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# Antimicrobial activity of endophytic fungal metabolites from breadfruit *(Artocarpus altilis)* leaves against multidrug-resistant *Staphylococcus aureus* and *Klebsiella* spp.

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**Abstract:** Antimicrobial resistance (AMR) has become a global concern due to the growing inability of medications to treat infections. Endophytic fungi produce secondary metabolites with antimicrobial properties, making them potential sources of new drugs. This study evaluates the antimicrobial activity of crude secondary metabolites produced by endophytic fungi isolated from the leaves of *Artocarpus altilis* against multidrug-resistant *Klebsiella* spp and *Staphylococcus aureus*. Fresh leaves of *Artocarpus altilis* (Breadfruit) were collected, authenticated, and surface sterilized for fungal isolation. Fungal isolates were purified and fermented on rice medium at 30°C for 21 days. Metabolites were extracted using ethyl acetate and tested against MDR-*Klebsiella* spp and MDR-*Staphylococcus aureus* using agar well diffusion. Phytochemical screening was also conducted. Fungal extracts showed moderate activity against MDR-*Klebsiella* spp, with Aa-MR exhibiting the highest effect (MIC: 37.5 mg/mL). No activity was observed against MDR-*Staphylococcus aureus*. Phytochemical analysis revealed alkaloids, flavonoids, saponins, tannins, terpenoids, and glycosides. These findings suggest that fungal metabolites from *A. altilis* may be useful in treating infections caused by gram-negative bacteria.

## Introduction

Microbial resistance has long been known as an imperative challenge in treating diseases caused by bacteria, viruses, fungi, and parasites. The global rise of antimicrobial resistance (AMR) has become a persistent public health challenge, resulting in the need for different antimicrobial agents [1, 2]. Antibiotic-resistant organisms in the critical priority group from the World Health Organization (WHO) include: third-generation cephalosporin-resistant, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant *Acinetobacter baumannii*, and carbapenem-resistant Enterobacteriaceae. The priority group includes *Enterococcus faecium, Staphylococcus aureus, Salmonella* spp., *Campylobacter, Helicobacter pylori,* and *Neisseria gonorrhoeae*, while *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Shigella spp*. are classified as middle priority [3]. Endophytic fungi can produce a wide range of secondary metabolites that function as antibiotics, antifungals, antivirals, and

anticancer agents. In addition, they enhance resistance of plants to herbivores and pathogens [4]. These make them attractive for the development of drugs [5]. Studies have shown that *Artocarpus altilis* (**Figure 1**) has anti-cancer, anti-ulcer, antioxidant, anti-inflammatory, anti-bacterial, and anti-atherosclerotic properties [6-9]. Their leaves are used as tea to treat high blood pressure, diabetes, and asthma [10]. Despite the rich biodiversity of breadfruit, the endophytic fungi associated with it remain underexplored. This presents a gap in our understanding of their diversity and potential to produce antimicrobial compounds. Addressing this gap is crucial for discovering new bioactive agents that could contribute to the fight against AMR. This study aims to empirically evaluate the antimicrobial activity of the crude secondary metabolites produced by the endophytic fungi isolated from the leaves of *Artocarpus altilis* against Multidrug Resistant *Klebsiella spp* and *Staphylococcus aureus.*

