

Screening of Endophytic Fungal Secondary Metabolites from *Garcinia kola* and *Cola nitida* for Antioxidant Properties

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Abstract

Objectives: Plant endophytic fungi are novel reservoir of bioactive compounds. The aim of this study was to explore endophytic fungi from *Garcinia kola* and *Cola nitida* for antioxidant secondary metabolites.

Methods: Six fungal endophytes, Gc1-4 and Cn1-3 were isolated from the medicinal plants *G. kola* and *C. nitida* respectively. The pure endophytes were fermented, their crude secondary metabolites extracted and screened for antioxidant properties using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activities and reducing power (RP) assay. The bioactive principles were identified by dereplication using High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) analysis

Results: Extracts of Cn3 (identified as *Trichophyton* sp.) and Gc3 (identified as *Collectotrichum* sp.) demonstrated the most potent antioxidant activity. In the DPPH scavenging assay, extracts of Cn3 and Gc3 showed an IC₅₀ of 50.7 and 69.1 µg/ml respectively compared to ascorbic acid (12.2µg/ml). Extracts of Cn3, Gc3 and positive control (ascorbic acid, 250µg/ml) showed reduction values of 1.112 ± 0.011, 1.075 ± 0.014 and 1.203 ± 0.004, respectively. There was positive correlation between total phenolic content (TPC) and results obtained in both assays (DPPH, R² = 0.7039; RP, R² = 0.9778), thus suggesting phenolics-related antioxidant activity. HPLC-DAD analysis revealed extract of Cn3 contain protocatechuic acid and p-hydroxybenzoic acid, while Gn3 contain 4-hydroxyphenyl acetic acid and indole-3-acetic acid. These compounds have been previously reported to exhibit potent antioxidant activity. In conclusion, this study reveals that endophytic fungi from both plants possess potential antioxidant properties. The secondary metabolites present in these endophytic fungi have potential for pharmaceutical and agrochemical applications.

Keywords: *Garcinia kola*; *Cola nitida*; Antioxidant; Endophytes; Phenolics; Climate change

Introduction

Oxidative stress, which arises from the presence of reactive oxygen species (ROS) has been established to be involved in the development of various health disorders [1]. Disorders linked to excessive generation of ROS in the human body include cancer, inflammation, Alzheimer's disease, dementia, cardiovascular diseases and atherosclerosis [2,3]. The consumption of fruits and vegetable known to possess antioxidant activity has proved useful in prevention and amelioration of some of these disease states. Recently, enormous research efforts have been directed towards the development of therapeutic antioxidant compounds from these medicinal plants. One of the end results of the commercialization of successful antioxidant compounds derived from plant source may be large scale harvesting and probably destruction of such plants, which in turn could lead to climate imbalance and environmental disruption [4]. This underscores the need to explore alternative and ecofriendly commercial sources of identified medicinal compounds.

Currently, endophytes are viewed as an outstanding and ecofriendly source of bioactive natural products. Endophytes and endophytic fungi refer to microorganisms which grow intercellularly and asymptotically within living tissues establishing mutual relationship with the host plant [5]. This group of organisms produces bioactive compounds that may provide defense for the host plant against pathogens, and some of these compounds are useful for novel drug discovery [5]. Most plant species harbour one or more endophytic organisms [5].

G. kola and *C.nitida* plants are reputed to exhibit several potent pharmacological activities such as antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties [6-9]. These plants belonging to the family *Guttiferae* (*G. kola*) and *Sterculiaceae* are popular in African traditional medicine. In our earlier reports, we have shown that Nigerian medicinal plants are good sources of endophytes that can generate secondary metabolites with proven therapeutic potentials [10-12]. In this study, we investigated the secondary metabolic extracts of endophytic fungi from *G. kola* and *C. nitida* for their antioxidant properties.

Materials and Method

Sampling/Plant Collection

Leaf samples of *Cola nitida* (PCG/474/A/050) and *Garcinia kola* (PCG/474/A/051) were collected from

mature healthy plants from Nsukka, in Enugu State located in South-Eastern part of Nigeria. They were identified by Mrs. Emezie Anthonia of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. Plant materials were brought to the laboratory in sterile bags and processed within a few hours after collection. Samples were also deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

Isolation and Purification of Endophytic Fungi

Fresh plant materials were used for isolation to reduce the risk of contamination. Plant leaves were surface washed in running tap water to remove dust and debris. After proper washing, leaf samples were cut into small segments (about 1-2 mm) and surface-sterilized. The process of surface sterilization involved sequential washing with the under listed solvents. First was in 70% ethanol for 2 min, second was 2% sodium hypochlorite (NaOCl) for 2-3 min. Third was in 70% ethanol for 2 min, and then double rinsing in sterile distilled water. The samples were surface-dried by blotting on sterile filter paper in a laminar flow [13].

