International Journal of Research Studies in Biosciences (IJRSB)
Volume 8, Issue 8, 2020, PP 48-56
ISSN No. (Online) 2349-0365
DOI: https://doi.org/10.20431/2349-0365.0808005
www.arcjournals.org

Antioxidant Activities of Extracts of Endophytic Fungi Isolated from Healthy Leaves of *Carica Papaya*

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Abstract: *The importance of the availability of good antioxidants for human health and the associated health challenges as a result of its deficiency calls for a search for newer bioactive compounds with the potentials to scavenge free radicals thus preventing oxidative stress. In this study, the antioxidant activities of some endophytic fungal extracts CP1, CP2, CP3, CP4, CP5, CP6 and CP7 were determined using DPPH radical scavenging assay and their respective percentage inhibition recorded. Also, the active constituents were detected using HPLC analysis. At a maximum concentration of 100 µg/mL, The fungal extracts Cp1, Cp5, Cp2 and Cp7 produced 54.7 % ± 3.2, 51.3 % ± 3.5, 51.0 % ± 2.0, and 50.0 % ± 3.6 inhibition of free radicals; IC50 85 ± 1.5, 97.5 ± 3.5, 98 ± 2.0 and 100 ± 3.6 respectively. These activities were observed to be moderate when compared to that of the standard ascorbic acid 75.0 ± 2.6 %. Four bioactive compounds beauvericin, p-methoxycoumarin, indol-3-carbaldehyde and pavetannin A2 were detected in the crude extract (fermentation product) of endophytic fungi isolated in this work. Based on their chemical structure having multiple hydroxyl groups and other substitutes, these compounds are suggested as possible lead compounds for the development of antioxidant drugs.*

Keywords: *Antioxidant, Phenolic compounds, Carica papaya, Endophytic fungi*

1. INTRODUCTION

Increased inflammation coupled with certain disorders and neurological diseases has been linked to oxidative stress. Several authors have confirmed this to be the result when reactive oxygen species attack biomolecules disrupting the balance between the formation and reduction of these reactive oxygen species (Xican *et al*., 2011; Igor *et al*., 2017). Administration of antioxidants is important in maintaining this balance and inhibiting the attack by these moieties (Marja P. Ka¨hko¨nen., 1999). Synthetic antioxidants used for this purpose have been reported to be toxic to the liver and this has triggered the search for a newer agent with good antioxidant capacities and at the same time not toxic (Xican *et al*., 2011).

Phenolic compounds such as phenolic acids, flavonoids, tannins have been observed to be the principal phytoconstituents produced by plants with good radical scavenging activities. They function by either preventing the formation of the free radicals or by inhibiting them before they can damage the cellular components (Igor *et al*., 2017).

Endophytic fungi which harbor internal tissues of plants have been shown to possess unprecedented chemodiversity and represent a reliable source of lead compounds needed for the development of antioxidant, antimicrobial, anti-inflammatory, anticancer agents, etc. (Vinton *et al*; 2001; Cannon, 2002; Filip *et al*; 2003; John *et al*., 2018; Okoye *et al*., 2015 & 2013; Okezie *et al*., 2017 & Okezie *et al*., 2015). 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 ofthe effects of the structure (substituents) of phenolic compounds on radical scavenging activities reveals a positive correlation (Igor *et al*., 2017).

Carica papaya belongs to the family of *Caricaceae* commonly called pawpaw (English), Ibebe (Yoruba– Nigeria), or Okroegbe (Igbo–Nigeria). Several species of *Caricaceae* have been used as

aremedy against a variety of infections (Alabi *et al*., 2012). *C. papaya* is a nutraceutical plant rich in vitamins and also having a wide range of pharmacological activities (Aravind *et al.*, 2013).Bioactive (phenolic) compounds such as protocatechuic acid, p-coumaric acid and caffeic acid have been detected in its leaf extract (Canini *et al*., 2007). Recent data gathered from previous studies targeted at identifying and isolating bioactive antioxidant agents reveals endophytic fungi as a promising and reliable source of antioxidants due to their untapped chemodiversity (John *et al.,* 2018). This study was therefore designed to investigate the antioxidant activity of endophytic fungal extracts isolated from healthy leaves of *C. papaya* and to determine the chemical constituents that may be present in the extracts. This formed the basis upon which this study was conducted.

