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ASSAY OF ANTIOXIDANT POTENTIAL OF TWO ENDOPHYTIC FUNGI ISOLATED FROM THE LEAVES OF *Psidium guajava*

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ABSTRACT
Free radicals in humans are the leading causes of several diseases such as diabetes, cancer, etc. Antioxidants are an interesting topic today because of their ability to protect the human body from attacks by several diseases caused by free radical reactions. The search for chemical compounds that have the potential to be antioxidants from nature continues, and the use of endophytic fungi from P. guajava leaves as a source of antioxidant compounds has not been widely explored. As such, this study was conducted to investigate the antioxidant activity of the endophytic fungal extracts isolated from the leaves of P. guajava. Endophytic fungi Alternaria spp.* (PG-2) and *Rhizoctonia spp.* (PG-10) isolated from healthy leaves of *P. guajava* were cultured in potato dextrose broth (PDB), tenellin production medium (TPM), and Rice medium (RM). The antioxidant activity of the crude extracts was observed using DPPH and ABTS assays. The result of the DPPH assay showed that the PG-2 extracts have the highest percentage inhibition (IC_{50} values: PDB-PG-2 16.28; TPM-PG-2 20.59; RM-PG-2 21.36 g/ml) than the PG-10 extracts (IC_{50} values: PDB-PG-10 118.22; TPM-PG-10 606.83; RM-PG-10 82.13 g/ml). The results were compared with three standards: ascorbic acid (IC_{50}: 1.41 g/ml); butylated hydroxytoluene (IC_{50}: 6.89 g/ml); and tannic acid (IC_{50}: 6.56 g/ml). The ABTS results obtained are expressed as Trolox equivalent antioxidant activity (TEAC) and Gallic acid equivalent (GAE). Both the extracts displayed a significant amount of Trolox and Gallic acid equivalence, which reflects their ability to donate hydrogen to scavenge radical formation. The results of the present study indicated that extracts with significant DPPH and ABTS activity are potential sources of natural antioxidants and pharmaceutical fungal-based products.
Keywords: Antioxidant, Radicals, Endophytes, Fungi, Guava.

INTRODUCTION

Among all the sources of secondary metabolites i.e., plants, animals and microorganisms, Fungi are an unsurpassed source of bioactive compounds and constitute a relevant economic resource for the pharmaceutical, cosmetic and food industry (Devi and Prabakaran, 2014).

Endophytic fungi are important components of plant micro-ecosystems which spend the whole or part of their lifecycle colonizing inter-and/or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease. Plant endophytic fungi have been recognized as an important and novel resource of natural bioactive products with potential applications in agriculture, medicine, and food industry (de Queiroz, *et al.* 2015; Ancheeva, *et al.,* 2020). Since the discovery of "gold" bioactive compound paclitaxel (Taxol) from the endophytic fungus *Taxomyces andreana*, the search for bioactive secondary metabolites from endophytic fungi became an innovative field of research. Several phenolic compounds have been isolated from fermentation products of several endophytic fungi with high throughput-screening establishing various biological activities of these classes of compounds. Also, analysis of the effects of the structure (substituents) of phenolic compounds on radical scavenging activities reveals a positive correlation (Ibrahim, *et al.,* 2019; Septiana, *et al.,* 2021).

Guava (*Psidium guajava* Linn.) Family Myrtaceae is important plant used traditionally for medicinal purposes. Guava is rich in antioxidants compounds and contains a high level of ascorbic acid. The pharmacological actions and the medicinal uses of aqueous extracts of guava leaves in folk medicine include the treatment of various type of gastrointestinal disturbances such as vomiting, diarrhoea, inhibition of the peristaltic reflex, gastroenteritis, spasmolytic activity, dysentery, abdominal distention, flatulence, and gastric pain (Miean and Mohamed, 2001).

Therefore, this study aimed to investigate the antioxidant activity of endophytic fungal extracts isolated from healthy leaves of *P. guajava* and to explore the diverse chemical constituents that may be present in the extracts.

MATERIALS AND METHODS

Plant Material

Fresh leaves of matured *Psidium guajava* were collected during winter season from the premises of Bayero University, Kano state, Nigeria. The plants were identified at the Department of Plant Biology Faculty of Life Sciences, Bayero University, Kano.

The samples were immediately transported to the tissue culture laboratory of Centre for Dry-land Agriculture, Bayero University for preparation and isolation of endophytic fungi. Samples were rinsed with tap water to remove debris, the cleaned leaves were sterilized by consecutive washes in 75% EtOH (1 min), 2.5% NaOCl (2 min) and 75% EtOH (30 s) and rinsed with sterile distilled water four times.

