultural characters, the fungi were grown on Malt Extract Agar medium. Cultural characters such as color, nature of the growth of the colony and texture were determined by visual observation.[24] The maximum growths of the fungi were also observed on Malt Extract Agar whereas, to produce metabolites, the starting material was taken from freshly sub-cultured plates.

Purification of fungi isolate

To prepare inocula for fermentation studies, cultivation of hyphae/mycelium to obtain pure cultures was done. Here, the hyphal tips of fungi, growing out of the previously sub cultured fungi was once more sub-cultured by picking the hyphal tip, placing on a fresh MEA, and incubating at 25^{o}C for 7 days. All transfers were done aseptically, to ensure purity of isolates. Upon incubation, it was discovered that the root, leaf blades and mid-ribs of the plant under study gave different fungi isolated during this research whereas, the stem did not show any form of growth after sufficient period of incubation.

Fermentation using pure isolates

The fungal metabolites used in this study were generated using the method described by Okezie *et al.*[23] with little modification. Local rice was used as the fermentation medium. The homegrown rice (100 g) was weighed into a sterilized conical flask and 200 mL of sterile water added. The content was sterilized at 121^{o}C for 30 min and allowed to cool. Subsequently, slices were aseptically cut from the actively growing pure isolates on MEA and inoculated into the now sterilized local rice fermentation medium contained in a 500-mL Erlenmeyer flask. This was properly sealed and kept on the shelf. The fermentation process was allowed for 21 days at 30^{o}C under static conditions.

Extraction of fungal metabolites

Extraction of metabolites generated from pure fungal isolates was carried out according to the established procedures of Okezie *et al.*[23] The fermentation process was stopped by the addition of the extraction solvent (ethyl acetate) and each of the fermented medium in the sterile Erlenmeyer flasks was made homogeneous. Here, fungal biomass together with the medium were cut into small lumps with a sterilized glass rod and the mixture was homogenized with 500 mL of ethyl acetate in a one liter Erlenmeyer flasks, and shaken intermittently for 2 days and then filtered with Whatman filter paper (pore size: 11 µm). The filtrate was concentrated at 50 ^{o}C under reduced pressure by means of a rotary evaporator. The concentrated extract was allowed to evaporate to dryness in a desiccator that contained sodium hydroxide. Then, the resultant extracts were weighed, and their corresponding percentage yields calculated. After evaporation, the dried fungal extracts were reconstituted in dimethyl sulphoxide (DMSO) and used for biological studies.

Biological studies

Test microorganisms

The test organisms used in this work were obtained from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka. These organisms were further confirmed by sub-culturing and subjecting pure isolates to specific pure culture identification techniques.

Identification and characterization of isolates

Identification of bacterial isolates was carried out based on their cultural, microscopic, and biochemical features. The different isolates obtained were characterized by carrying out on them morphological (colonial morphology), physiological and biochemical tests.

(a) Morphological characterization

The isolated colonies were examined and their form of growth, elevation, size, color, margin edge, consistency, opacity, and change in medium established.

(b) Microscopy - gram staining
A thin smear of each isolate was prepared on clean glass slide, air dried and heat fixed by passing it slightly through the flames, about 3 to 4 times. The slide was flooded with crystal violet and allowed for 1 min. It was washed in tap water and excess water was drained off. It was flooded with Iodine solution (a mordant) for 1 min, to retain the dye even when washed. This was decolorized with ethanol, added drop wise on the tilted slide until all free color (purple) had been removed, and then washed with water after which Safranin (2-3 drops) was applied and allowed for 3 min. Finally, the slide was washed with water and air dried. One drop of immersion oil was added to the slide which was then viewed under the microscope. Gram positive cells stained purple while Gram negative cells stained pink.

(c) Biochemical tests
Some biochemical assessments were carried out to further identify the organisms and some of the tests include: Catalase test, Coagulase test, Indole test and Oxidase test.

