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EVALUATION OF ANTIOXIDANT POTENTIAL OF CRUDE EXTRACT OF METABOLITES FROM ENDOPHYTIC FUNGI ISOLATED FROM *ANNONA SENEGALENSIS* PERS

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ABSTRACT

Degenerative diseases resulting from free radicals can be prevented through the use of natural antioxidants. This study aimed to assess the free radical scavenging potential of the underexplored endophytic fungal metabolites from AnnonasenegalensisPers(wild custard apple). Freshly collected root, midrib, and leaf tissues were planted on malt extract agar following surface sterilization with 70% ethanol for 3 minutes, washed twice with distilled water, immersed in a sodium hypochlorite solution (4%) for 5 minutes, and subsequently rinsed with sterile water. Seven endophytic fungi were isolated, and the antioxidant activity of their metabolites was evaluated using the 2,2-diphenyl-1-picrylhydrazyl free radical scavenging method, with ascorbic acid serving as a reference antioxidant. The crude extract from the seven endophytic fungal isolates demonstrated concentration-dependent antioxidant activities. LB2 exhibited a strong antioxidant activity with an IC_{50} value of 38.15 µg/ml. Extracts from RT1 and LB1 showed antioxidant activities, with the highest inhibition of 60% and 65%, respectively, observed at a concentration of 1000 µg/ml. This was compared favorably to ascorbic acid, which exhibited 94% inhibition. This indicates that the crude extract from endophytic fungi isolated from A. senegalensis possesses free radical scavenging properties. Further purification and elucidation of the crude extract will reveal the bioactive compounds responsible for the antioxidant activities.

Keywords: Antioxidant activity, endophytes, *Annonasenegalensis*, bioactive compounds, Free radical scavenging

INTRODUCTION

In normal cellular metabolic activities, free radicals are produced (Ibrahim *et al.*, 2021). The imbalance between the rate of production and the efficient endogenous mechanism of elimination of these free radicals leads to oxidative stress (Omeke*etal.*,2019). The impact of reactive oxygen species (ROS) on biomolecules such as proteins, lipids, glycoproteins, and phospholipids causes certain degenerative diseases in humans, as well as rancidity and food spoilage in the food industries (Nadri*etal.*,2014).The toxic effect of free radicals on cells causes the activation of certain genes and abnormal enzymes that lead to cancer, diabetes, and cardiovascular diseases (Omeke*etal.*,2019; Nadri*etal.*,2014).It has been widely reported that antioxidants can be used to suppress, prevent, or eliminate oxidative processes caused by free radicals (Irène *et al.*,2022; Habisukan *et al.*,2022).The prolonged use of synthetic antioxidants such as benzoic acid, butylatedhydroxyanisole (BHA), or butylatedhydroxytoluene (BHT) has been associated with mutagenic and hepatic disorders (Irène *et al.*,2022; Habisukan *et al.*,2022; Oktiansyah *et al.*,2023). The rising need for antioxidants of natural origin in the prevention and management of the impact of free radicals on the human body and in industries has stimulated the quest for safe, effective, and accessible antioxidants from renewable sources such as plants and their microbial diversity.

Annonasenegalensis is a known medicinal plant used in the traditional treatment of malaria, sexually transmitted diseases, snake bites, cancer, and hepatitis (Omeke *et al.*,2019).

Fig 1: fresh leaves of *Annonasenegalensis*.

A review of the plant extract has shown that it has good antioxidant activities and contains flavonoids, alkaloids, fatty acids, tannins, and sterols (Omeke*et al.*,2019; Samuel *et al.*,2016).In a related study, it was reported that endophytic fungi were isolated from *A.senegalensis*, and the extract from the isolates showed antibacterial and antiplasmodial activities (Onah *et al.*,2021).

Endophytes are microorganisms such as bacteria, fungi, or viruses that live wholly or part of their lives in the tissues of their host plant without causing any obvious harm to

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the plant (Ibrahim *et al.*,2021). These organisms live and maintain a symbiotic relationship with their host plant, where they enjoy shelter and nutrient support from the plant and, in turn, synthesize plant hormones as well as metabolites that enable the plant to survive stress and attacks from pathogenic microbes (Prastya*etal.*,2023). Through gene reuptake mechanisms, endophytes have been able to synthesize secondary metabolites that are more diverse or similar to those of their host plant (Onah *et al.,* 2020; Germaine *et al.*,2004; Arun, *et al.*,2015).