Isolation of Endophytic Fungi

The samples were dried in the laminar flow on a sterile filter paper, then cut into 1.0 × 1.0 cm pieces, and the tissues were deposited on a petri dish containing 20 mL potato dextrose agar (PDA) medium (39 g in 1000 mL with Streptomycin antibiotic) and incubated at 26 °C temperature for a maximum of 4 weeks. The emerged growing fungi from the plant sample inoculated were transferred to fresh sterile PDA plate to obtain a pure fungal isolate. The isolated fungi were identified using microscope at Department of Microbiology, Bayero University.

Fermentation of Fungal Isolates

The isolated fungal strains were fermented in two liquid culture media; potato dextrose broth (PDB), tenellin production medium (TPM) (Yakasai*.,* 2011), and one solid medium; rice medium (RM). To each 500 mL flask, 150 mL of the liquid media was added while 80 g in 150 mL of the solid media was used for the fermentation. The fermentation was done on a small scale i.e., one fermentation medium per fungus.

Extraction of Fungal Metabolites

The broth of the fermented liquid medium products was recovered by the addition of 500 mL ethyl acetate into each of the fermentation cultures, followed by homogenization and allowed to stay for 3 days. The mycelia and fermented rice were soaked in methanol for one week each and filtered using vacuum pump and concentrated at reduced pressure to yield the methanol extracts. The concentrated methanol extracts were further partitioned in water and ethyl acetate of equal ratio. The ethyl acetate extracts obtained were partition with brine solution and then dried with anhydrous magnesium sulphate ($MgSO_4$) and finally concentrated at reduced pressure to obtain the fungal extract. The extracts obtained were analysed for their antioxidant activity using DPPH and ABTS radical scavenging assays.

Molecular Diversity of the Extracts

The endophytic fungal extracts were subjected to HPLC analysis to study the molecular diversity of each extract of the fungi and its reproducibility. Each extract was dissolved in MeOH (1 mg/mL) and the supernatant was analysed by HPLC using an Agilent HPLC system sampling from Agilent 1200 auto-sampler. Detection was achieved by UV between 220 and 360 nm using Agilent 1200 diode array detector. The chromatogram of all the extracts were obtained using a high resolution Kinetex column (5 µm, C18, 100 A°, 10 × 250 mm) equipped with a Kinetex Security Guard pre-column. A 15 min acetonitrile method was used in achieving the chromatography of the samples which involves a gradient of solvents described in Table 1.

Table 1: HPLC solvent system/method development

Time (min)	A (%)	B (%)	C (%)	D (%)	Flow (mL/min)	Max Pressure (bar)
0.00	35.00	0.00	65.00	0.00	1.00	570.00
10.00	10.00	0.00	90.00	0.00	1.00	570.00
12.00	5.00	0.00	95.00	0.00	1.00	570.00
15.00	95.00	0.00	5.00	0.00	1.00	570.00

Key: A; HPLC grade H_2O containing 0.05 % tetrafluoro acetic acid **B**; HPLC grade CH_3CN in 0.04 % tetrafluoro acetic acid

DPPH Radical Scavenging Assay

The free radical scavenging activity of the plant extracts against 2,2-diphe-nyl-1-picrylhydrazyl (DPPH) radical was determined according to the method described by de Oliveira and his co-workers, with slight modification (Ibrahim, *et al.,* 2019; Okezie, *et al.,* 2020). The stock solutions of both standard control (i.e., ascorbic acid (AA) and butylated hydroxytoluene (BHT)) and each sample were prepared (2.0 mg/mL) and diluted to final concentration of 1000, 500, 250, 125, 31.3, 15.63 and 7.82 µg/mL. 100 µM DPPH was also prepared by diluting 1 mg of the DPPH in 50 mL of methanol. 40 µL of the standards and samples were added to the microplate and their absorbance was measured at 517 nm. Then, a total of 160 µL of the DPPH was added to positive controls and sample solutions and allowed to react at room temperature for 30 min in dark. The absorbance of the mixtures and that of blank DPPH were measured at 517 nm. A sample is considered active if its reaction changes the colour of the DPPH from purple to yellow. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity, and vice versa. Inhibitions of DPPH radical in percent (I%) were calculated using the formula:

$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$

ABTS Radical Scavenging Assay

In addition to DPPH radical scavenging assay, ABTS assay in employed to fully characterize the antioxidant properties of the extracts ability to scavenge the 2,2'-azino-bis (ethylbenzthiazoline-6-sulfonic acid ABTS radical cation ($ABTS^{\cdot+}$)$\cdot$ The assay is based on the ability of an antioxidant compound to quench the ABTS+ relative to that of a reference antioxidant such as Trolox/Gallic acid. The radical cation was prepared by mixing 7 mM $ABTS^{\cdot+}$ solution with 2.45 mM potassium persulfate (1/1, v/v) and leaving the mixture for up to 16 hrs for a complete reaction. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of approximately 0.700 ± 0.050 for the assay measurements. The working ABTS+ solution was produced by dilution in 10 mM PBS (pH 7.4) incubated at 30 °C of the stock solution to achieve an absorbance value of 0.7 (±0.02) at 734 nm.

An aliquot of 20 µL of diluted extract was added to ABTS+ working solution (180 µL). For the blank and standard curve, 20µL of PBS or Trolox solution were used, respectively.

Absorbance (for both DPPH and ABTS assays) was measured by means of a UV–vis spectrophotometer (Thermo Scientific *Multiskan Go*) at 734 nm. The photometric assay was conducted by 0.9 mL of $ABTS^{\cdot+}$ solution with 0.1 mL of tested samples (Shalaby, *et al.,* 2013). A standard reference curve was built by plotting absorbance value against Trolox/Gallic acid concentrations (31.2–1000µg/ml). The equations from the graph were used to calculate Trolox/Gallic acid equivalence from the samples and were expressed as mg of standards equivalence per gram of the samples tested.

Statistical Analysis

All the photometric/colorimetric data obtained were reported as mean±SD (Standard deviation) and were compared with each other; one-way analysis of variance (ANOVA) was performed using SPSS software.

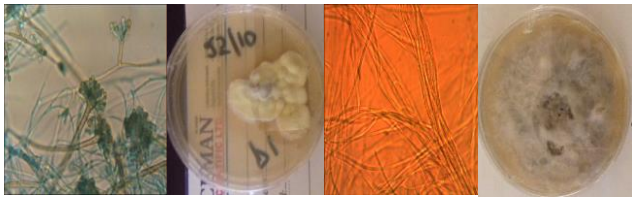
RESULTS AND DISCUSSION

Isolation of Endophytic Fungi

The results of the isolation of endophytic fungi from *P. guajava* leaves obtained a total of 2 isolates, coded PG-2 and PG-10 (Figure 1). Classification of isolate codes based on host type and emergence during the isolation process. The microscopic morphological characters were involved in the identification of the fungal strain including the shape of colony growth and spores. The isolates were then prepared for fermentation in different culture medium.

Fermentation and Extraction of Endophytic Fungi

Two endophytic fungal isolates (**PG-2** and **PG-10**) yield of the extracts (fermentation product) recovered was observed to vary from one medium to another. The rice medium of both PG-2 and Pg-10 were observed to produce the highest content of secondary metabolites (390.7 and 267.1 mg respectively) while the TPM extracts produced the least contents (85.5 and 26.3 mg) (Table 2).



PG-2 PG-10

Figure 1: Endophytic Fungal Isolates obtained from the Leaves of *P. guajava.*

Table 2: Identification of Endophytic Fungi and weight (mg) of their extracts

S/N	Sample ID	Fungal Strain	Wt of Extract in PDB	Wt of Extract in TPM	Wt of Extract in RM
1	PG-2	*Alternaria* spp.	155.7	85.5	390.7
2	PG-10	*Rhizoctonia spp.*	87.9	26.3	267.1

Key: PG= *Psidium guajava*; Wt= weight; PDB= potato dextrose broth; TPM= tenellin production media; RM= rice media

Molecular Diversity of the Extracts

Detection of the bioactive compounds produced by the fungi isolates in this work was achieved using HPLC analysis. The detected compounds are presented in Figure 2. Each Peak at a certain retention time in the chromatogram represent a particular compound. These compounds are largely responsible for the biological activity of the extracts.