(i) Catalase test
This was carried out on all Gram-positive cocci to distinguish Staphylococcus species from Streptococcus species. Using a sterilized syringe, 2 drops of hydrogen peroxide (H_2O_2) were placed on a clean glass slide. A sterile cooled wire loop was used to pick a small portion of the test bacteria, placed in the drop of H_2O_2 on the slide and emulsified. Positive result shows effervescence, while negative outcome gives no effervescence.

(ii) Coagulase test
This was done on all *Staphylococcus* species for the identification of pathogenic *Staphylococcus aureus*. The slide technique was adopted. A drop of distilled water was placed on a clean glass slide using a sterilized syringe. The wire loop was sterilized (by flaming) and used to lift a colony of the test bacteria from the plate. The organism was emulsified in the drop of distilled water on the slide and a drop of undiluted plasma was added to it. This was mixed by tilting the slide to and fro while watching out for coagulation. A positive result displays instant clumping within 10 sec, while a negative outcome shows no clumping.

(iii) Indole test
This was carried out on all Gram-negative bacilli for the identification of *Enterobacteriaceae*: *Escherichia coli*, *Proteus vulgaris/mirabilis*, and *Klebsiella spp*. The test organism was inoculated in a test tube containing 3 mL of sterile tryptone water and incubated at 37^{o}C for 48 h. Kovac's reagent (4 p-dimethylaminobenzaldehyde), (0.5 mL) was added and shaken lightly. The product was observed for a red coloration in the surface layer within 10 min. A positive result provided red surface layer while a negative result does not.

Determination of antimicrobial activity
The antimicrobial activities of the endophytic fungal extracts were tested using the agar well diffusion technique.[25] The activities were tested against vancomycin resistant *Staphylococcus aureus* (VRSA) and extended spectrum β-lactamase *Escherichia coli*. These were typical laboratory cultures whose resistance to frequently used antibiotics was previously established and generously provided by Okezie, M.U of Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka.
The bacterial suspensions were adjusted to 0.5 McFarland turbidity standard and inoculated onto previously sterilized Mueller-Hinton Agar plates (diameter: 90 mm). A sterile cork borer was used to make five wells (6 mm in diameter) on each of the MHA. Aliquots of 50 µl of each extract dilution, reconstituted in DMSO at concentrations of 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL, was dropped in each of the wells in the culture plates earlier seeded with the test bacteria. Ciprofloxacin single disc (5 µg) served as the positive controls against the organisms. The cultures were incubated at 37 ^{o}C for 24 h. The antimicrobial activity of each extract was established by measuring the zone of inhibition around each well (without the diameter of the well). The test was done in triplicates for each organism. Each extract was tried against all the bacterial strains.

In vitro test for anti-malaria activity
Using an *in vitro* method described by Rini *et al.*[26] with slight modifications, several graded doses (ranges) of the test extracts were prepared in 5 ml of dimethyl sulfoxide. From the stock solution, two-fold serial dilutions were made into Petri dishes. At the start of the experiment, seven Petri-dishes were selected, one serving as the control while the remaining 6 contain various concentrations of test fungal metabolites. Next, 1.8 mL of the medium was added to each of the seven dishes followed by 0.2 mL of parasitized Red Blood Cells for continuous culture. The Petri dishes were covered, shaken gently, and placed in a candle-jar desiccator. The jar was placed in the incubator for 24-30 h at 37^{o}C. After incubation, thick and thin films were made from the contents of each dish and stained with 4 % Leishman's stain for 15-20 min. Asexual parasite and schizont counts were made against 500 leucocytes (White Blood Cell) both in the control and in the test dishes. The values for samples containing the test drug were expressed as a percentage of the control sample. Then, % maturation of schizonts was plotted against the test compound doses to determine the activity of the plant extract.
From the thin films, different parasites were observed for ring forms, trophozoites and schizonts. Parasites counts were determined for each dose level by comparison with the control smears thus indicating the % inhibition of parasitic growth.

