

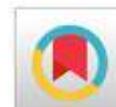


The anti-pseudomonal potentials of metabolites from some endophytic fungi isolated from *Garcinia kola* leaves

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Received: 17 April, 2020; Accepted: 14 May, 2020; Published online: 12 June, 2020

Abstract

The morbidity and mortality rates from *Pseudomonas aeruginosa* infections are increasing, due to the development of drug-resistant strains. This study aimed to explore the secondary metabolites of endophytic fungi of *Garcinia kola* for their antibacterial activities against *P. aeruginosa*. The endophytic fungi associated with healthy leaves of *G. kola* were isolated using the standard methods. These fungi were subjected to solid-state fermentation on rice media at 28°C for 21 d. The fungal secondary metabolites were extracted using ethyl acetate, and then concentrated under vacuum. The fungal crude extracts were screened for their antibacterial activities against clinical and laboratory strains of *P. aeruginosa*, using the agar diffusion method. The bioactive components of the fungal extracts were identified using High-Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis. Three endophytic fungi mainly; *Aspergillus* sp., *Fusarium* sp. and *Colletotrichum* sp. were isolated. At concentration of 1 mg/ml, extracts of the three fungi displayed anti-pseudomonal activities against all the isolates, except for a *P. aeruginosa* isolate recovered from urine. Results of the HPLC-DAD analysis revealed the presence of several active compounds such as; indole-3-acetic acid, p-hydroxybenzoic acid, and protocatechuic acid, among others in the fungal extracts. These compounds have been previously reported to have significant antimicrobial properties. This study reveals that endophytic fungi associated with *G. kola* leaves possess promising anti-pseudomonal potential.

Keywords: *Pseudomonas aeruginosa*, *Garcinia kola*, Endophytic fungi, Anti-pseudomonal, Secondary metabolites

1. Introduction

Pseudomonas aeruginosa is a major etiological agent of healthcare-associated bacterial infections and is responsible for 11 % of the hospital-acquired infections, which results in high mortality and morbidity rates (Haque *et al.*, 2018). Unfortunately, *P. aeruginosa* is susceptible to a limited number of antibacterial agents, suggesting that the high mortality rate associated with this bacterium could be attributed not only to its virulence, but also to the administration of ineffective empirical antibacterial therapy. The morbidity and mortality from *P. aeruginosa* infections are increasing, due to these drug-resistant strains. Several previous studies of Ozer *et al.*, (2009); Marilyn *et al.*, (2012); Egbujor *et al.*, (2020) reported that resistance development in *P. aeruginosa* is multifactorial, with mutations in several genes contributing for resistance to β -lactams, carbapenems, aminoglycosides, fluoro-quinolones and sulphonamides. Accordingly, it has become of utmost importance to develop new antibacterial agents to inhibit or completely kill this bacterium, thus saving our society from its deleterious effect. A previous study of Petri, (1991) defined endophytes and endophytic fungi as microorganisms that grow intercellularly and asymptotically within the plant living tissues, thus establishing a mutual relationship with this host plant. Yan *et al.*, (2011); Okoye *et al.*, (2013); Nwobodo *et al.*, (2017) highlighted that the endophytic fungal species are considered as exciting novel sources of new bioactive compounds for drug discovery. A large number of the world's populations especially from developing countries rely mainly on traditional medicines derived from plants for their primary health care. A recent study conducted by Lawrence and Vandecar, (2015) revealed that as the search for more effective and safer bioactive compounds from natural sources continues, products derived from plant sources require large scale harvesting and probably destruction of such plants, which in turn may lead to climatic imbalance and environmental disruption. The study of plant-associated endophytes could provide an alternative

way of discovering novel active metabolites with anti-pseudomonal properties. Several previous studies of Tende *et al.*, (2011); Aljabry *et al.*, (2017) demonstrated that *G. kola* (Heckel) plant is reputed to exhibit several potent pharmacological activities such as; antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties. This plant belongs to the family *Guttiferae* that is popular in African traditional medicine (Indabawa and Arzai, 2011). Considering the numerous therapeutic potentials of this plant, as well as the eco-friendly properties of the endophytic fungi drug discovery approach, the objective of this study was to screen for the *in-vitro* anti-pseudomonal potency of the endophytic fungal extracts of *G. kola*, against the clinical and laboratory strains of *P. aeruginosa*.