About 4 segments from each plant was selected and placed on each Petri dish containing Malt Extract Agar (MEA) supplemented with Chloramphenicol 500 mg/L. The efficacy of surface sterilization was tested by pressing the sterilized segments on to the surface of MEA medium, and removed immediately. The absence of growth of any fungi on the medium after incubation, confirmed that the surface sterilization procedure was effective [14]. The plates were sealed and incubated at $27 \pm 2^\circ\text{C}$. Periodically, fungal growth from the leaf segments were monitored and hyphal tips from distinct colonies emerging from leaf segments were sub-cultured on fresh MEA plates to obtain pure colonies.

Fermentation and Extraction of Secondary Metabolites

Pure isolates of the fungi were cultured in rice media for the production of secondary metabolites. Small scale fermentation was carried out primarily to perform bioassay for the detection of active metabolites. Solid state fermentation was carried out in 1000 ml Erlenmeyer flasks containing 100 g of rice media (200 ml of water was added to the rice and then autoclaved at 121°C at 15 psi for 30 min). The flasks were inoculated with 3 mm diameter agar blocks containing pure fungi endophyte and incubated at $25-27^\circ\text{C}$ for 30 days. The culture media

and the mycelia were extracted in ethyl acetate and separated by filtration. The organic phase was vacuum-concentrated at 40°C under reduced pressure using a rotary evaporator.

Sample Preparation

A stock solution (1 mg/ml) of each of the extract was prepared by dissolving each extract in 5% Tween 80. From the respective stock solutions, two-fold serial dilutions (250, 125, 62.5, 31.3, 15.6, and 7.8 µg/ml) of each of the extracts were prepared.

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) Free Radical Scavenging Assay

The measurement of the DPPH radical scavenging activity was performed using a previously described method [15]. The free radical scavenging ability of the samples was determined by measuring the absorbance of DPPH at 517 nm using a spectrophotometer. Ascorbic acid was used as the standard for the assay, and was prepared using same procedure as the test samples. The experiment was carried out in triplicate for each test sample or standard. The percentage (%) of DPPH scavenging activity was calculated using the formula below:

$$\% \text{ scavenging} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

Where

Abs of control = Absorbance of DPPH solution without extract or standard

Abs of sample = Absorbance of DPPH solution with extract or standard

IC₅₀ values, which denote the concentration of test sample required to scavenge 50% of DPPH free radicals were calculated [16].

Reducing Power (RP) Assay procedure

The reducing capacity of the extracts was determined using the potassium ferricyanide reduction method as reported by Oyaizu [17]. The experiments were done in triplicates and ascorbic acid was used as the reference standard. An increase in absorbance indicated higher reductive ability.

Total Phenolic Content (TPC) Assay

The total phenol content of the extracts was evaluated using a modified colorimetric method described by Ghasemi, et al. [18]. The values obtained were compared

to a standard curve prepared with gallic acid solution (Sigma Chemical). The total phenolic content was expressed as milligrams of gallic acid equivalents (mg GAE).

HPLC Analysis

HPLC analysis was carried out on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, DionexSoftron GmbH, Germering, Germany). The HPLC-DAD analysis was carried out to identify the active compounds contained in the respective endophytic fungal extracts. Detection was at 235, 254, 280 and 340 nm. The separation column (125x4 mm; length x internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent.