In recent times, endophytic fungi have attracted more scientific research interest due to their bioactive metabolites that have been explored and found to possess diverse novel chemical substances that can be useful in agricultural, pharmaceutical, and biotechnological industries (Okezie *et al.*, 2023). However, the endophyticmycoflora of *Annonasenegalensis* has not been exhaustively explored for its antioxidant potential. Thus, the present study was designed to evaluate the free radical scavenging activities of the crude extract of metabolites from endophytic fungi from *Annonasenegalensis*.

MATERIALS AND METHODS

Cultivation and Isolation of Endophytic Fungi

The various plant parts of *Annonasenegalensis*used in this study (root, stem, and leaves) were collected from a non-diseased, mature plant in September 2017, at Mbu-Akpoti, in Isi-Uzo local Government Area of Enugu State, Nigeria. The freshly collected plant samples were duly identified by a taxonomist, Mr. Alfred Ozioko of the botany department, University of Nigeria, Nsukka. Further authentication of the samples was conducted by botanist Mr. OnyeukwuChijioke John of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka. The samples were assigned voucher number "UNH NO 9a" and deposited at the herbarium collection center of the Department of Plant Science and Biotechnology, University of Nigeria.

Surface Sterilization and Sample Processing

The methods of Okezie*et al*.(2017) were adopted with slight modifications. All the freshly harvested samples were washed thoroughly in running tap water followed by sterile double-distilled water before processing. To eliminate epiphytic microorganisms, all the samples underwent a four-step surface sterilization process, which included washing under running tap water, ethanol, sodium hypochlorite, and distilled water. The samples were washed in running tap water, after which they were immersed in 70% ethanol for 2 min and washed twice with distilled water. Additionally, the already washed samples were immersed in a 2%sodium hypochlorite solutionfor 5 minand washed thoroughly thrice in distilled water. Subsequently, the samples were rinsed in 70% ethanol for 2 min, before a final rinse in sterilized double-distilled water. The washed samples were dried in the laminar flow on a sterile filter paper. A sterile knife was used to cut the samples to approximately 1 cm in length.

Inoculation and Incubation

Segments (a total of 30, with three to six segments per Petri dish) of samples were inoculated on previously sterilized malt extract agar (MEA) incorporated with chloramphenicol (500 mg/L). The cut end of the material was made to contact the media. The Petri dishes were properly sealed using parafilm and then incubated at 25°C, checked on alternate days. After 7 days, hyphal tips of actively growing fungi from the plant material were sub-cultured to other sterile MEA plates and incubated for 5 to 7 days. The purity of the cultures was checked periodically, and sub-culturing was done at an interval of two weeks to maintain pure cultures. Cultural characteristics, such as color, nature of the growth of the colony, and texture, were determined by visual observation (Simth and Black, 1990).

Additionally, the maximum growth of the fungi was observed on MEA. For the production of metabolites, the starting material was taken from freshly sub-cultured plates.

Fermentation and Extraction of Fungal Metabolites

Solid-state fermentation and extractionof the fungal metabolites were carried out as describedbyNwobodo*et al.*(2020). Rice medium was prepared in 1000 ml Erlenmeyer flasks asfollows: approximately 200 ml of distilled water wasadded to 100 g of rice, and then autoclaved at 121 °Cfor 30 min. The flasks were inoculated individuallywith 4 agar blocks (3 mm diameter), cut from eachpureendophytic fungal culture using a sterile corkborer, and then incubated at 28°C for 21 days. Afterincubation, the culture media and the growing myceliawere extracted using ethyl acetate and then separatedby filtration. The organic phase was vacuumconcentrated at 50°C under reduced pressure, using arotary vacuum evaporator to obtain the crude extracts

Measurement of Antioxidant Property of the Fungal Extracts

The antioxidant activity of the extracts was studied using a slightly modified method of free radical scavenging, as described by Brand-Williams *et al.*(1995), with ascorbic acid employed as a reference antioxidant. The free radical scavenging properties of the extracts against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were measured at 490 nm, serving as an index of their antioxidant activity. The concentrations of the extracts and ascorbic acid used were 20, 40, 60, 80, and 100 µg/mL. The samples were reacted with the stable DPPH radical in a methanol solution. The reaction mixture, consisting of 25 µl of the stock, 25 µl of DPPH 0.1 mol/L, and 150 µl of the methanol solution, was added to the respective wells in the microtiter plate and then incubated at 27°C for 30 min.After this incubation period, the absorbance of the mixtures was measured at 490 nm using a UV-Vis spectrophotometer. The percentage of antioxidant activity (AA%) of each extract was assessed by the DPPH free radical assay. The antioxidant capacities of the extracts were compared with those of ascorbic acid. Free radical scavenging activities were expressed as the percentage inhibition of each extract and calculated using the following formula:

$$AA\% = \frac{Ao-A1}{Ao} X100 \ldots\ldots\ldots\ldots\ldots \text{ eqn. 1}$$

where: AA% = the percentage of antioxidant activity, A0 = absorbance in the presence of the extract (test), A1 = absorbance in the presence of the positive control (ascorbic acid).

Statistical Analysis

Results were analyzed using the Statistical Package for Social Science (SPSS-20) software and presented as Mean ± Standard Error of Mean (SEM) of sample replicates (n = 3). Significant differences between control and treatment groups were compared using a one-way analysis of variance (ANOVA) followed by a Post hoc Dunnett (two-sided) test. A significance level of $p< 0.05$ was considered statistically significant.

RESULTS

From the cultured tissues of the root, leaf blades, and mid-ribs of *A.senegalensis*, seven endophytic fungi were isolated. The root produced three; RT1, RT2, RT3, the leaf blades two; LB1, LB2, and the mid-ribs two; MR1, MR2. The ethyl acetate extracts from these isolates demonstrated concentration-dependent free radical scavenging activities, as shown in **Figures** 2-4. The order of activity, based on their IC_{50} for the ethyl acetate extract from each isolate, was LB2>RT1>MR2>MR1>RT2>RT3>LB1. In the figures below, the extracts showed good antioxidant activities compared to ascorbic acid.

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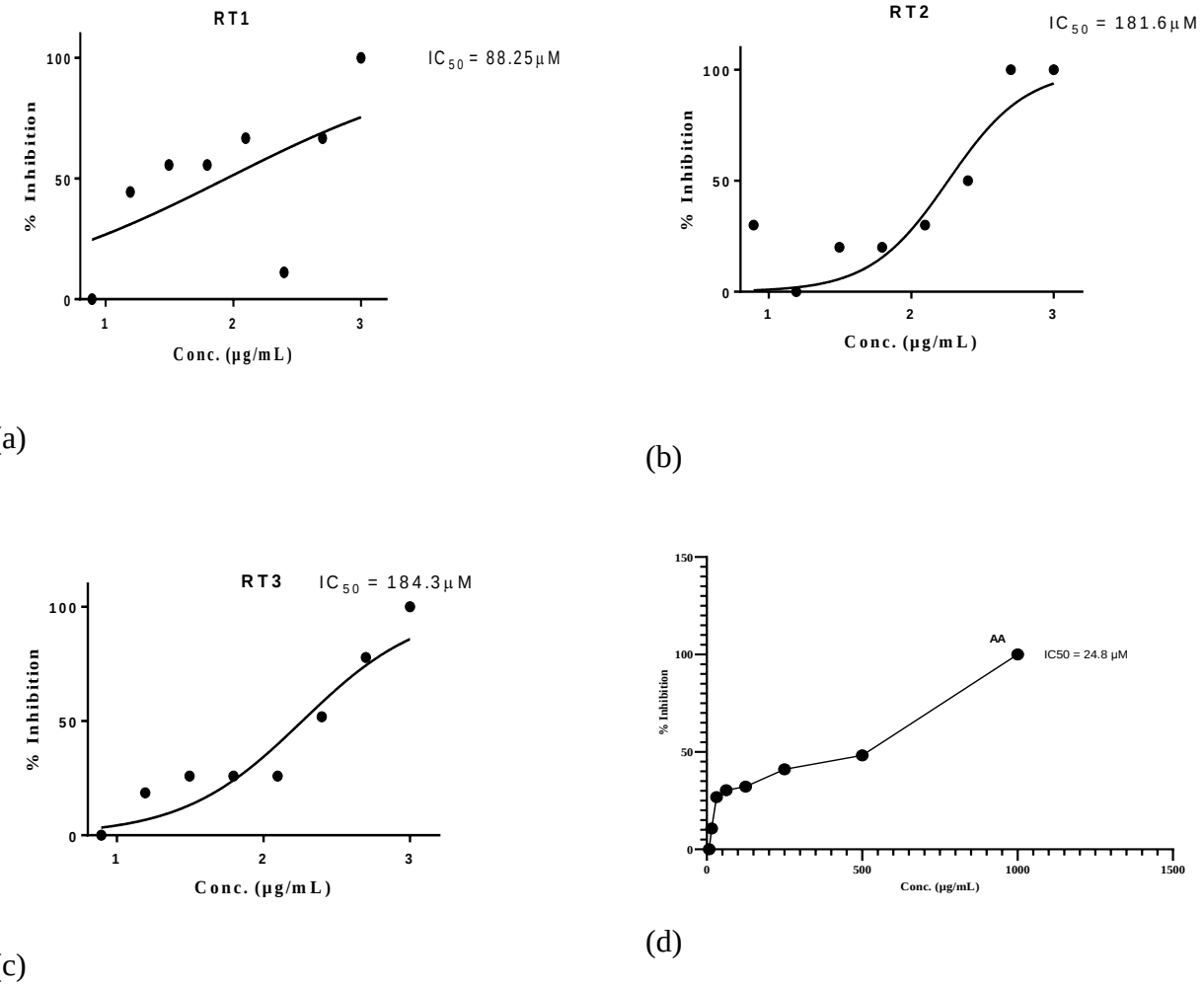