2. Material and methods

2.1. Isolation and purification of the endophytic fungi

Samples of fresh leaves of mature healthy *G. kola* plants were collected from Nsukka, Enugu State, South-Eastern Nigeria. Isolation of endophytic fungi from the plant leaves was carried out as described by Eze *et al.*, (2018). The leaves were washed thoroughly in running tap water, and then cut into small fragments (about 1 cm²). The leaf fragments were surface-sterilized by immersion in 2 % sodium hypochlorite solution for 2 min., 70 % ethanol for nearly 2 min., before a final rinse in sterile water for 5 min. These leaf fragments were transferred into malt extract agar (MEA) plates, supplemented with chloramphenicol (500 mg/ l). The Petri plates were then incubated at 27°C for 7 d. Hyphal tips of fungal colonies emerging from the leaf segments were sub-cultured on fresh MEA plates, and then purified using single spore technique.

2.2. Solid state fermentation and extraction of the fungal secondary metabolites

Massive production of the isolated endophytic fungi through solid-state fermentation, and extraction of the fungal metabolites were carried out as described

by [Okoye *et al.*, \(2013\)](#); [Nwobodo *et al.*, \(2017\)](#). Rice medium was prepared in 1000 ml Erlenmeyer flasks as follows: approximately 200 ml of dist. water was added to 100 g of rice, and then autoclaved at 121°C for 30 min. The flasks were inoculated individually with 4 agar blocks (3 mm diameter), cut from each pure endophytic fungal culture using a sterile cork borer, and then incubated at 28°C for 21 d. After incubation, the culture media and the growing mycelia were extracted using ethyl acetate, and then separated by filtration. The organic phase was vacuum-concentrated at 40°C under reduced pressure, using a rotary vacuum evaporator to obtain the crude extracts.

2.3. *In vitro* anti-pseudomonal activity assay

2.3.1. Test bacteria

Various clinical isolates of *P. aeruginosa* were obtained from orthopedic wound infections; urine, sputum, and a vaginal swab, provided by the National Orthopedic Hospital Enugu, Nigeria, and a laboratory strain were used for the study. The identities of the bacterial isolates were confirmed at the Pharmaceutical Microbiology Laboratory, Nnamdi Azikiwe University, Agulu, using the standard morphological and biochemical characteristics of the bacteria, according to [Bergey and Holt, \(2000\)](#).

2.3.2. The *in vitro* bioassay

The preliminary screening of the endophytic fungal extracts for their anti-pseudomonal activity was carried out using the agar well diffusion assay of [Onyegbule *et al.*, \(2014\)](#). Stock concentrations (1 mg/ml) of the fungal extracts were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO 100 % v/v). About 0.1 ml (1×10^5 cells/ml) of test bacterial isolates (*P. aeruginosa*) was spread aseptically and individually onto the surface of Mueller Hinton Agar (MHA) plates. All plates were allowed to dry for about 5 min., and then agar wells were made by using a sterile cork-borer (6 mm in diameter). These wells were individually inoculated with 20 µl of each of the fungal extracts and the controls. The plates were then kept at room temperature for 1 h, and then incubated at

37°C for 24 h. Gentamicin antibiotic disc (10 µg/ml) and DMSO (100 % v/v) were used as the positive and negative controls, respectively. The inhibition zones diameters (IZDs) were measured using a calibrating ruler. The assay was conducted in triplicates, and repeated twice.

2.4. High performance liquid chromatography (HPLC) analysis

The HPLC analysis was carried out on the fungal extracts as described by [Eze *et al.*, \(2018\)](#). A Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany) was used during the analysis. The separation column (125 × 4 mm; length × internal diameter) was pre-filled with Eurospher-10 C18 (Knauer, Germany), a linear gradient of nano-pure water (adjusted to pH 2 by addition of formic acid), and methanol was used as eluent. A weight of 2 mg of each fungal extract was reconstituted with 2 ml of HPLC grade methanol, the mixture was sonicated for 10 min., and thereafter centrifuged at 3000 rpm for 5 min. A volume of 100 µl of the dissolved sample was transferred to a vial containing 500 µl of HPLC grade methanol, and then the vial was put in the HPLC machine for analysis. Detection was carried out at 235 nm. The absorption peaks of the fungal extracts were analyzed by comparing with those in the HPLC-UV/Vis database.

