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# Antibacterial and Antioxidant Activity of Bioactive Compounds Produced by Endophytic Fungi from some Nigerian Medicinal Plants

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**A B S T R A C T**

Endophytes' natural habits are within plant tissues. The study aims to evaluate the antibacterial and antioxidant activity of bioactive compounds synthesized by endophytic fungi obtained from leaves of *Azadirachta indica* (AI), *Talinum fruticosum* (TF), *Telfairia occidentalis* (TO), *Mangifera indica* (MI) and *Vernonia amygdalina* (VA). The compounds produced by the isolated endophytic fungi from the medicinal plants used in this study were extracted with ethylacetate and subjected to antibacterial using agar well diffusion method while the antioxidant activities was determined using theor ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Four fungal species were isolated from the plants and were identified to be *Mucor plumbeus*, *Rhizopus*, *Aspergillus niger* and *Penicillium roquefortil*. Alkaloids, saponins and steroids were detected in all the extracts while tannin was not detected. Flavonoids were detected in AI, TO and MI; flavonoids was detected in TO, TF and VA. All the extracts obtained from this study showed good antioxidant activity with TO having the highest diphenyl 2-picryhydrazyl (DPPH) radical scavenging activity compared to the other extracts. All the extracts inhibited the growth of both *E. coli* and *S. aureus* with TO having the highest diameter zone of inhibition of 26±0.88 and 35±1.78 respectively. The effect of AO, SJ1, SJ2 was bacteriocidal against *S. aureus* and *E. coli.* TC1 was bacteriocidal against *S. aureus* but bacteriostatic against *E. coli* while TC2 was bacteriostatic for both *S. aureus* and *E.* coli. The compounds produced by the isolated fungi possess both antioxidant and antibacterial activity which can be used for development of new therapeutics.

## Introduction

Medicinal plants are rich sources of secondary metabolites with vast biological activities which informed their use as medicines by man. They are also regarded as precursors for the synthesis of useful drugs for various therapeutic purposes (Sofowora *et al.,* 2013). Numerous are scientific reports validating the medicinal properties of plants including *in-vitro* and *in-vivo* assays. Successes have been achieved in isolating the active principles in various plants and have been used in drug

development. However, the process of drug development is tedious due to the complexity of the compounds present in the different plant species (Melaku, 2021). Also, some of these medicinal plants serve as vegetables and can be easily cultivated in homes while others are trees of economic value found in wildlife.

Thus, due to the ever-increasing population characterized by rapid urbanization and industrialization, the natural habitats of these plants is destroyed leading to extinction of several plant species (Olaleye *et al.,* 2015). In addition, non-principled use of these resources including poor agricultural and conservation practices leads to extinction of plant species (Jamshidi-Kia *et al.,* 2018). This could pose a problem to the healthcare system, especially due to the increasing emergence of resistance to existing drugs by infectious organisms and the increased rate of non-communicable diseases across the world. This informed the intensive research on endophytes and their biological activities.

Endophytes are microorganisms housed within the internal tissues of plants. They are either bacteria or fungi; however, this study focus on fungi isolation. Endophytic fungi have been greatly researched and discovered to pose no harm to the host plant but protect them (Sandhu *et al.,* 2014) and evolve the ability to produce secondary metabolites similar to their host plant; therefore, they can be exploited for drug developments (Alvin *et al.,* 2014; Praptiwi *et al.,* 2018). The biological activity of endophytic fungi isolated from various medicinal plants have been reported including antibacterial, antioxidant, anti-cancer, anti-filarial and anti-inflammatory (Ilyas 2009; Khiralla *et al.,* 2015; Kumari *et al.,* 2018; Praptiwi *et al.,* 2015; Ukwatta *et al.,* 2020).

The present study aims to evaluate the antibacterial and antioxidant activity of bioactive compounds synthesized by endophytic fungi obtained from leaves of *Azadirachta indica*, *Talinum fruticosum*, *Telfairia occidentalis*, *Mangifera indica* and *Vernonia amygdalina*.

## Materials and Methods

### Collection and Identification of Plant Samples

The leaves of *Azadirachta indica*, *Talinum fruticosum*, *Telfairia occidentalis*, *Mangifera indica* and *Vernonia amygdalina* were collected from Lagos State Polytechnic farm with the coordinates Lat/Long: [6.64766583/ 3.52038056].