2. MATERIALS AND METHODS

2.1. Plant Material

C papaya leaves were collected from farmland located in Nneogidi village in Agulu, Anambra State, Nigeria and were immediately transferred in a polythene bag. They were identified by a botanist Mrs. Emezie Anthonia of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

2.2. Isolation and Purification of Endophytic Fungi

All the harvested leaves were subjected to appropriate disinfection processes before culturing. The leaves were thoroughly washed in running tap water, followed by washing with sterile double distilled water to remove particles of dust. To eliminate epiphytic microorganisms, all the samples were further immersed in 70% ethanol and 2% sodium hypochlorite solution for 3 and 5 min respectively, before a final rinse in sterilized double-distilled water. The samples were dried in the laminar flow on a sterile filter paper (blotting). After the disinfection processes were carried out, a sterile knife blade was used to cut the samples to approximately 2 cm in length. Then the segments were inoculated into the previously sterilized agar fortified with chloramphenicol 500 mg/L using sterile forceps and applying the minimum amount of pressure. The Petri dishes were properly sealed using parafilm, then incubated at 25^{o}C in the incubator for 5-7 days, while observations for the emergence of hypha were done on alternate days. During the incubation period, emerged hyphal tips of actively growing fungi from the plant material inoculated were then isolated and transferred to fresh sterile MEA plate (purification) and were incubated appropriately (Okezie *et al*., 2017). Seven pure isolates were subjected to fermentation.

2.3. Fermentation of Pure Isolates

Local rice served as the fermentation medium. Here, 100 g of the local rice was weighed into a sterile conical flask and 200 mL of sterile water added and sterilized appropriately at 121^{o}C for 30 mins. After sterilization, the mediums were then allowed to cool properly. The segments were aseptically cut using a sterile spatula from the actively growing pure isolates on MEA and transferred into the fermentation medium contained in a 1000-mL Erlenmeyer flask, this was properly sealed and kept on the shelf for a maximum of 21 days at 30^{o}C under static conditions (Okezie *et al*., 2017).The fermentation was done on a small scale i.e. one fermentation medium per fungus.

2.4. Extraction of Fungal Metabolites

The fermentation products were recovered by the addition of 500 mL ethyl acetate into each of the fermentation cultures, followed by homogenization using a sterile glass rod. The flask was agitated at an interval of 1 hr for 2 days and then filtered using Whatman filter paper (size: 188 mm)**.** The filtrates were concentrated at 50^{o}C under reduced pressure using a rotary evaporator. The concentrated extract was further left to evaporate to dryness in a desiccator containing sodium hydroxide (Okezie *et al*., 2017).

After evaporation, the corresponding extracts were weighed and their respective percentage yieldswere recorded in milligram. In other to test for biological activities, the dried fungal extracts were reconstituted in dimethyl sulphoxide (DMSO).

2.5. Analytical HPLC of the Extracts

This was carried out according to the methods of Ajaegbu *et al.*, 2016 & Ajaegbu *et al.*, 2020. This was carried out with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S,

Dionex Softron GmbH, Germering, Germany) at different lambda max (235, 254, 280 and 340 nm). Each sample (fraction) was dissolved using 2 ml of HPLC grade methanol, and 100 µl of the dissolved samples were each transferred into the vials of HPLC. The separation column

(125 x 0.4 cm; length x internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of Nano pure water (adjusted to pH 2 by addition of formic acid) using methanol as the eluent. The absorption peaks for the fractions were analyzed by comparing it with those in the HPLCUV/Visible library.

2.6. Antioxidant Propertiesof the Extracts

The free radical scavenging potentials of the endophytic fungal extracts were carried out as described by Chigozie*et al*., 2020, with some modifications. The free radical scavenging properties of the extracts against 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical were measured at 490 nm. The concentrations of the extracts and ascorbic acid used were 20, 40, 60, 80, and 100 µg/mL The reaction mixture consists of 25 µl of the stock, 25 µl of DPPH (0.1 mol/L) and 150 µl of methanol solution. These were added into their respective wells in the microtiter. The plate was incubated at 27^{o}C for 30 min**.**The absorbance of the mixtures was measured at 490 nm using a UV-vis spectrophotometer (06452; USA). The experiment was done in triplicate for each fungal extract.

Free radical scavenging activities were expressed as the percentage inhibition of each extract and calculated using the formula: [(Ao – A_1)/ Ao] * 100; Ao is the absorbance of the blank solution and $A_1$is the absorbance of the positive control.

2.7. Statistical Analysis

The results are expressed as mean ± standard deviation. Analysis of variance (one-way ANOVA) was used to check the significant mean difference and was achieved using SPSS 20. The measures were done in triplicate (n = 3). The obtained results were considered significant at $P \leq 0.05$.