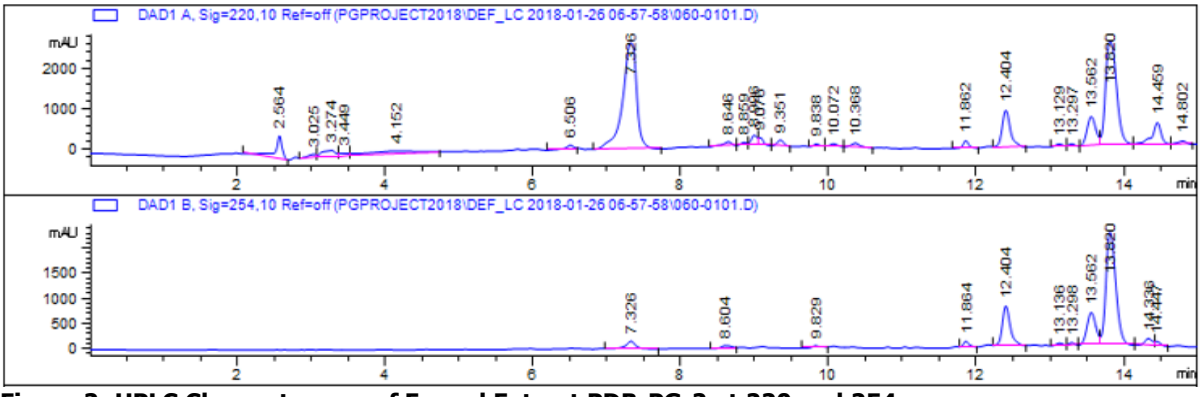


Figure 2: HPLC Chromatogram of Fungal Extract PDB-PG-2 at 220 and 254 nm

Antioxidant Activity of the Extracts

DPPH Scavenging Activity

The antioxidant activities demonstrated by the fungal extracts in this study were observed to be concentration dependent (i.e. higher concentration leads to higher antioxidant percentage inhibition).

Table 3: IC_{50} values of the DPPH antioxidant activity

Sample	AA	BHT	TA	TPM-PG-2	PDB-PG-2	RM-PG-2	TPM-PG-10	RM-PG-10	PDB-PG-10
IC_{50} (µg/mL)	1.41±0.013	6.89±0.021	6.56±0.03	20.59±0.01	16.28±0.02	21.36±0.03	606.83±0.03	82.13±0.02	118.22±0.01

The fungal extract PG-2 and displayed the highest inhibition activity (TPM-PG-2: 94.22, 91.02, 90.66, 87.22, 83.60, 80.15, 79.75, 66.89; PDB-PG-2: 86.65, 85.47, 78.17, 65.05, 54.46, 47.80, 43.90; RM-PG-2: 90.20, 87.47, 83.14, 72.61, 61.22, 51.79, 44.11, 41.09) at 1000, 500, 250, 125, 62.5, 31.3, 15.63 and 7.82 µg/mL respectively (Figures 3). The strength of antioxidant activity can be grouped into very active categories if they have IC_{50} 100 µg/ml (Septiana, *et al.,* 2021). From the test results, it can be seen that the antioxidant activity of all endophytic fungi extracts is in the active category except TPM-PG-10 and PDB-PG-10 because IC_{50} value is higher than 100 µg/ml (Table 3). Although the activities demonstrated by these extracts can be said to be comparable to one another, but moderate when compared with the activity of the standards (AA: 1.41; BHT: 6.89; TA: 6.56 µg/ml). Scheme 1 shows how the DPPH radical scavenging occurs *via* chemical reaction.