Identification of the plant specimens were conducted at the Herbarium of Botany Department University of Lagos State.

### Isolation of Endophytic Fungi

Fresh Leaves were collected from the field, cleaned under tap water and immersed in 70% ethanol for 1 minute, then immersed in 5.3% Na-hypochlorite for 5 mins and finally immersed in 70% ethanol for 30 secs. Samples were dried under aseptic conditions.

The sterilized samples were cut aseptically into small pieces ($1 \times 1$ $cm^2$), and then, placed on top of the Potato Dextrose Agar (PDA) growth medium containing chloramphenicol 0.05 mg/mL, and incubated at room temperature for 1 week.

The emerging colonies were sub-cultured several times on Potato dextrose agar (PDA) to obtain pure isolates (Pessini *et al.,* 2003).

### Identification of Endophytic Fungi

The isolated endophytic fungi were identified based on their morphology features and spores at the hyphal tips (Barnett and Hunter, 1998; Gilman, 2001) while the microscopic examination was conducted to study their reproductive spores (Anitha *et al.,* 2013).

The identified fungal isolates from respective plants were isolated sub-cultured in a Petri dish containing sterile PDA media. The pure cultures of the endophytic fungi were preserved in PDA slant and incubated at $4^oC$.

## Secondary Metabolites Extraction from Endophytic Fungi

Pure isolate of endophytic fungi was cultured on broth medium (Potato dextrose broth) and incubated in dark condition, at room temperature for 3 weeks. After the incubation period, growth media and endophytic fungi biomass were extracted three times with ethyl acetate. The extraction solvent was evaporated by rotary evaporation and the concentrated extracts were stored in glass vials (Suryanarayanan and Thennarasan, 2004).

## Phytochemical Screening of Compounds Produced by Endophytic Fungi

Phytochemical constituent of the extracts were determined according to the method of Sofowora, (2014).

### Test for Alkaloids

A quantity (3mL) of concentrated extract was taken into a test tube and 1ml HCl was added. The mixture was heated gently for 20mins cooled and filter, the filtrate was used for following test.

Wagner test: 1mL of the extract was treated with Wagner's reagent; formation of brown reddish precipitate indicates presence of alkaloids.

Hager's test: 1mL of the extract was treated with Hager's reagent, presence of alkaloids confirmed by the yellow colored precipitate.

### Test for Saponin

The extract (5 mL) was mixed with 20 mL of distilled water then agitated in graduated for 15 minutes, formation of foam indicates Saponin.

### Test for Steroid

The extract was dissolved in 10 mL of chloroform & equal volume of concentrated $H_2SO_4$ acid was added from the side of test tube. The upper layer turns red and $H_2SO_4$ layer showed yellow with green fluorescence. This indicates the presence of steroid.

### Test for Tannin

The extract (4 mL) was treated with 4 mL $FeCl_3$ formation of green colour indicates that presence of condensed tannin.

### Test for Flavonoid

Alkaline reagent test: Extract was treated with 10 % NaOH solution, formation of intense yellow colour indicates the presence of Flavonoid.

$NH_4OH$ test: 3 mL of the extract was mixed with 10 % $NH_4OH$ solution development of yellow fluorescence indicates positive test.

### Test for Phenol

Ferric Chloride test: Test extracts were treated with 4 drops of alcoholic $FeCl_3$ solution. The formation of bluish black colour indicate the presence of phenol

## Detection of Antioxidant Activity by Spectrophotometric

The free-radical scavenging activity of the extracts was measured as decrease in the absorbance of methanol solution of DPPH using the method of Omotayo *et al.*, (2017). DPPH solution (0.033 g/L in methanol; 1 mL) was added to 1 mL of extract solution at different concentrations. After 30 min, absorbance was measured at 517 nm and compared with standards ascorbic acid and gallic acid. The DPPH scavenging activity was expressed as the percentage inhibition extrapolated using the formula:

$$\% \text{ DPPH activity} = \frac{Ac - As}{Ac} \times 100$$

Ac = Absorbance of Control, As = Absorbance of sample

## Evaluation of Antibacterial Activity

The antibacterial activity of the extract was determined by the agar well diffusion method using the well techniques. Briefly, all the extracts were dissolved in ethyl acetate in order to obtain concentration of 20mg/mL. Inoculums of the bacterial strain ($10^6$CFU/mL) was then plated using sterile swabs into sterilized Petri dishes containing 25mL of Potato Dextrose Agar. Wells with diameter 6mm wells were cut and filled with 0.1mL of extract into respective plates. The Petri dishes were pre-incubated at room temperature for 3 hrs in order to allow complete diffusion of the extracts before incubating at 37$^o$C for 24 hrs (Das *et al.,* 2010).

## Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

A modification of the dilution method for the determination of MIC and MBC was used. Briefly, stock solution of extract was diluted into various concentrations in sterile nutrient broth in test tubes. After the dilution process, 0.5 mL of bacterial suspension ($10^6$ CFU/mL) was added to the test tubes. The tubes were incubated at 37°C for 18 to 24 hr and thereafter observed for growth or turbidity. Subsequently, a loopful of broth from each test tube not showing growth, was inoculated into nutrient agar plate.

## Results and Discussion

Endophyte is any bacterium or fungus that lives in the internal tissues of plants parts including leaf, stem and roots. Various reports are available to support the isolation of endophytes from leaves of plants. In this study, five plant species including *Azadirachta indica, Talinum fruticosum, Telfairia occidentalis, Mangifera indica* and *Vernonia amygdalina* were used for the isolation of endophytes. According to Lu *et al.,* (2012), endophytic fungi are hosted in nearly 300,000 land plant species and each plant can host more than one fungal species. From table 1, single colonies were observed on the culture plates for all the plant samples. The organism isolated from *Azadirachta indica* is *Mucor plumbeus*; *Rhizopus stolonifer* was isolated from *Talinum fruticosum* and *Telfairia occidentalis* while *Aspergillus niger* and *Penicillium roquefortil* was obtained from *Mangifera indica* and *Vernonia amygdalina* respectively

Fungi have been employed for various industrial processes including Single cell protein production. The dried cell mass of fungi, moulds and bacteria, sometimes called biomass are used as protein supplement in animal and human feed to augment their diet (Mahan *et al.,* 2018; Olaleye *et al.,* 2015).

In this study, fungi were isolated from the internal tissues of the test plant and were cultured in Broth to produce bioactive compounds for three weeks. Maximum mycelia weight of 2.01g was achieved for TF (figure 1). The bioactive compound was extracted using ethylacetate and were concentrated to dryness in water bath at 50$^O$C to give maximal yield of 1.61g for MI. The work of Akinsanya *et al.*, (2017), informed the choice of ethylacetate as the solvent of choice for extracting bioactive compounds from the broth culture. He reported higher extract yield and bioactivity of bioactive compounds for ethylacetate compared to diethylether and n-hexane.

Plants are regarded as a store house of compounds with numerous biological importance to man especially as medicines (Omotayo *et al.,* 2020). Phytochemicals are plants' secondary metabolites with diverse functional group and function responsible for their medicinal properties (Saxena *et al.,* 2013). From table 2, alkaloids, saponins and steroids were detected in all the extracts while tannin was not detected. Flavonoids were detected in AI, TO AND MI; flavonoids was detected in TO, TF and VA. Saponins are a group of glycosides with foaming and detergent properties. They are reported to possess antimicrobial, anti-malarial, anti-allergic, anti-diabetic, insecticidal, and anti-inflammatory activities (Elekofehinti, 2015; Maatalah *et al.*, 2012).