**Figure 1:** *Artocarpus altilis* (breadfruit) leaves

## Materials and methods

*Test microorganism and processing:* The test organisms used in this work include *Staphylococcus aureus* and *Klebsiella spp*. They were collected from the Laboratory of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. Culture media used were malt extract agar (MEA), Mueller-Hinton agar (LS BIOTECH, UK), Mac Conkey agar, mannitol salt agar, and Sabouraud agar (LS BIOTECH, UK). Nutrient Broth (LS BIOTECH) was the broth media used. The bacterial isolates were reconfirmed by sub-culturing them onto a nutrient agar plate and incubating at 37ºC for 18-24 hrs. Pure isolates were subjected to specific identification techniques. The organisms were standardized by transferring the pure isolates using a sterile wire loop into 3.0 mls of sterile nutrient broth and grown in an oxygen-rich shaker water bath at 37ºC for 3.0 hrs to a cell density equivalent to the turbidity of 0.5 McFarland [11]. The susceptibility tests for the organisms were performed following the M2A6 disc diffusion method described as follows. The standardized organisms were swabbed onto a Mueller Hinton agar plate and the discs were placed on the inoculated plates and pressed firmly onto the agar plate for complete contact. The bacterial strains were tested against the following discs; ceftriaxone (30 µg), amoxicillin & clavulanic acid (30 µg), imipenem (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), aztreonam (30 µg), cefoxitin (30 µg), cefpirome (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), gentamicin (10 µg), sulphamethoxazole-trimethoprim (30 µg), tetracycline (10 µg), meropenem (10 µg), ertapenem (10 µg), piperacillin (75 µg), nitrofurantoin (200 µg), ticarcillin/clavulanic acid (85 µg), nalidixic acid (30 µg), chloramphenicol (10 µg), tobramycin (10 µg), and cefotetan (30 µg). The plates were left for 30 min to allow for pre-diffusion of antibiotics into the agar and then incubated at 37°C for 18-24 hrs. The susceptibility of each isolate to the antibiotic disc was shown by a clear zone of growth inhibition, and the diameter of the zones of inhibition was measured and interpreted using a standard chart [12].

*Collection and processing of plant materials:* Fresh leaves of *Artocarpus altilis* were collected at the medicinal garden school of Pharmacy Agulu and were authenticated by Taxonomist from the Department of Pharmacognosy

and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria. The harvested leaves were washed thoroughly under running tap water followed by sterile double distilled water before processing. For isolation of endophytic fungi from the leaves, all the samples were subjected to three step surface sterilization, using ethanol, sodium hypochlorite and distilled water to eliminate epiphytic microorganisms. They were immersed in 70.0% ethanol for three minutes and transferred to sodium hypochlorite solution (2.0%) for five minutes before a final rinse in sterile double distilled water and then dried in the laminar flow on a sterile filter paper. The disinfected samples were cut aseptically to 1.0 cm and inoculated into previously sterilized MEA media incorporated with chloramphenicol 500 mg/l. The plates were properly sealed and incubated at 25ºC for seven days while being checked for mycelium development on alternate days [13]. The isolation of pure cultures was achieved through multiple subculturing of isolates on fresh MEA. Examination of the morphological/cultural characteristics of the pure isolates was carried out by noticing visible phenotypic character keys like colony texture, pigmentation and color mycelium [13].

*Fermentation and extraction of fungal metabolites:* Each pure fungal isolate was grown in 1000 ml Erlenmeyer flasks containing sterilized rice medium, previously autoclaved at 121ºC at 15 psi for one hour [6]. The flasks were properly sealed and incubated under static conditions at 30°C for 21 days. Extraction of biosynthesized fungal metabolites was done using 500 ml of ethyl acetate. The filtrates were concentrated by evaporating the solvent at 50ºC using a rotary evaporator.

*Antimicrobial evaluation of the fungal extracts:* The antibacterial assay for the fungal extract was carried out using the agar well diffusion assay described by [14, 15] with small alterations. The standardized isolates were inoculated onto sterilized Mueller-Hinton agar plates using a swab stick. A sterile cork borer was used to make five wells (6.0 mm in diameter) on each of the MHA plates. Aliquots of 80 µl of each fungal extract dilution, reconstituted in DMSO at concentrations of 150, 75, 37.5, and 18.75 mg/ml, were applied in each of the wells. Ciprofloxacin (8.0 µg/mL) served as the positive control. The cultures were incubated at 37ºC for 24 hrs. The antimicrobial potential for each fungal extract was determined by measuring the zone of inhibition for each of the test organisms.

*Phytochemical analysis of the fungal extracts:* The crude extracts were tested for the presence of various phytoconstituents like alkaloids, flavonoids, reducing sugars, saponins, proteins, tannins, amino acids, steroids, triterpenoids and glycosides using the following tests: Dragendoff's and Wagner's tests for alkaloids; lead acetate and alkaline reagent test for flavonoids, Fehling's test for reducing sugar; frothing test for saponins; precipitation test for protein; ferric chloride test for tannins; ninhydrin test for amino acid; Liebermann-Burchard test for steroid; Salkowski test for triterpenoid and general test for glycosides [16-18].