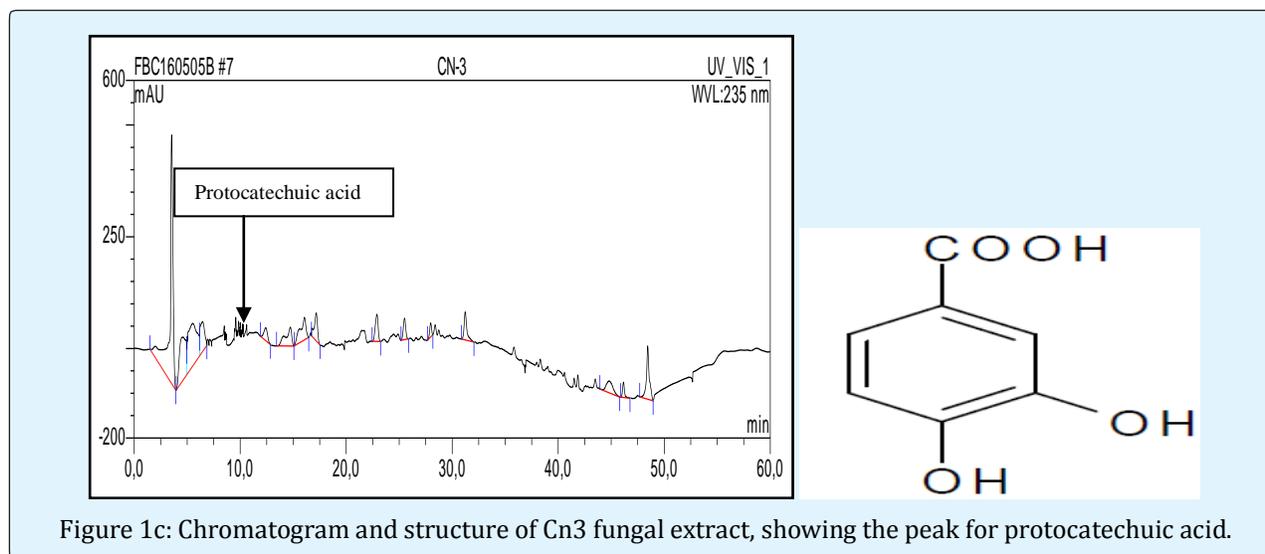
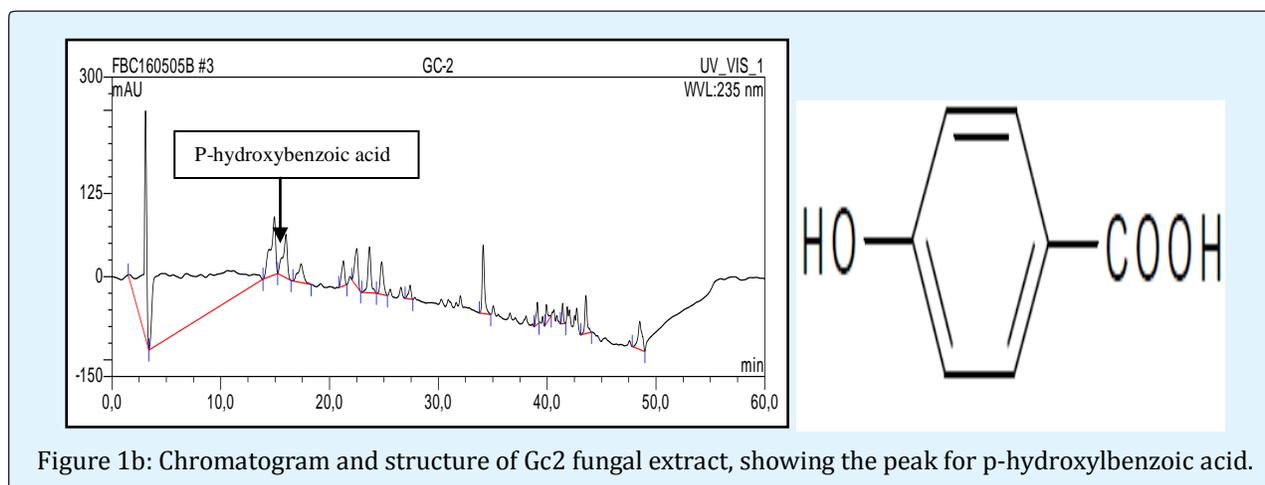
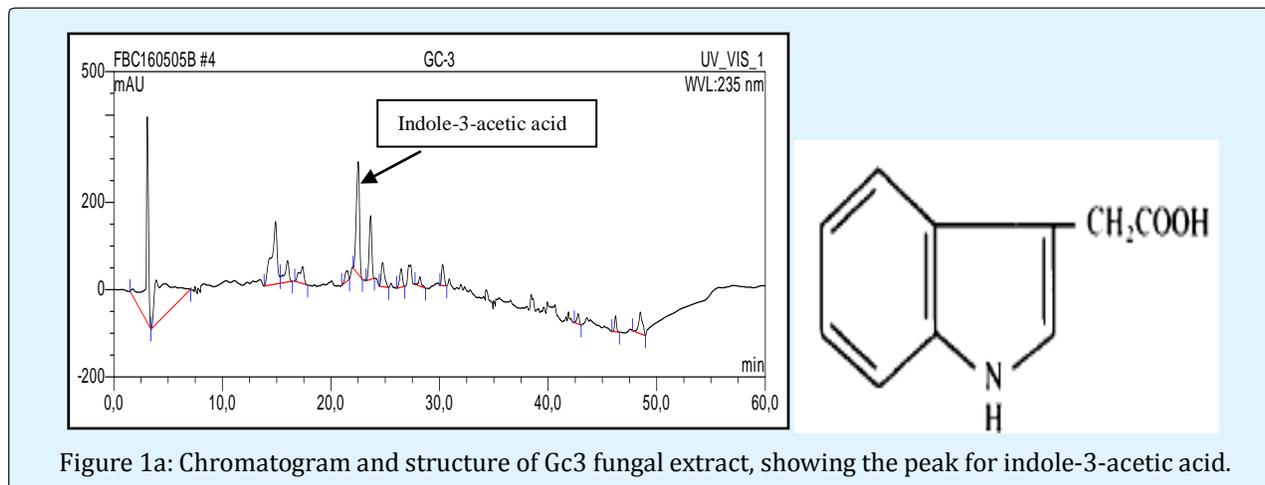
Statistical Analysis