**Table.1** Morphological and Microscopy features of isolated fungi

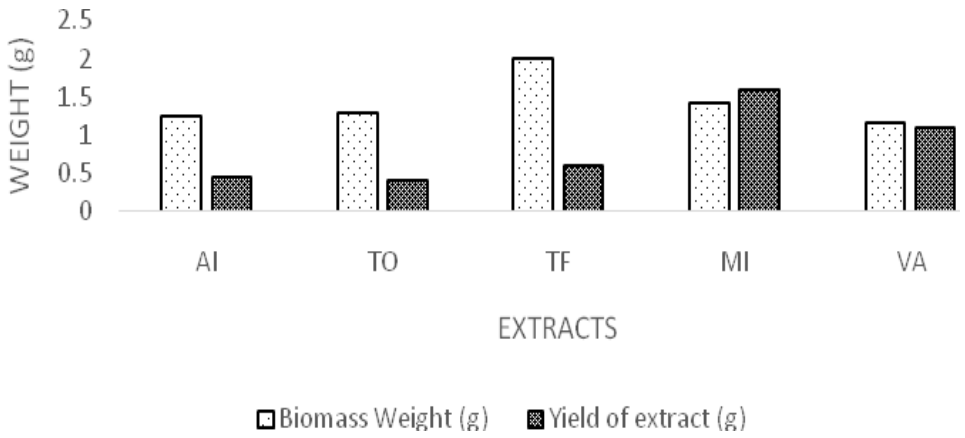
| | |
|---|---|
| ***Azadirachta indica* (AI)** | **Number of colonies: 1** |
| | **Morphological features on Agar:** Colonies growth spreads rapidly well within 2-3days of incubation period and most frequently only a few milliliters high. Colonies at first white then dull brown and finally brownish black with reverse slightly brownish. |
| | **Microscopy Morphology:** Sporangiophore symbolically branched above 0.5mm high. Sporangia up to 80µm in diameter, the wall encrusted and appearing spiny, rupturing at maturity, colonialae ovoid or pyriform with projection at the top. Spores glabose smaller and veruculose. |
| | **Organism:** *Mucor plumbeus* |
| ***Talinum fruticosum* (TF)** | **Number of colonies: 1** |
| | **Morphological features on Agar:**Colonies growth spreading very rapidly and completely filling dishes within two days of incubation. Stolons clearly differentiated, arising from and terminating in strong tufts of brown rhizoids (root-like hyphae) colony grows with dense cottony masses of myceline white with black spores after maturity. |
| | **Microscopy Morphology:** Sporangiophores, erect, arising in groups opposite the rhizoids sporangia globose, shinning white with black spores after maturity. Spores variously shaped, ellipsoidal angular and striate in long axis. |
| | **Organism:** *Rhizopus stolonifer* |
| ***Telfairia occidentalis* (TO)** | **Number of colonies: 1** |
| | **Morphological features on Agar:**Colonies growth spreading very rapidly and completely filling dishes within two days of incubation. Stolons clearly differentiated, arising from and terminating in strong tufts of brown rhizoids (root-like hyphae) colony grows with dense cottony masses of myceline white with black spores after maturity. |
| | **Microscopy Morphology:** Sporangiophores, erect, arising in groups opposite the rhizoids sporangia globose, shinning white with black spores after maturity. Spores variously shaped, ellipsoidal angular and striate in long axis. |
| | **Organism:** *Rhizopus stolonifer* |
| ***Mangifera indica* (MI)** | **Number of colonies: 1** |
| | **Morphological features on Agar:** Colonies growth spreading within 2-3days of incubation with fluffy and velvety texture. The aerial mycelium white at first frequently developing into dark brown to black conidial heads with no reverse colour. |
| | **Microscopy Morphology:** Colonial heads are round or globose, large and also radiate or as they grow splitting into loose columns of conidia chains with conidiophores arising from the subotration mostly colourless to brown, smooth splitting when crushed like a pieces of cone. Vesticles globose while phialides borne directly on the vesicle metulae and foot cells are usually present. |
| | **Organism:** *Aspergillus niger* |
| ***Vernonia amygdalina* (VA)** | **Number of colonies: 1** |
| | **Morphological features on Agar:** Colonies growth usually broadly spreading within 2-3days of incubation. Colony blue-green to deep green, smooth, velvety texture with reverse almost colourless. |
| | **Microscopy Morphology:** *Penicillin* districtly assymmetile, commonly with three stages of branching, slopes rough, mitulae often rough, phialites, conidia globose, but occasionally large, smooth, borne in loose columns or tangled chains condiophores smooth. |
| | **Organism:** *Penicillium roquefortil* |

**Table.2** Phytochemical constituent of compounds

| Plant | Alkaloids | Saponin | Steroid | Tannin | Flavonoid | Phenol |
|---|---|---|---|---|---|---|
| **AI** | + | + | + | - | + | - |
| **TO** | + | + | + | - | + | + |
| **TF** | + | + | + | - | - | + |
| **MI** | + | + | + | - | + | - |
| **VA** | + | + | + | - | - | + |