Percentage parasitemia was calculated with the formula:

$$\% \text{ Parasitemia} = \frac{\sum Infected\ Erythrocytes}{\text{No of erythrocytes}} \text{ X } 100 \text{ ..... Equation 1 [26]}$$

The percentage inhibition of parasite growth was calculated using the formula:

$$\text{Percentage of Inhibition } (\%) = \frac{\text{Parasitemia in test samples}}{\text{Parasitemia in the control plates}} \text{ X } 100 \text{ ..... Equation 2 [26]}$$

Finally, in a graph, percentage inhibition of parasitic growth was plotted against test doses (concentrations) in order to show the activity of each test dose against *P. falciparum*.

Statistical analysis
Results collected from the study were presented as mean ± standard error of mean (SEM) of sample replicates (n = 3). Significant differences between control and treatment groups were compared by means of one-way analysis of variance (ANOVA) followed by post hoc Dunnett (2-sided) test. $P < 0.05$ was statistically significant, while $p > 0.05$ was statistically non-significant. Statistical Package for Social Science version 20 (SPSS-20, for windows) was the software used for data analyses. In graphical presentations of parameters, calculation of fifty percent effective concentration (EC_{50}) of the extract was done with logarithmic equation using Microsoft Excel, 2010.

Results and Discussion

Recent increase in microbial resistance to the already existing antimicrobial agents has necessitated diverse exploration of natural resources, including endophytic metabolites for novel drug discovery and development. Endophytes are known to be valuable source of pharmacologically active plant-related compounds.[27-30] The antibacterial activities observed from the endophytic fungal metabolites from *Annona senegalensis* can be attributed to the synergy from the various compounds present in the ethyl acetate extract. It has been reported earlier that ethyl acetate has high extraction capacity for flavonoids, tannins, alkaloids and saponins which had been adjudged to have high antimicrobial activities.[31]In a related study, Dikko *et al.*[32] further observed that the ethyl acetate fraction of their endophytic metabolites showed higher antimicrobial activities against their test

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organisms than other fractions of same metabolites. The various inhibition halos shown in Figure 1, clearly depicts susceptibility of the test bacteria to the endophytic fungal metabolites. In figure 1 A-C, the RT1 and MR1 extracts produced more inhibition against *ESβL* than *VRSA.* This variation in susceptibility may be due to the difference in the cell wall composition of the test Gram negative and Gram-positive organisms. Similarly, Ibtisam *et al.*[27] reported varied susceptibility of their test organisms to endophytic fungal extract and attributed the difference in susceptibility to the microbial cellular structure. On the contrary, in Figure 1 D, *VRSA* showed little or no zone of inhibition from RT1 and RT3 metabolites. This low inhibition suggests that the tested doses of the endophytic fungal extracts were below the minimum inhibitory concentrations for the organism.