Statistical Program for Social Sciences (SPSS) version 20 was used for all statistical analysis. Data were expressed as mean ± standard deviation (SD) for three parallel measurements. One way analysis of variance (ANOVA) was carried out to compare the mean obtained in the DPPH scavenging assay and the ferric reduction power assay. Correlation and linear regression were carried out on the results obtained in the DPPH scavenging assay, reduction power assay and total phenolic content, with the total phenolic content serving as the independent variable. The results obtained were considered significant at P ≤ 0.05.

Results

Identification of Principal Secondary Metabolites

The chromatograms and structures of the compounds identified from the extracts of Gc3, Gc2 and Cn3 are presented in Figures 1a-c. The compounds indole-3-acetic acid, p-hydroxybenzoic acid and protocatechuic acid were detected by dereplication. Non-phenolic compounds, namely, 4-hydroxyphenyl acetate acid and Indole-3-acetic acid were also identified in Gc3.



DPPH Scavenging Activities of Fungal Endophytic Extracts

The results of the DPPH scavenging activities and the corresponding IC₅₀ values are shown in Table 1 and Figure 2, respectively. All the extracts demonstrated a

dose-dependent scavenging activity. The highest percent inhibitions were recorded at 250 µg/ml by Cn3 (59.4 ± 1.8) and Gc3 (57.2 ± 1.8). Cn3 showed the lowest IC₅₀ value (50.7 µg/ml), followed by Gc3 (69.1 µg/ml), Gc4 (161.6 µg/ml), Gc1 (172.4 µg/ml), Cn2 (230.4 µg/ml) and Cn1 (281.5 µg/ml).

Extract	Concentration (µg/ml)					
	250µg/ml	125µg/ml	62.5µg/ml	31.3µg/ml	15.6µg/ml	7.8µg/ml
Gc1	53.0 ± 2.1	48.3 ± 1.6	40.4 ± 3.9	37.5 ± 3.7	34.2 ± 0.6	32.8 ± 1.1
Gc2	49.6 ± 2.3	47.0 ± 0.0	44.1 ± 1.4	40.7 ± 1.6	36.8 ± 2.2	33.2 ± 1.4
Gc3	57.2 ± 1.8	54.0 ± 1.4	49.4 ± 1.6	45.6 ± 1.8	40.9 ± 2.7	37.0 ± 1.3
Gc4	51.8 ± 3.4	49.3 ± 1.1	44.4 ± 3.2	41.6 ± 0.4	36.9 ± 2.5	32.8 ± 1.1
Cn1	50.3 ± 3.6	47.1 ± 0.2	44.6 ± 2.4	40.7 ± 1.1	38.3 ± 1.1	34.5 ± 1.4
Cn2	49.2 ± 1.8	46.9 ± 0.8	45.0 ± 1.1	39.4 ± 1.1	37.8 ± 0.9	35.6 ± 1.8
Cn3	59.4 ± 1.8	54.4 ± 1.9	51.6 ± 0.7	48.2 ± 1.8	42.7 ± 3.7	39.0 ± 0.9
Control	69.3 ± 2.1	63.5 ± 1.6	61.1 ± 1.3	57.0 ± 2.3	50.6 ± 1.9	47.8 ± 0.4

Table 1: Percentage (%) DPPH Scavenging activity.