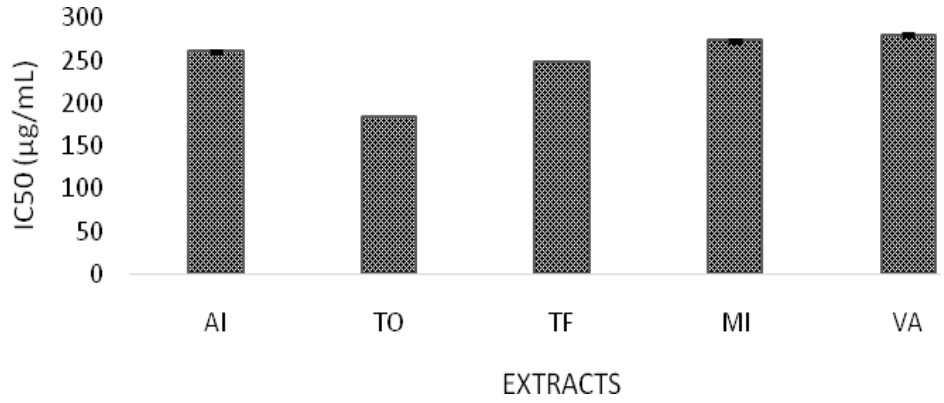
Key: + means detected – means not detected; AI-*Azadirachta indica*, TO-*Talinum fruticosum*, TF-*Telfairia occidentalis*, MI-*Mangifera indica* and VA-*Vernonia amygdalina*

**Fig.1** Weight of biomass and extract



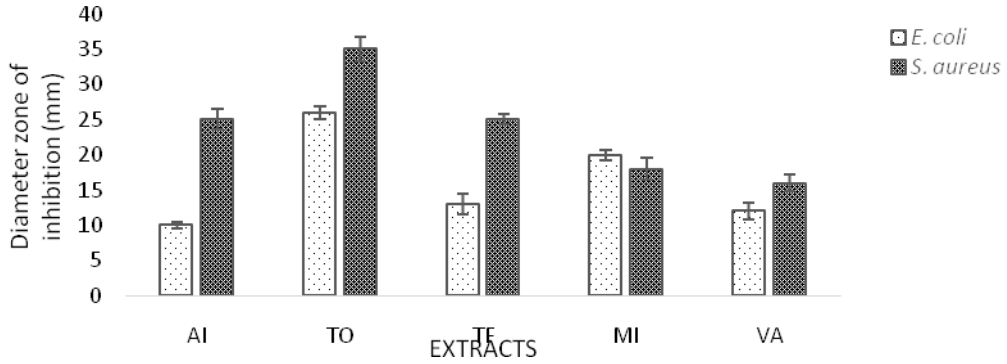

AI-*Azadirachta indica*, TO-*Talinum fruticosum*, TF-*Telfairia occidentalis*, MI-*Mangifera indica* and VA-*Vernonia amygdalina*

**Fig.2** DPPH radical scavenging activity of the extracts



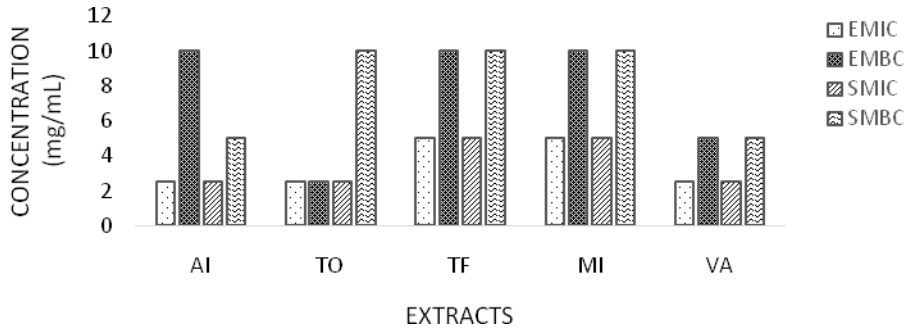

AI-*Azadirachta indica*, TO-*Talinum fruticosum*, TF-*Telfairia occidentalis*, MI-*Mangifera indica* and VA-*Vernonia amygdalina*

**Fig.3** Antibacterial activity of extracts against test organisms



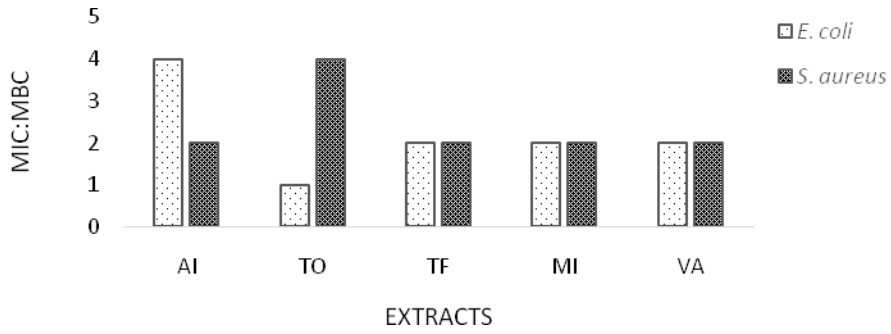

AI-*Azadirachta indica*, TO-*Talinum fruticosum*, TF-*Telfairia occidentalis*, MI-*Mangifera indica* and VA-*Vernonia amygdalina*