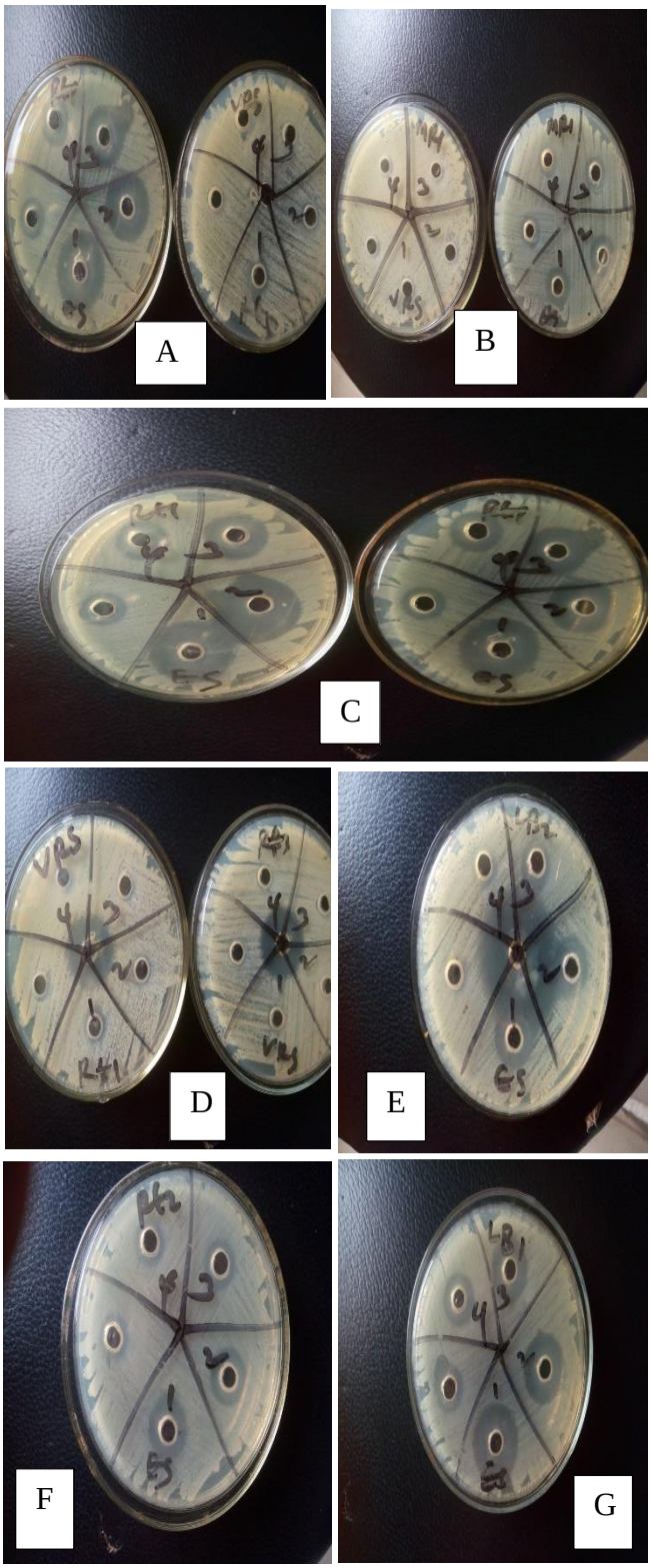
The fungal metabolite in RT1 was very active against extended β lactamase producing *E. coli*. At 10 mg/ml of the bioactive metabolites, the inhibition zone diameter was approximately 18 mm, a value that is comparable to that of ciprofloxacin (control drug). Again, the organism was also sensitive to the least concentration (0.63 mg/ml) of the extract. On the other hand, vancomycin resistant *Staphylococcus aureus* was sensitive to the extract at 10 and 5 mg/ml concentration, respectively. Interestingly, at 2.5, 1.25 and 0.63 mg/ml, the fungal metabolite exerted activity against extended β lactamase producing *E. coli* but not vancomycin resistant *Staphylococcus aureus*. In both organisms, the activity of the metabolites is directly related to the concentration of the extracts. The activity of RT1 extract seen in ESβL *E. coli* may be ascribed to the inhibition of the β – lactam enzymes in addition to any other mechanism of activity the extract may have. Albeit the control exerted marked activities against test organisms as shown in Figure 2.

In Figure 3, the extract demonstrated good antibacterial activities against the two test organisms. At 2.5, 1.25 and 0.63 mg/ml, the test organisms maintained a unique equal response to each concentration of the fungal metabolites. Generally, the inhibitory zone diameter of the extract was directly proportional to the various extract concentrations for each test organism. At 10 and 5 mg/ml, vancomycin resistant *Staphylococcus aureus* showed more sensitivity than the extended spectrum β lactamase *Escherichia coli* to the bioactive compounds. However, at other concentrations, the extract exerted equipotent activity on the organisms. It is of interest to note that all the concentrations of the extract were active against the test organisms. The broad-spectrum nature of the bioactive compound if elucidated will be very useful in drug therapy design.