Fig 2: Percentage inhibition of free radicals by crude endophytic fungal extracts of:(a) RT1 (b) RT2 (c) RT3 (d) AA

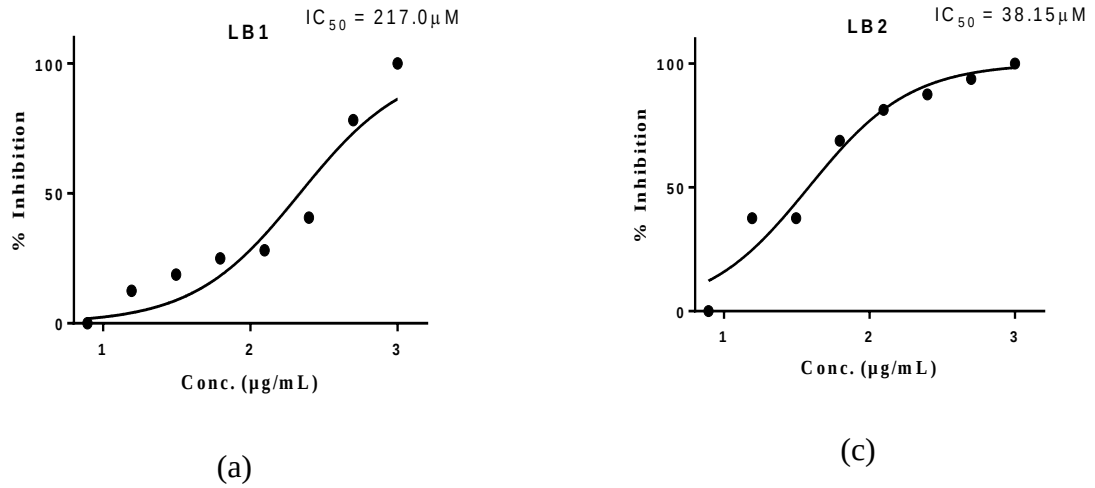
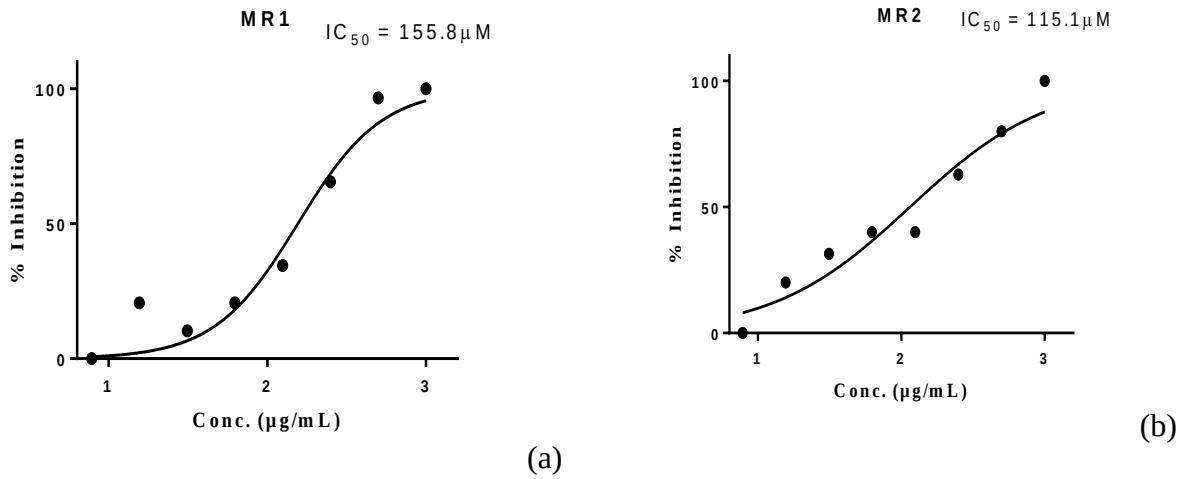