2.5. Statistical analysis

Data obtained were presented as means of experiments that were carried out in triplicates. The mean inhibition zones diameter of the fungal extracts against the various *P. aeruginosa* isolates were compared using one way ANOVA. Statistical significance was considered at $p \leq 0.05$. Analysis of data and graph were made using Microsoft Excels 2013 software and SPSS version 20.

3. Results

3.1. Isolation and identification of the endophytic fungi

A total of three endophytic fungi were isolated from the leaf segments of *G. kola* plant, and labeled as: Gc1-3. The three isolates exhibited different colony morphologies on MEA on (Fig. 1). These isolates were

identified according to their morphological and microscopic characters as; Gc1: *Aspergillus* sp., Gc2: *Fusarium* sp. and Gc3: *Colletotrichum* sp. (Table 1).



Fig 1: Macroscopic colony morphologies of the three endophytic isolates. Gc1: *Aspergillus* sp., Gc2: *Fusarium* sp., and Gc3: *Colletotrichum* sp.

Table 1. Cultural morphological and microscopic features of the endophytic fungi isolated from *G. kola* leaves

Isolates	Color	Reverse	Growth Rate	Texture	Hyphae	Spores	Identified fungus
Gc1	Brownish Green, with a white border	Hyaline	Moderate	Dry and Hard	Septated	Micro conidia	<i>Aspergillus</i> sp. (Afzal et al., 2013).
Gc2	White	Colorless	Rapid	Cottony Smooth	Septated	Micro and Macro conidia	<i>Fusarium</i> sp. (Tayung et al., 2011).
Gc3	White/orange	Whitish grey	Moderate	Cottony Rough	Septated	Macro conidia with ascus/ascospores	<i>Colletotrichum</i> sp. (Arivudainambi et al., 2011).

3.2. *In vitro* anti-pseudomonal activity of the fungal extracts

The *in vitro* antibacterial potential of the fungal extracts against the clinical isolates of *P. aeruginosa* are demonstrated in Fig. (2). The laboratory isolate (*P. A*) and the vaginal isolates (*P. E*) are susceptible to 100 % of the tested fungal extracts at a concentration of 1 mg/ ml. While the *P. aeruginosa* isolate from urine is resistant to all fungal extracts. The orthopedic wound isolate (*P. B*) is susceptible only to the extracts of *Colletotrichum* sp., recording an IZD of 2 ± 0 at 1 mg/ ml. All the tested fungal extracts inhibited at least 60 % of the *P. aeruginosa* isolates under study. The mean values of the zones of inhibition obtained for the three fungi are statistically not significant as $p > 0.05$. However, when compared to Gentamicin, $p < 0.001$.

3.3. Separation and identification of the compounds present in the fungal extracts

Results present in Table (2) demonstrate the active compounds present in the individual fungal extracts identified through the HPLC, and their previously reported biological activities. About six known compounds were identified as; Enniatin A, Protocatechuic acid, P-hydroxybenzoic acid, P-methoxycumarin, Indolyl-3-acetic acid, and 4-hydroxyphenyl acetic acid. The chromatogram and structures of *Fusarium* sp. and *Colletotrichum* sp. extracts are represented in Fig 3(a, b), respectively. They reveal the peaks and structures for P-methoxycumarin and indole-3-acetic acid, respectively.