Figure 4 shows concentration-dependent activity of the extract against extended spectrum β lactamase *Escherichia coli*. The organism showed sensitivity at 10, 5 and 2.5 mg/ml relative to the control drug. The organism was susceptible to all the concentrations of the extract. Similarly, Eze *et al.*[33] reported high susceptibility of both Gram positive and Gram negative test bacteria to their endophytic fungal extracts. However, vancomycin resistant *Staphylococcus aureus* did not show any form of sensitivity to all the tested concentrations of the bioactive compounds in RT3. This selective non inhibition of vancomycin resistant *Staphylococcus aureus* may be attributed to varied uptake mechanisms and protective biochemical endowment that probably limited the entry of the fungal metabolites into the organism.

The metabolites from the leaf blade isolate (LB1) had marked activity against extended spectrum β lactamase *Escherichia coli* as shown in Figure 5. At 10 mg/ml concentration of the fungal metabolites, the inhibitory zone diameter of the ESβL.*E. coli* was 18.5 mm. Again, this organism was also susceptible to the least concentration of the extract (0.63 mg/ml). This shows high activity relative to the control drug. In a related investigation, Caio *et al,* [34] while studying the effect of fungal mediated biosynthesis of silver nanoparticles, observed that *Fusarium oxysporum* mediated extract was highly active against ESβL *E. coli*. This and other scientific breakthroughs in research have rekindled the hope of surmounting the challenges posed by antimicrobial resistance. On the contrary, VRSA was resistant to all tested concentrations of the LB1 extract. Nonetheless, the control was equally active on the test organisms.

The mid Ribs Isolate (MR1) from figure 6, had a marked activity against extended spectrum β lactamase *Escherichia coli.* The organism was sensitive to all the tested concentrations of the extract and the activity increased directly with increase in concentration of the extract.



Figures 1: (A-G): Inhibition halos produced by endophytic fungal extracts against Vancomycin resistant *Staph aureus (VRSA)* and extended spectrum βeta-lactamase producing *E. coli (ESβL)*. Both RT1 and MR1 in A-C produced more inhibition against *ESβL* than *VRSA.* In D and E, *VRSA* and *ESβL* showed little or no zone of inhibition from RT1 and LB2 respectively. Similarly, RT2 and LB1 in F and G had obvious zones of inhibition against *ESβL.*

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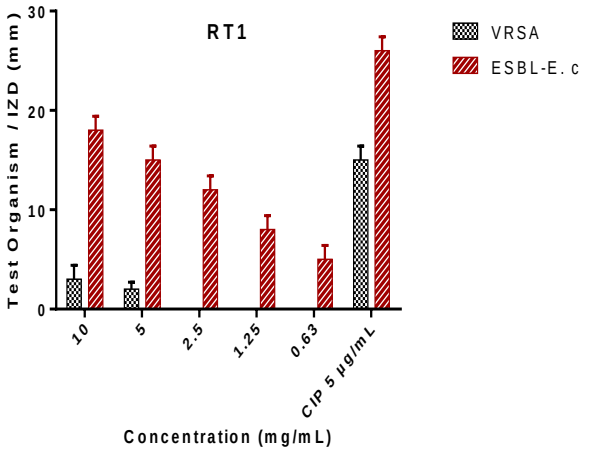

Figure 2: Antimicrobial activities of RT1 against vancomycin resistant *Staphylococcus aureus* and extended β-lactamase producing *E. coli.*

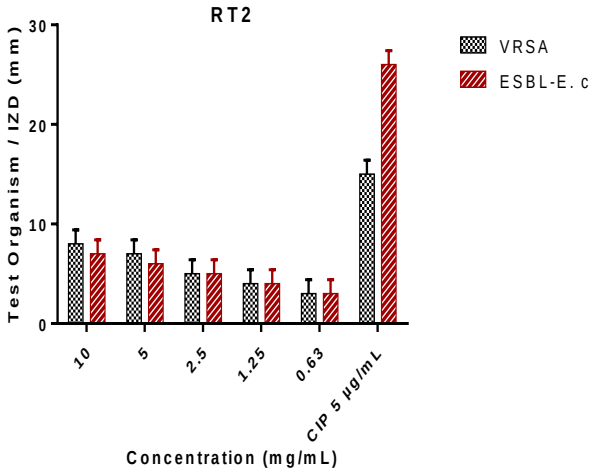

Figure 3: Antimicrobial activities of RT2 against vancomycin resistant *Staphylococcus aureus* and extended β-lactamase producing *E. coli.*