## Results

*Antibiotic susceptibility test for Staphylococcus aureus isolates.* The susceptibility test carried out on different isolates of the test organisms revealed that they are multidrug-resistant (**Tables 1** and **2, Figure 2**).

*Isolation of endophytic fungi and extraction of metabolites*. A total of 14 endophytic fungal isolates were obtained from the leaves of *A. altilis* (**Figure 3**). Nine originated from the leaf blade and five originated from the midrib. The number of endophytic fungi in the leaf blade was significantly higher than that in the midrib of the leaves, indicating that the distribution of endophytic fungi differs among the different parts of the leaves. After further sub-culturing, three pure isolates were obtained from the leaf blade while a single isolate was obtained from the midrib (**Figure 4**). The yields of crude fungal metabolites obtained from various fungal isolates showed that Aa-Lb3 recorded the highest yield of 2.42 g, while Aa-Lb1 had the lowest yield of 1.00 g (**Table 3**).

**Table 1:** Antibiotic susceptibility test for *Staphylococcus aureus* isolates

| Isolate code | Antibiotics Used / Inhibition zone diameter (mm) | | | | | | | | | | | | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AUG | IMP | OFX | CIP | LBC | CTX | CRO | CXM | ZEM | GN | ERY | AZN | |
| H10 | 0 | 0 | 20 | 16 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 10 | MDR |
| H29 | 0 | 0 | 30 | 0 | 34 | 0 | 0 | 0 | 0 | 22 | 0 | 26 | MDR |
| H35 | 0 | 0 | 9 | 0 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | MDR |
| H38 | 0 | 0 | 0 | 28 | 34 | 0 | 0 | 0 | 0 | 20 | 0 | 26 | MDR |
| H48 | 0 | 0 | 16 | 22 | 0 | 0 | 0 | 0 | 0 | 12 | 0 | 0 | MDR |

***Key:*** AUG-amoxicillin clavulanate (30 mcg); CTX-cefotaxime (25 mcg); CRO-ceftriaxone sulbactam (45 mcg); ZEM-cefixime (5 mcg); LBC-levofloxacin (5 mcg); CIP-ciprofloxacin (5 mcg); IMP-imipenem/cilastatin (10/10 mcg); CXM-cefuroxime (30 mcg); OFX-ofloxacin (5 mcg); ERY-erythromycin (15 mcg); GN-gentamicin (10 mcg); AZN-azithromycin (15 mcg) MDR-multidrug resistant

**Table 2:** Antibiotic susceptibility test for *Klebsiella spp*

| Isolate code | Antibiotics used / Inhibition zone diameter (mm) | | | | | | | | | | | | Status |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OFX | LBC | AUG | IMP | ACX | CTX | CXM | CRO | ZEM | GN | NF | NA | |
| K32 | 0 | 28 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 24 | 14 | 0 | MDR |
| K28 | 18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 27 | 17 | 0 | 17 | MDR |
| K43 | 25 | 21 | 0 | 0 | 10 | 0 | 0 | 10 | 0 | 20 | 10 | 0 | MDR |
| K31 | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 22 | 0 | 15 | MDR |
| K38 | 0 | 30 | 0 | 0 | 0 | 0 | 0 | 12 | 14 | 0 | 16 | 0 | MDR |

***Key:*** AUG-amoxicillin clavulanate (30 mcg); CTX-cefotaxime (25 mcg); CRO-Ceftriaxone sulbactam (45 mcg); ZEM-cefixime (5 mcg); LBC-levofloxacin (5 mcg); NA-nalidixic Acid (30 mcg); NF-nitrofurantoin (30 mcg); IMP-imipenem/cilastatin (10/10 mcg); CXM-cefuroxime (30 mcg); OFX-Ofloxacin (5 mcg); GN-gentamicin (10 mcg); ACX-ampiclox (10 mcg) MDR-multidrug resistant