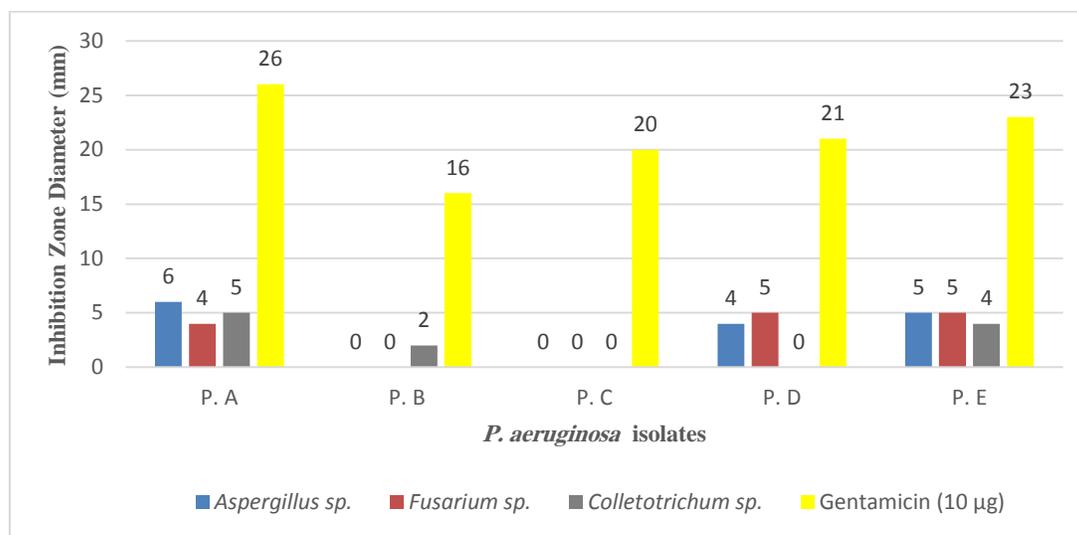
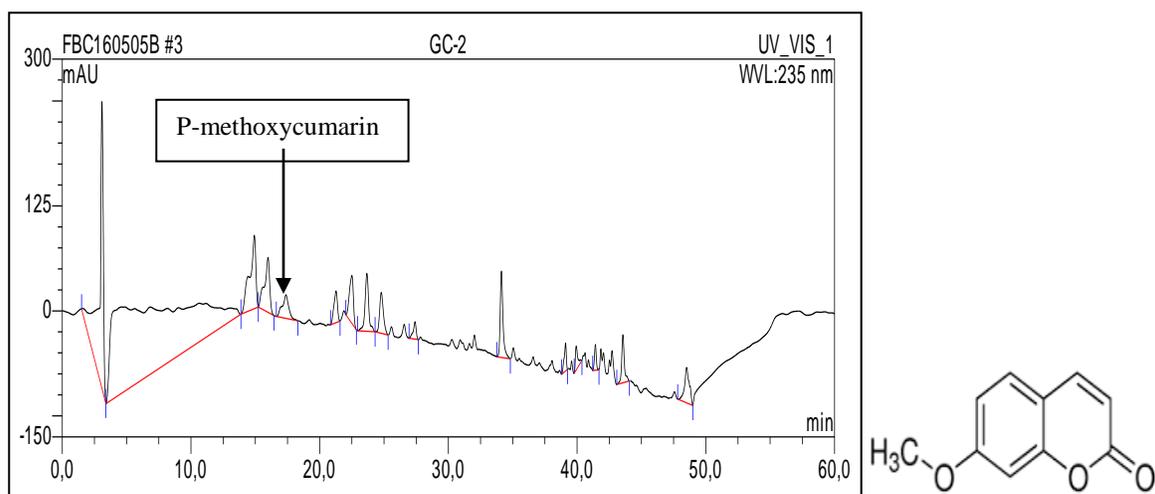


Fig. 2: Inhibition zones diameters (mm) of the different clinical *Pseudomonas* isolates, caused by treatments with the three fungal extracts and the antibiotic (Gentamicin). Where; *P. A*= Laboratory isolate, *P. B*= Orthopedic wound infection isolate, *P. C*= Urine isolate, *P. D*= Sputum isolate, *P. E*= Vaginal swab isolate. Values above the columns represent diameters of the inhibition zones (mm). The mean values of the inhibition zones are statistically not significant as $p > 0.05$ for the fungal extracts; however, for Gentamicin, $p < 0.001$.