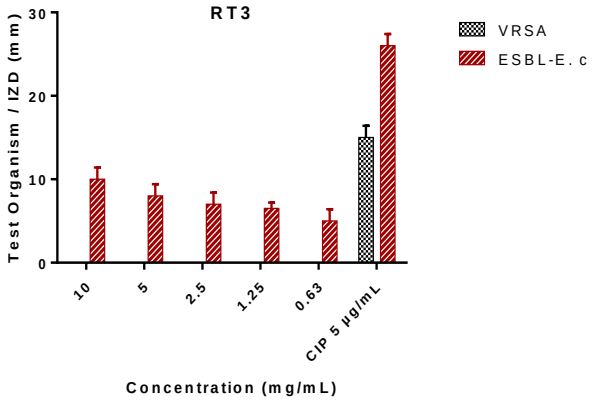

Figure 4: Antimicrobial activities of RT3 against vancomycin resistant *Staphylococcus aureus* and extended β-lactamase producing *E. coli.*

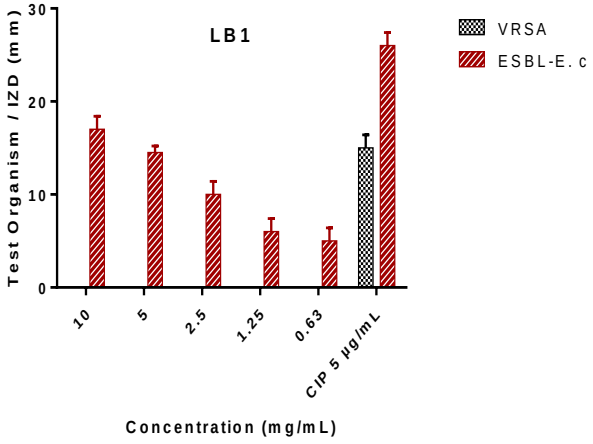

Figure 5: Antimicrobial activities of LB1 against vancomycin resistant *Staphylococcus aureus* and extended β-lactamase producing *E. coli.*

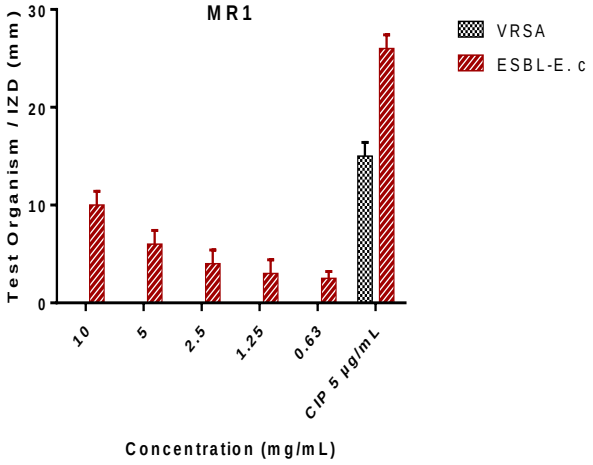

Figure 6: Antimicrobial activities of MR1 against vancomycin resistant *Staphylococcus aureus* and extended β-lactamase producing *E. coli.*