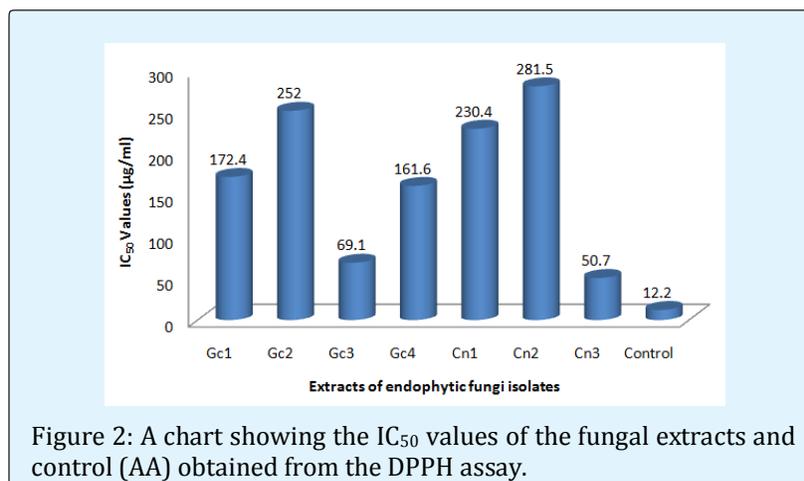


Figure 2: A chart showing the IC₅₀ values of the fungal extracts and control (AA) obtained from the DPPH assay.

Reducing Power (RP) Assay

The result of the RP (Table 2) shows the absorbance value increases as the concentration of each extract increases. Each extract recorded its highest absorbance at

250 µg/ml. Cn3 showed the overall highest value of (1.112 ± 0.011), followed by Gc3 (1.075 ± 0.014). Both values were lower than the positive control (1.203 ± 0.004).

Extract	Concentration of extract (µg/ml)					
	250 µg/ml	125 µg/ml	62.5 µg/ml	31.3 µg/ml	15.6 µg/ml	7.8 µg/ml
Gc1	1.032 ± 0.006	1.019 ± 0.010	1.002 ± 0.010	0.986 ± 0.007	0.942 ± 0.027	0.911 ± 0.007
Gc3	1.075 ± 0.014	1.032 ± 0.006	1.019 ± 0.004	0.993 ± 0.021	0.971 ± 0.007	0.912 ± 0.018
Gc4	1.004 ± 0.003	0.993 ± 0.001	0.952 ± 0.013	0.930 ± 0.006	0.906 ± 0.011	0.817 ± 0.102
Cn1	1.063 ± 0.014	1.023 ± 0.010	1.001 ± 0.013	0.972 ± 0.000	0.899 ± 0.030	0.859 ± 0.003
Cn2	1.031 ± 0.004	1.020 ± 0.006	0.990 ± 0.024	0.973 ± 0.000	0.956 ± 0.008	0.816 ± 0.054
Cn3	1.112 ± 0.011	1.075 ± 0.013	1.048 ± 0.008	1.012 ± 0.010	0.998 ± 0.003	0.897 ± 0.018
Control	1.203 ± 0.004	1.157 ± 0.016	1.089 ± 0.010	1.030 ± 0.014	1.012 ± 0.003	0.978 ± 0.016

Table 2: Mean Absorbance Values for RP Assay.

Determination of Total Phenolic Content (TPC)

The total phenolic content as well as the variation among the samples analyzed is presented in Figure 3. The highest values were 470 mg/gGAE (Cn3) and 400 mg/gGAE (Gc3), while the lowest value was 180 mg/gGAE

(Gc4). On statistical analysis, the result showed a positive correlation ($R^2 = 0.978$) between results obtained from TPC and RP. Similarly, there was also a strong correlation ($R^2 = 0.704$) between TPC and DPPH scavenging activity.