Table 2. The compounds detected in the fungal extracts and their antimicrobial properties

Fungi Extract	Identified compounds	Reported biological activities	References
<i>Aspergillus</i> sp.	Enniatin A	Inhibition of drug efflux pump, Antimicrobial	Firáková <i>et al.</i>, (2007)
	Protocatechuic acid	Antimicrobial	Kakkar and Bais, (2014)
<i>Fusarium</i> sp.	Enniatin A	Inhibition of drug efflux pump, Antimicrobial	Firáková <i>et al.</i>, (2007)
	<i>P</i> -hydroxybenzoic acid	Antimicrobial	Manuja <i>et al.</i>, (2013)
	<i>P</i> -methoxycumarin	Antimicrobial	Malhotra <i>et al.</i>, (2008)
<i>Colletotrichum</i> sp.	Indole-3-acetic acid	Antimicrobial	Trinagaraju <i>et al.</i>, (2015) ; Arnao <i>et al.</i>, (1996)
	4-hydroxyphenyl acetic acid	Antimicrobial	Zuo <i>et al.</i>, (2014)

**Fig. 3a:** HPLC chromatogram of *Fusarium* sp. extract, showing UV spectrum and structure of P-methoxycumarin

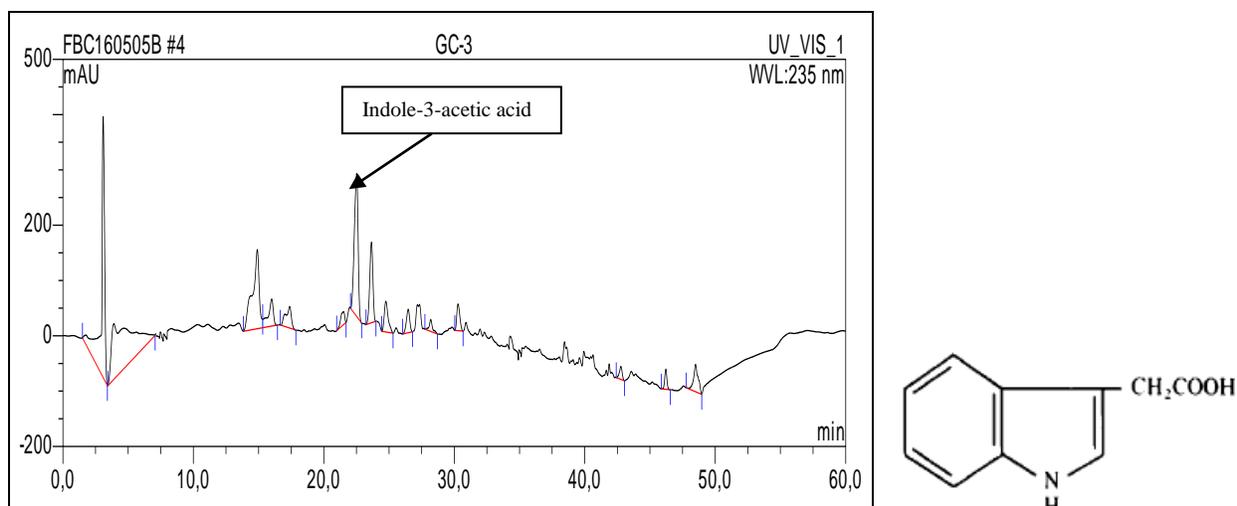


Fig. 3b: HPLC chromatogram of *Colletotrichum* sp. extract, showing UV spectrum and structure of indole-3-acetic acid

4. Discussion

In accordance with our results, the fungal endophytes isolated from *G. kola* in this study have been previously isolated as endophytes in several previous studies; *Aspergillus* sp., *Fusarium* sp. (Phongpaichit *et al.*, 2006; Zhao *et al.*, 2010) and *Colletotrichum* sp. (Lu *et al.*, 2000). The majority of the compounds identified in the endophytic fungal extracts in this study, have been previously reported to be produced by fungi of endophytic origins. Lu *et al.*, (2000) reported the production of indole acetic acid and hydroxyphenyl derivatives by *Colletotrichum* sp.; in addition, two new indole alkaloids were isolated from *Aspergillus* sp. by Hasan *et al.*, (2015). Likewise, in other different independent and separate studies, *Fusarium* sp. was reported to produce Enniatin A (Firáková *et al.*, 2007; Lucasz and Waskiewicz, 2013).