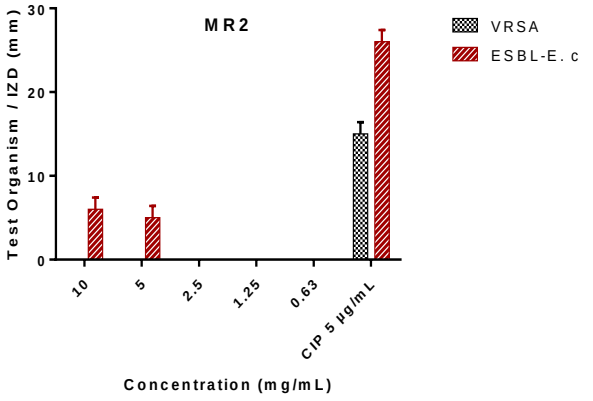

Figure 7: Antimicrobial activities of MR2 against vancomycin resistant *Staphylococcus aureus* and extended β-lactamase producing *E. coli.*

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This quite agree with the reports of Etidal and Tawheeda[35] who observed concentration dependent antibacterial activities of endophytic fungal metabolites from four medicinal plant in Sudan on their test organisms.

On the other hand, vancomycin resistant *Staphylococcus aureus* was very resistant to all the tested concentrations of the extracts.

The optimum concentration that gave the highest inhibitory zone diameter was 10 mg/mL whereas 0.63 mg/ml was the least concentration of the extract that ESβL *E. coli* was susceptible to. The two organisms were however, highly susceptible to ciprofloxacin.

In Figure 7, metabolites from mid ribs isolate (MR2) demonstrated good activity against extended spectrum β lactamase *Escherichia coli*. At 10 and 5 mg/ml, the organism was susceptible. However, other concentrations of the extract (2.5, 1.25 and 0.63 mg/mL) exerted no activity on any of the test organisms. In the same vein, VRSA was resistant to all the tested extract concentrations.

In Figure 8, various concentrations of the fungal metabolites from different parts of *Annona senegalensis* Pers were tested against *Plasmodium falciparum*. These concentrations were compared with the arthemeter/lumefantrine drug as control. Generally, both the *Annona senegalensis* extract and the control (arthemeter/lumefantrine) showed concentration-dependent inhibition of *P. falciparum*. In a related study, *Plasmodium falciparum* has been observed to be highly susceptible to metabolites from *Annona spp*.[36,37] Metabolites from RT1 and RT2 showed marked anti-plasmodial activities comparable to the control drug. The highest concentration of RT2 gave 58 % inhibition of the parasite whereas that of the control was 66 %. Similarly, RT1, LB1 and MR1 were equally active against the parasite. On the contrary, RT3 and LB2 did not show marked anti plasmodial activity. Several research reports have observed and recorded antimalarial properties of some endophytic fungal metabolites. Indeed, Elfita *et al.*[38] isolated seven fungi from the stem and leaves of *Tinaspora crispa*. In their report, they noted that the extracts had great anti plasmodial activities against chloroquine resistant *Plasmodium falciparum*. Further Joel *et al.*[39] studied the anti-plasmodial activity of extract from *Symphonia globulifera*, a plant popularly used in Cameroun for malaria treatment. The metabolite from their fungal isolates recorded antiplasmodial activity with IC_{50} >10 µg/ml. Again, in a related study, an *in vivo* antimalarial evaluation of the leaves of *Annona senegalensis* was carried out by Ajaiyeoba *et al.*[22] They noted that the leaf extract has marked antimalarial potential that was concentration dependent. They reported significant chemo-suppression of parasitemia of 91.1 % by their extract relative to chloroquine which they used as a standard drug. In the present work, seven different fungal isolates from the root, mid-rib and leaf blade produced concentration dependent *P. falciparum* inhibition as shown in Figure 8. Here, isolate from root (RT2) had the highest percentage inhibition among all the extracts relative to the artemether/lumefantrine (control).

Roots are known to interface with the soil and that provides the ease at which minerals, water and other essential nutrient requirement of endophytes are met. Some endophytes utilize these nutrients to boost their synthesis of metabolites. This may have accounted for the marked antimalarial activity shown in RT2 with very low EC_{50} comparable to the control drug. On the other hand, isolate from the leaf-blade (LB2) and root (RT3) gave the highest effective concentration values whereas the EC_{50} of RT1, LB1 and MR1 were significantly ($p < 0.05$) higher than that of the control drug against the parasites as shown in Figure 9. The impressive activities of all the fungal metabolites against *P. falciparum* calls for further studies on these metabolites to purify and elucidate the bioactive component of each compound.