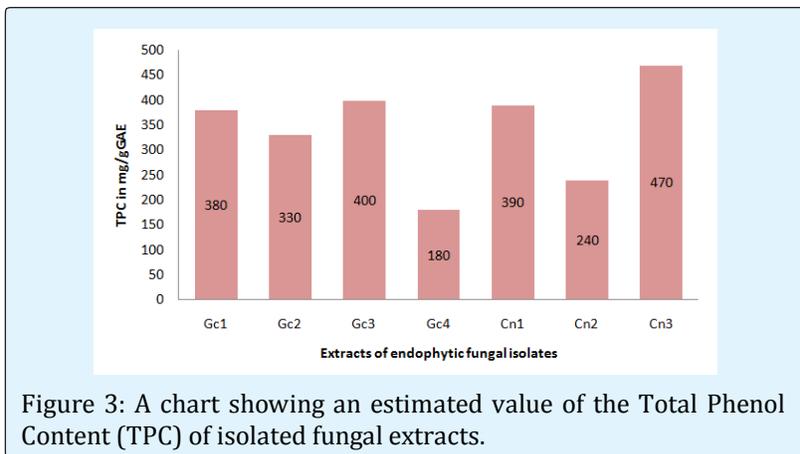


Figure 3: A chart showing an estimated value of the Total Phenolic Content (TPC) of isolated fungal extracts.

Discussion

Radical scavenging activity is very crucial to the survival of living organisms, since their presence results in several damages to the body systems. As earlier said, consumption of antioxidants, either as fruits or vegetables is useful for prevention and amelioration of some health conditions. Interestingly, extracts from fungal isolates of *G. Kola* and *C. nitida* demonstrated a marked radical scavenging activity.

Phenolic compounds possess antioxidant properties [15]. The highest scavenging activity was exhibited by Cn3 and it is interesting to observe that all the compounds identified in Cn3 are phenolic compounds. The reducing capacities of the extracts of Cn3 and Gc3 could as well be attributed to the principal compounds identified in the extracts by HPLC analysis. P-hydroxybenzoic acid, protocatechuic acid, and indole-3-acetic acid have all been reported to possess antioxidant effects [19,20].

IC₅₀ values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals [16]. From this study, we observed that as the concentration increases, the absorbance value also increases (Table 2). Similarly, using this same assay method, Ayebe, et al. [21] reported the presence of antioxidant compounds in *C. nitida*. A close look at the overall results of the antioxidant assay from both methods, show the extracts of the fungi Cn3 and Gc3 as the most potent of the

test samples. Though phenolic acid and its esters particularly p-hydroxybenzoic acid and protocatechuic acid possess good antioxidant effect [19], indole-3-acetic acid is also reported to scavenge free radicals [20].

Even though DPPH scavenging and reducing powers were based on different mechanisms of antioxidant assay, results from both methods did not show any difference when compared statistically. The similarity observed signifies results from both models are comparable. Furthermore, it provides more reason to believe the extracts truly possess antioxidant activity since it has been suggested that antioxidant activity should not be concluded based on a single test model [22].

It is widely accepted that the antioxidant activity of a plant extract is correlated to its phenolic content [23]. In the present study, strong correlations were observed between phenolic content and antioxidant activity.

Determination of Total Phenolic Content (TPC)

These findings are in line with previous works [19,24] that demonstrated a positive correlation between phenolic content and free radical scavenging activity. In addition, they agree with Ayebe, et al. [21], which attributed the antioxidant activity of *C. nitida* to the presence of phenolic acids. The high phenolic contents of extract from Cn3 are prime suspects likely to be responsible for the observed antioxidant activity.

As described previously, besides phenolic compounds, other compounds were identified in the fungal extracts, particularly in Gc3. Investigations revealed that those compounds possess different pharmacological properties. Definitely, the pharmacological effects of the fungal extracts are due to the varying phytoconstituents they contain. These observed pharmacological properties could be a consequence of phytoconstituents acting in isolation or in synergy with others. Conversely, the antioxidant activities demonstrated by the fungal extracts in this study can be regarded as moderately potent, when compared to other already known available high natural antioxidants. This may be due to the amount or purity of the actual active compounds in the various fungal extracts.

In conclusion, the results from these *in vitro* experiments demonstrated that active compounds in the endophytic fungal extracts from *C. nitida* and *G. kola* possess significant antioxidant activities. The secondary metabolites present in these endophytic fungi have potential for pharmaceutical and agrochemical applications. However, large-scale harvesting of these plants could untowardly affect climate change. Alternatively, endophytic fungi from these plant leaves could serve as an ecofriendly sustainable source of natural antioxidants for pharmaceutical industries. Furthermore, there may be need to conduct more investigation on these fungal extracts. These would involve compounds purification and characterization, as well as *in vivo* research techniques.

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