The recorded antibacterial activity displayed by the endophytic fungal extracts in this study could be attributed to the presence of antimicrobial compounds in their metabolic extracts. Several previous studies of Anthony, (2009); Carolina *et al.*, (2010); Oksana *et al.*, (2012), reported that 4-hydroxy benzoic acid is an organic chemical that exhibits antimicrobial activity against a number of microorganisms such as Gram

positive as well as Gram negative bacteria, including *P. aeruginosa*. Moreover, Manuja *et al.*, (2013); Eze *et al.*, (2019) added that Protocatechuic acid (3, 4-dihydroxybenzoic acid) is a natural phenolic acid found in most edible and medicinal plants. This compound has also been reported to be produced by several species of bacteria and fungi (Nguyen *et al.*, 2015; Eze *et al.*, 2018). Previous studies conducted by Kakkar and Bais, (2014); Nwobodo *et al.*, (2017) revealed that protocatechuic acid exhibited varying biological activities including; antibacterial, antioxidant, anticancer, and anti-inflammatory potentials. On the other hand, indole-3-acetic acid is a plant hormone, which regulates various aspects of plant growth and development (Fu *et al.*, 2015). There are many reports on the production of indole-3-acetic acid by certain bacterial and fungal species (Kakkar and Bais, 2014; Fu *et al.*, 2015; Nwobodo *et al.*, 2017). A previous study of Trinagaraju *et al.*, (2015) described the metal complexes of 3-indole acetic acid as having good inhibitory activity against the *Bacillus subtilis* as well as *Candida* sp. The other compounds recorded in this study including Enniatin A, P-methoxycoumarin, P-hydroxybenzoic acid and 4-hydroxyphenyl acetic acid, have all been reported to possess antimicrobial activities (Table 2.).

Extracts of the three fungal spp. isolated in this study displayed varying anti-pseudomonal activities against all the bacterial isolates, except *P. aeruginosa* isolate that was recovered from urine. The extract of *Aspergillus* sp. exhibited the best inhibitory activity, with an average inhibition zone of 5 mm at 1 mg/ml. [Brown and Izundu, \(2004\)](#); [Garba *et al.*, \(2012\)](#) reported that *P. aeruginosa* wound isolates are highly resistant, when compared to the same isolates from other sources.

An emerging problem with *P. aeruginosa* infection is that this pathogen exhibits a high degree of resistance to a broad spectrum of antibiotics. Interestingly, *P. aeruginosa* isolate from wound regarded to be a very resistant strain, is currently observed to be susceptible to the extract of *Colletotrichum* sp. This may be due attributed to the presence of 4-hydroxyphenyl acetic acid and indole-3-acetic acid identified in the fungal extract, as revealed by the HPLC results. Since only the extract of *Colletotrichum* sp. reported to contain both 4-hydroxyphenyl acetic acid and indole-3-acetic acid, and had an activity against the wound isolate, thus the combination of both of these principal compounds could produce synergistic mechanisms, responsible for the inhibitory activity observed against the wound isolate. Moreover, 4-hydroxyphenyl acetic acid is a phenolic compound, and phenolics have been previously reported by [Merkl *et al.*, \(2010\)](#); [Bouarab-Chibane *et al.*, \(2019\)](#) to possess antimicrobial properties. This is an important observation, and suggests that the extract of *Colletotrichum* sp. possesses potential as an antibacterial agent against the possible drug-resistant strains of *P. aeruginosa*. A previous study of [Webber, \(1981\)](#) documented that the presence of bioactive compounds confer resistance to plants against the bacteria, fungi, and several other pests. This probably further explains the antibacterial potency demonstrated by the extracts of the plant endophytic fungi recorded in the current study. It is noteworthy that, the compounds responsible for the observed antibacterial activities may have a structural analogue to the previously established drugs, known to

show such effective anti-pseudomonal activity. This can be ascertained through purification and further structural elucidation of the identified compounds, which may be considered as promising compounds for the development of anti-pseudomonal drugs.

Conclusions

The current recorded *in vitro* anti-pseudomonal activity of the endophytic fungal extracts of *G. kola* appears promising. This may be attributed to the presence of several active compounds identified in these fungal extracts. Thus they could be used as promising natural sources of anti-pseudomonal agents, for treatment of infections caused by the pathogenic strains of *P. aeruginosa*.

Acknowledgments

The authors are grateful to the Departments of Microbiology, Renaissance University; Pharmaceutical Microbiology and Biotechnology; Pharmaceutical and Medicinal Chemistry, Nnamdi Azikiwe University, Nigeria, for provision of laboratory space and instrument.

Conflict of interest

The authors declare that they have no any competing interests.

Funding source

There was no financial aid received concerning this research work.

Ethical approval

Non-applicable.

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