*Klebsiella spp Isolates* *Staphylococcus aureus* Isolates

**Figure 2:** Antibiotic susceptibility test of the test microorganisms

**Figure 3**: The Endophytic fungi isolated from the midrib and leaf blades of *A. altilis*

**Figure 4**: The 4 purified fungal isolates from the midrib and leaf blade *A. altilis*

**Table 3:** The yield of the fungal metabolites from the different isolates

| Fungal isolate | Yield (g) |
|---|---|
| Aa-Mr | 2.33 |
| Aa-Lb2 | 2.10 |
| Aa-Lb1 | 1.00 |
| Aa-Lb3 | 2.42 |

***Key:*** Aa-*Artocarpus altilis;* Lb-Leaf Blade; Mr-Mid rib

*Determination of the antibacterial potential of the metabolites:* **Tables 4** to **7** revealed the antibacterial potential of crude endophytic fungal extracts against the multi-drug resistant *Klebsiella spp.* and *Staphylococcus aureus.*

*Phytochemical analysis of the crude fungal extract:* The phytochemical analysis of the leaves showed the presence of alkaloids, flavonoids, saponins, tannins, glycosides, steroids, and terpenoids. None contained reducing sugars (**Table 8**).

**Table 4:** Antibacterial activity of Aa-Lb3 against MDR test organisms

| Concentration (mg/mL) | Test organisms / inhibition zone diameter (mm) | | | | | |
|---|---|---|---|---|---|---|
| | K28 | K32 | K38 | K43 | H10 | H35 |
| **150** | 3.0±0.0 | 4.0±0.0 | 3.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **75** | 0.0±0.0 | 2.0±0.0 | 3.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **37.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **18.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CIPRO (8µg/mL)** | 12.0±0.0 | 15. 0±0.3 | 17.0±0.0 | 8.0±0.0 | 11.0±0.0 | 5.5±0.2 |

**Key:** Aa-*Artocarpus altilis;* Lb-Leaf blade; K *Klebsiella spp*; H-*Staphylococcus aureus*

**Table 5:** Antibacterial activity of Aa-Lb2 against MDR test organisms

| Concentration (mg/mL) | Test organisms / inhibition zone diameter (mm) | | | | | |
|---|---|---|---|---|---|---|
| | K28 | K32 | K38 | K43 | H10 | H35 |
| **150** | 4.0±0.0 | 8.0±0.0 | 5.0±0.0 | 3.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **75** | 2.0±0.0 | 4.0±0.0 | 4.0±0.0 | 2.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **37.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **18.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CIPRO (8 µg/mL)** | 12.0±0.0 | 15.5±0.3 | 17.0±0.0 | 8.0±0.0 | 11.0±0.0 | 5.5±0.2 |

***Key:*** Aa-*Artocarpus altilis;* Lb-Leaf blade; K-*Klebsiella spp*; H-*Staphylococcus aureus*

**Table 6:** Antibacterial activity of Aa-Lb1 against MDR test organisms

| Concentration (mg/mL) | Test organisms / inhibition zone diameter (mm) | | | | | |
|---|---|---|---|---|---|---|
| | K28 | K32 | K38 | K43 | H10 | H35 |
| **150** | 8.0±0.0 | 9.0±0.0 | 8.0±0.0 | 5.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **75** | 4.0±0.0 | 5.0±0.0 | 6.0±0.0 | 2.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **37.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **18.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CIPRO (8 µg/mL)** | 12.0±0.0 | 15.5±0.3 | 17.0±0.0 | 8.0±0.0 | 11.0±0.0 | 5.5±0.2 |

***Key*:** Aa-*Artocarpus altilis;* Lb-Leaf blade; K-*Klebsiella spp*; H-*Staphylococcus aureus*

**Table 7:** Antibacterial activity of Aa-Mr against MDR test organisms

| Concentration (mg/mL) | Test organisms / inhibition zone diameter (mm) | | | | | |
|---|---|---|---|---|---|---|
| | **K28** | **K32** | **K38** | **K43** | **H10** | **H35** |
| **150** | 7.0±0.0 | 8.0±0.0 | 11.0±0.0 | 6.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **75** | 6.0±0.0 | 5.0±0.0 | 5.0±0.0 | 3.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **37.5** | 4.0±0.0 | 3.0±0.0 | 3.0±0.0 | 2.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **18.5** | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| **CIPRO (8µg/mL)** | 12.0±0.0 | 15.5±0.3 | 17.0±0.0 | 8.0±0.0 | 11.0±0.0 | 5.5±0.2 |