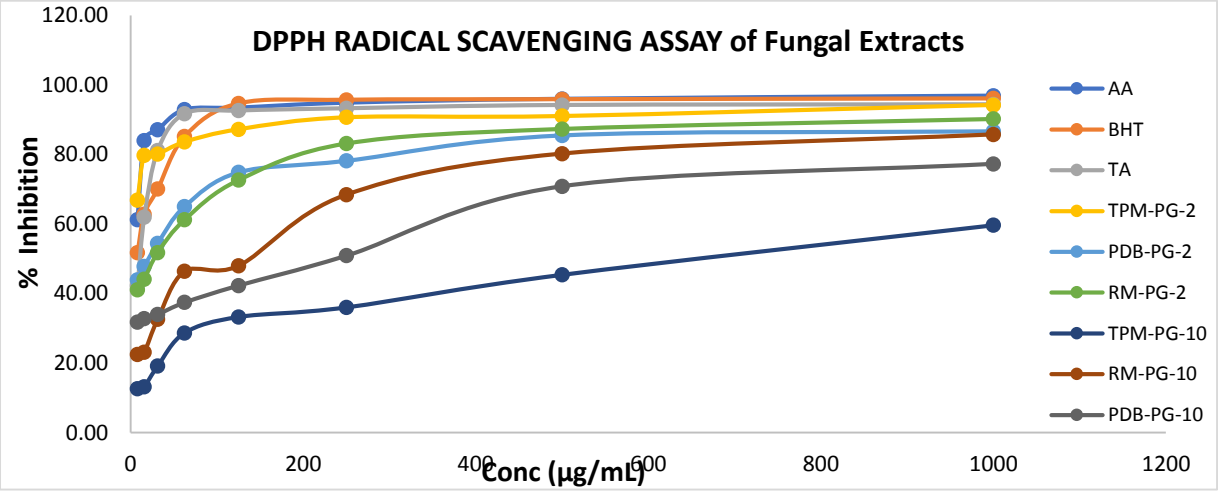
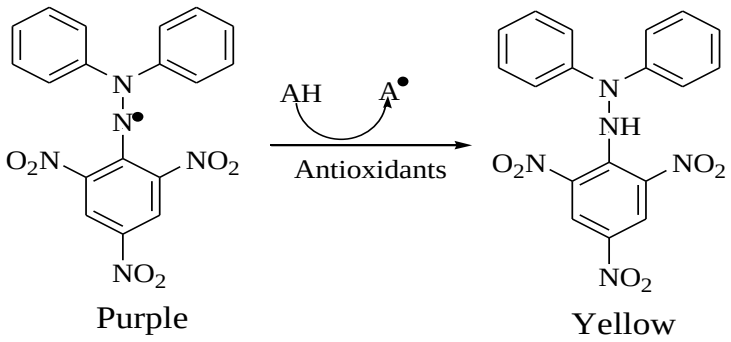


Figure 3: % Inhibition of DPPH radical scavenging Activity of Fungal Extracts



Scheme 1: DPPH radical scavenging assay reaction.

ABTS Scavenging Activity

The antioxidant capacity ($ABTS^+$ radical scavenging activity) of the fungal isolates is presented in Table 4. Using ABTS radical scavenging method, for the determination of antiradical and antioxidant activities, the ABTS assay measures the relative ability of antioxidant to scavenge the ABTS generated in aqueous phase as compared with a water-soluble vitamin E analogue (Trolox) and gallic acid standards (Shalaby, *et al.,* 2013). Higher contents of Trolox and gallic acid in the extract is an indication of more antiradical scavengers in the extract. PDB-PG-2 and RM-PG-10 showed the highest equivalence of Trolox and gallic acid (305.77 mg TE/g; 368.13 mg GAE/g and 395.83 mg TE/g; 520.58 mg GAE/g respectively). Other extracts also showed significant equivalent of trolox and gallic acid which is consistent with the antioxidant acitvity recorded. The mechanism of the ABTS assay involves the reduction of blue-green ABTS radical colored solution by hydrogen donating antioxidant (Scheme 2).

Table 4: ABTS Assay of Fractions

Extract	TEAC (mg TE/g)	GAE (mg GAE/g)
PDB-PG-2	305.77 ± 0.21	368.13 ± 0.16
TPM-PG-2	107.42 ± 0.03	169.79 ± 0.03
RM-PG-2	218.50 ± 0.03	260.08 ± 0.04
PDB-PG-10	289.75 ± 0.03	331.33 ± 0.03
TPM-PG-10	198.17 ± 0.21	334.21 ± 0.16
RM-PG-10	395.83 ± 0.03	520.58 ± 0.04

Scheme 2: ABTS radical scavenging assay reaction.

CONCLUSION

Two endophytic fungi PG-2 and PG-10 isolated from fresh-healthy leaves of *Psidium guajava* which were identified to be *Alternaria spp.* and *Rhizoctonia spp.* were fermented in three different culture media (PDB, TPM, and RM). The fermentation extracts obtained were subjected to molecular diversity analysis using HPLC, and antioxidant activity study using DPPH and ABTS radical scavenging assays. The results obtained further validates the fact that endophytic are reliable source of bioactive compounds with interesting chemo-diversity that can be utilised as single strain with an excellent prospect for discovery and development of novel effective bioactive drugs. These results could justify the use of these plants in traditional pharmacopoeia practice, thus further investigation on bioactive antioxidant compounds of endophytic fungi is important. Ethnomedicinal plants in the northern region of Nigeria have not widely been investigated for molecular diversity studies of bioactive metabolites from their endophytic fungi. The results further suggest that apart from the actions of endophytic fungi to liberate antioxidant compounds from plant cell walls, endophytic fungi themselves could be sources of compounds associated with antioxidant activity *via* their own biosynthetic process.

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