Fig 3: Percentage inhibition of free radicals by crude endophytic fungal extracts of:(a) LB1, (b) LB2



(a) (b)

Fig 4: Percentage inhibition of free radicals by crude endophytic fungal extracts of: (a) MR1 and, (b) MR2

DISCUSSION

Annonasenegalensis, a renowned medicinal plant, has stood the test of time as a valuable resource for addressing human healthcare needs. The escalating utilization of such medicinal plants, however, raises legitimate concerns regarding the depletion of natural resources (Alamgir, 2018). In response to this challenge, the exploration of endophytic microorganisms emerges as a promising avenue, offering a sustainable solution by regenerating metabolites akin to or even broader in their spectrum to those of the host plant. This approach not only contributes to the conservation of natural resources but also opens avenues for the discovery of novel compounds with diverse applications in the pharmaceutical, agricultural, and biomedical sectors (Brand-Williams *et al.*,1995; Alamgir, 2017; Alamgir, 2018; Thangaraj,2016).

In the current study, the crude endophytic fungal extracts demonstrated concentration-dependent antioxidant activities. This finding aligns with previous studies highlighting the antioxidant properties of ethyl acetate extracts from crude endophytic fungi metabolites (Okezie *et al.,* 2023; Brand-Williams *et al.*,1995). Ethyl acetate, as noted by Nouari*et al*. (2013), stands out as a preferred solvent for selectively extracting low molecular weight phenols and higher molecular weight polyphenols. According to them, phenols are emphasized for their presumed crucial role in high antioxidant activities. Similarly,in the course of studying the antioxidant properties of fungal endophytes associated with*Garcinia cola* and *Cola nitida*, Nwobodo *et al* (2017) also reported a high value of phenolic compounds in the fungal extracts and attributed the free-radical scavenging capacity of their extract to the phenols. However, GC-MS analysis in a separate study revealed a notable presence of fatty acids and phenolic compounds. This suggests a potential synergistic or additive antioxidant effect, challenging the notion that phenols solely dictate the observed antioxidant activities.

The definition of an extract as an antioxidant hinges on its ability to neutralize DPPH free radicals (Fathoni *et al*., 2022), a process contingent on the antioxidant's capability to donate either an electron or a hydrogen atom to stabilize DPPH free radicals (Dhankhar *et al.*,2012; Prima *et al.,* 2022).In our study, LB2 and RT1 isolates had IC_{50} values of 38.15 and 88.3 µg/ml, respectively. Previous studies have indicated that an IC_{50} value of less than 50 µg/ml in the DPPH test is considered very strong (Prima *et al.,* 2022; Rustamova *et al.,* 2020). This implies that the

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extract from LB2 isolate possesses a very strong antioxidant activity. The extract highest inhibition of 60% was observed in the RT1 isolate at the concentration of 1000 µg/ml, comparable to ascorbic acid,which had 94% at 1000µg/ml. The lowest concentration of 7.8 µg/ml showed an inhibition of 51%forRT1,whereas ascorbic acid had 38% as positive control This indicates that the crude endophytic fungal extract from *A.senegalensis* could be further explored and harnessed as a natural source of antioxidants.

CONCLUSION

In conclusion, our findings underscore the importance of *A. senegalensis*associated fungal endophytes as reservoirs of valuable bioactive compounds. The synergy of these compounds within endophytic fungal extracts showcases a rich source of antioxidants with promising applications in various industries. The observed antioxidant activities, particularly the potent effects of LB2 and RT1 isolates, warrant further exploration and harnessing of these natural resources for potential therapeutic and industrial benefits.

Conflict of Interest

The authors have no conflict of interest to declare.

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