**Fig.4** MIC and MBC of the extracts against test organisms




AI-*Azadirachta indica*, TO-*Talinum fruticosum*, TF-*Telfairia occidentalis*, MI-*Mangifera indica* and VA-*Vernonia amygdalina*

**Fig.5** MBC:MIC ratio of extracts against test organisms




AI-*Azadirachta indica*, TO-*Talinum fruticosum*, TF-*Telfairia occidentalis*, MI-*Mangifera indica* and VA-*Vernonia amygdalina*

Alkaloids are plants' important bioactive substances that display wide therapeutics properties including antimicrobial (Deng *et al.*, 2011), cytotoxic, antioxidant (Khan *et al.*, 2015), analgesic activities, narcotics, antimalaria, topical anesthetic and used in treatment of hypertension, neuralgia, rheumatism and motion sickness (Ngoci *et al.*, 2011). Plants or extracts rich in phenolics and flavonoids exhibit

antioxidant activities due to their chemical structures and redox properties (Shoib *et al.,* 2014). The presence of these phytochemicals in the compounds produced by these fungal endophytes may be responsible for their activity observed in this study.

The TLC-bioautography is a simple method for the detection of antioxidant activity of plant extracts and plant products (Takao *et al.,* 1994; Dewanjee *et al.,* 2015). The DPPH radical scavenging assay is a widely used method employed to assess the propensity of plant extracts to scavenge free radicals attributed to their ability to donate hydrogen showed the least $IC_{50}$ of 184.51±0.24 µg/mL and this indicates higher activity compared to the other extracts.

The agar well diffusion assay is a method used commonly to determine the antibacterial activity of plants and plants' products (Valgas *et al.,* 2007). The results obtained in this study showed that the test organisms, *E. coli* and *S. aureus* were susceptible to the plant extract, however, it was observed that TO had highest diameter zone of inhibition (figure 3). Also all the extract showed higher inhibition against *S. aureus* than *E. coli*. The lower activity observed for *E. coli*, a Gram negative bacteria, is due to the outer membrane, which acts as a barrier to many environmental substances including antibiotics (Tuney *et al.,* 2006).

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) tests are used to determine the activity of the extracts or antimicrobial drugs on test species of bacteria. MIC is therefore defined as the lowest concentration of antimicrobial agent or plant extract that will inhibit the growth of bacteria (Levison, 2004), while MBC is the lowest concentration of antimicrobial agent or plant extract needed to kill the test bacterium (Wiegand *et al.,* 2008). The lower the MIC and MBC, the more effective the compound is, thus, TO is the most effective in the inhibition of the growth of both *S. aureus* and *E. coli* (figure 4). The MBC:MIC ratio was deduced to ascertain the tolerance of the extract against the test organism (Mogana *et al.,* 2020). From the results obtained in this study (Figure 4), the MBC: MIC of AO, SJ1 and SJ2 was 2.0 for both *S. aureus* and *E. coli* while that of TC2 was 10. TC2 had value of 10 for *E. coli* but 1 for *S. aureus*. According to Benjamin *et al.,* (2012), if the ratio MBC/MIC ≤4, the effect was considered as bactericidal but if the ratio MBC/MIC > 4, the effect was defined as bacteriostatic. On this basis, the effect of AO, SJ1, SJ2 was bacteriocidal against *S. aureus* and *E. coli*. TC1 was bacteriocidal against *S. aureus* but bacteriostatic against *E. coli* while TC2 was bacteriostatic for both *S. aureus* and *E. Coli*.

The present study justified the presence of endophytes in the leaves of five Nigerian medicinal plants. The bioactive compounds produced by the enphytic fungi showed good antibacterial and antioxidant activities, thus can be explored for new drugs with potentials to treat old and emerging infectious and oxidative stress related diseases.

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