**Table 8:** Phytochemical analysis of the crude fungal extract

| **Phytoconstituents** | **Test** | **Aa-MR** | **Aa-LB** | | |
|---|---|---|---|---|---|
| | | | Aa-$LB_1$ | Aa-$LB_2$ | Aa-$LB_3$ |
| Alkaloids | Dragendorff's/Wagner's | + | + | + | + |
| Flavonoids | Magnesium Ribbon Test | + | + | + | + |
| Saponins | Frothing | + | + | + | + |
| Tannins | Ferric Chloride | + | + | + | + |
| Steroids | Salkowski | + | - | - | + |
| Terpenoids | Liebermann–Burchard | + | + | - | - |
| Glycosides | Keller-Killiani | + | - | + | - |
| Reducing Sugars | Benedict | - | - | - | - |

***Key*:** Aa-*Artocarpus altilis;* Mr-Mid rib; K-*Klebsiella spp*; H-*Staphylococcus aureus*
Aa-MR: Crude fungal extract obtained from the midrib of *Artocarpus altilis* leaves; Aa-LB: Crude fungal extract obtained from the leaf blade of *Artocarpus altilis*; + = present; - = absent

## Discussion

The isolation of 14 fungal strains from the leaf blade and midrib of *A. altilis* indicates the diverse presence of endophytic fungi in the plant. The higher fungal prevalence in the leaf blade suggests it may provide a more conducive microenvironment for fungal colonization, due to differences in nutrient availability or surface area compared to the midrib. This aligns with findings from the previous study [20], where distinct fungal distributions were observed across various plant tissues including the roots, stems and leaves. The variability in yields highlights differences in metabolite production capacities among the isolates. Factors such as genetic differences and substrate utilization efficiencies could explain this variation. Similar observations were reported in studies by [21], where endophytic fungi exhibited diverse metabolic profiles and extractive yields depending on isolation sources and growth conditions. The susceptibility testing confirmed the multidrug resistance of *Klebsiella spp.* and *Staphylococcus aureus* isolates. Such resistance is a significant public health concern and supports the necessity of exploring alternative antimicrobial agents, including bioactive compounds from endophytic fungi [2, 22, 23]. The fungal extracts demonstrated moderate activity against MDR-*Klebsiella spp.* but exhibited no activity against MDR-*Staphylococcus aureus* at the tested concentrations. The antibacterial efficacy exhibited by Aa-MR against *Klebsiella spp.* suggests that their metabolites possess significant potential for combating these gram-negative bacteria even at relatively low concentrations, though their inactivity against MDR-*Staphylococcus aureus,* a gram-positive strain may indicate a narrower spectrum of activity. The differential efficacy observed across extracts is consistent with the previous study [19], which noted that fungal metabolite activity can vary significantly depending on both fungal strain and bacterial target. This is in line with various works which reported that endophytic fungi isolated from various plant tissues demonstrated activities against known bacterial pathogens [23-28]. This is a novel report on the antimicrobial activity of an endophytic fungi extract isolated from *A. altilis*. The observed antibacterial activity of fungal extracts from *A. altilis* can be attributed to the presence of

various bioactive phytochemical constituents such as alkaloids, flavonoids, saponins, tannins, glycosides, steroids, and terpenoids. None of the extracts contained reducing sugars, possibly indicating the fungi's preference for producing more complex, bioactive compounds. This is a promising development, though; there is a need for further exploration of this extract. Moreso, MDR *Klebsiella spp.* infections are challenging to treat with conventional antibiotics, and the demonstrated efficacy of these fungal extracts could serve as the basis for developing new therapeutic agents.

*Conclusion:* The current findings uncovered novel strains from *Artocarpus altilis* leaves that produce bioactive compounds with antimicrobial properties which could lead to the development of new antimicrobial agents for effective treatments against some gram-negative pathogens.

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