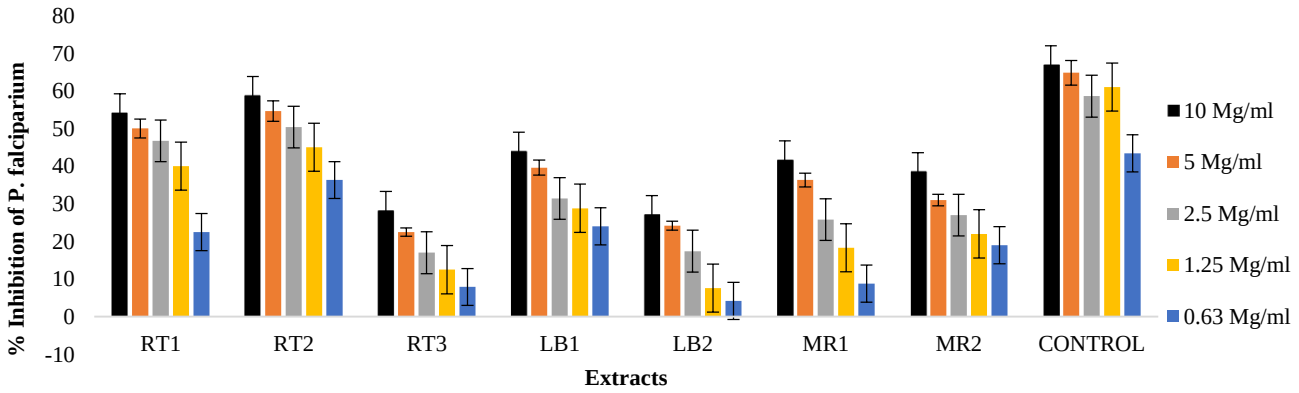

Figure 8: Percentage *P. falciparum* parasite inhibition by various concentrations of endophytic fungal metabolite from *Annona senegalensis* Pers

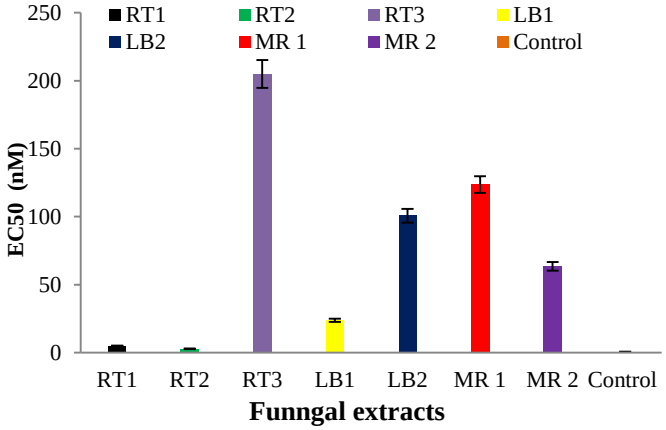

Figure 9: Effective concentration (EC_{50}) of endophytic fungal extract against *P. falciparum* parasite

Conclusion

The incidence of microbial resistance to the currently available antibiotics is on the increase with its attendant burden on human health and world economy. It is therefore imperative that exploration of various sources for possible drug discovery which includes endophytic microbial diversity can afford an opportunity to discover novel moieties that can be useful in combating the challenges of microbial resistance. Data generated from the present study, has shown the susceptibility pattern of the test organisms to different concentrations of the endophytic fungal extracts. This study has further provided baseline information on the isolation of endophytic fungi from *Annona senegalensis* Pers. Further studies are however, recommended in order to purify and isolate the various chemical compounds responsible for the antimicrobial activities.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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