3. RESULTS

3.1. Isolation and Extraction

On completion of the isolation and purification processes of the endophytic fungi from the plant part under study, 7 fungal isolates (Cp1-Cp7) were recovered. The percentage yield of the extracts (fermentation product) recovered was observed to vary from one fungus to another. Cp2 was observed to have produced the highest amount of secondary metabolites (126.6 mg) small-scaled fermentation under static conditions, while Cp7 produced the least amount (70.1 mg) (Table 1).

Table1. *Yield of extracts of the isolated endophytic fungi*

Fungal extract	Yield (mg)
Cp1	71.1
Cp2	126.6
Cp3	78.2
Cp4	84.4
Cp5	98.9
Cp6	124.9
Cp7	70.1

Cp: *Carica papaya*

3.2. HPLC Analysis

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 and Table2. These compounds have been reported by several authors to exhibit varying biological activities.

Include the HPLC chromatogram here

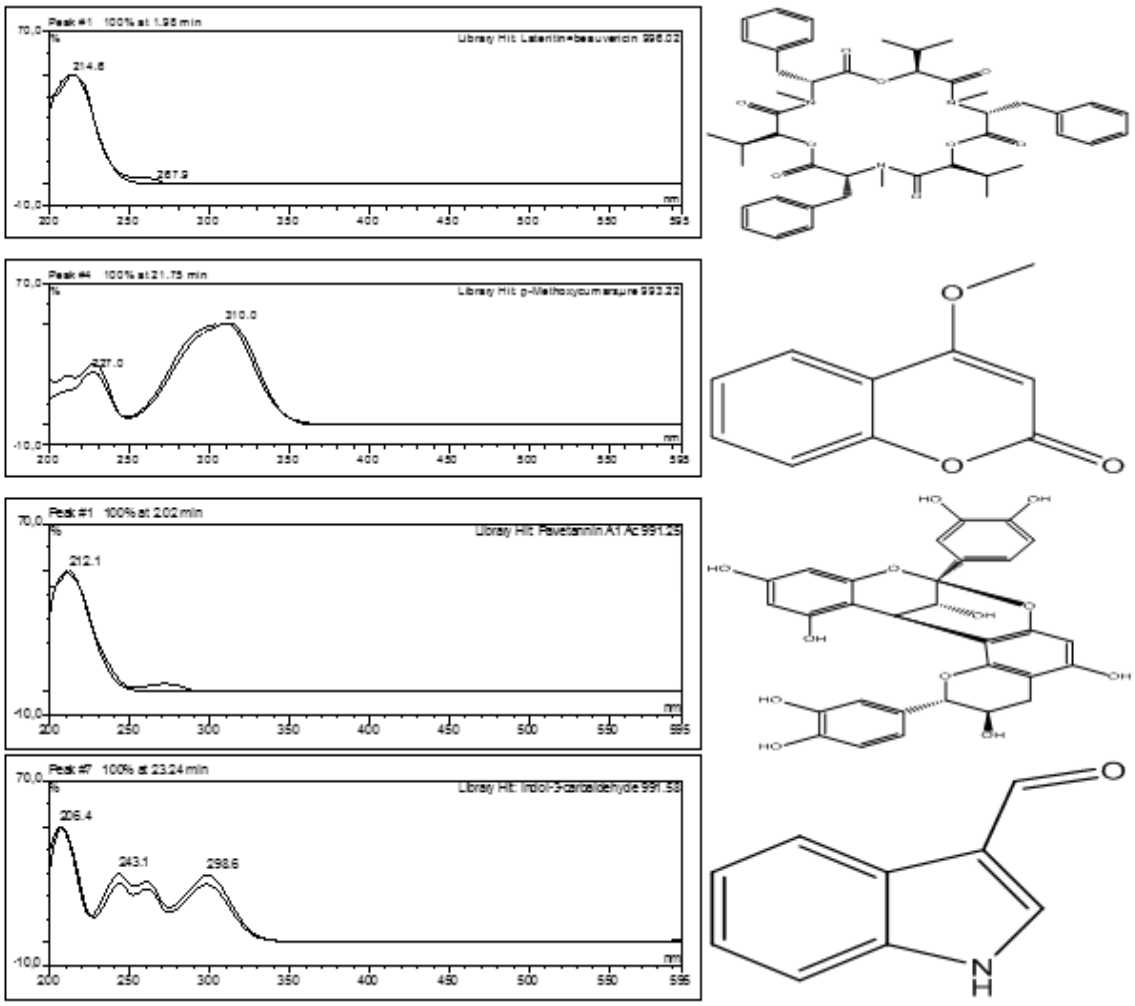


Figure2. ***HPLC chromatogram of the detected compounds showing*** *beauvericin, p-methoxycoumarin, pavetannin A2 and Indole carboxaldehyde****; their UV spectra; and structures***

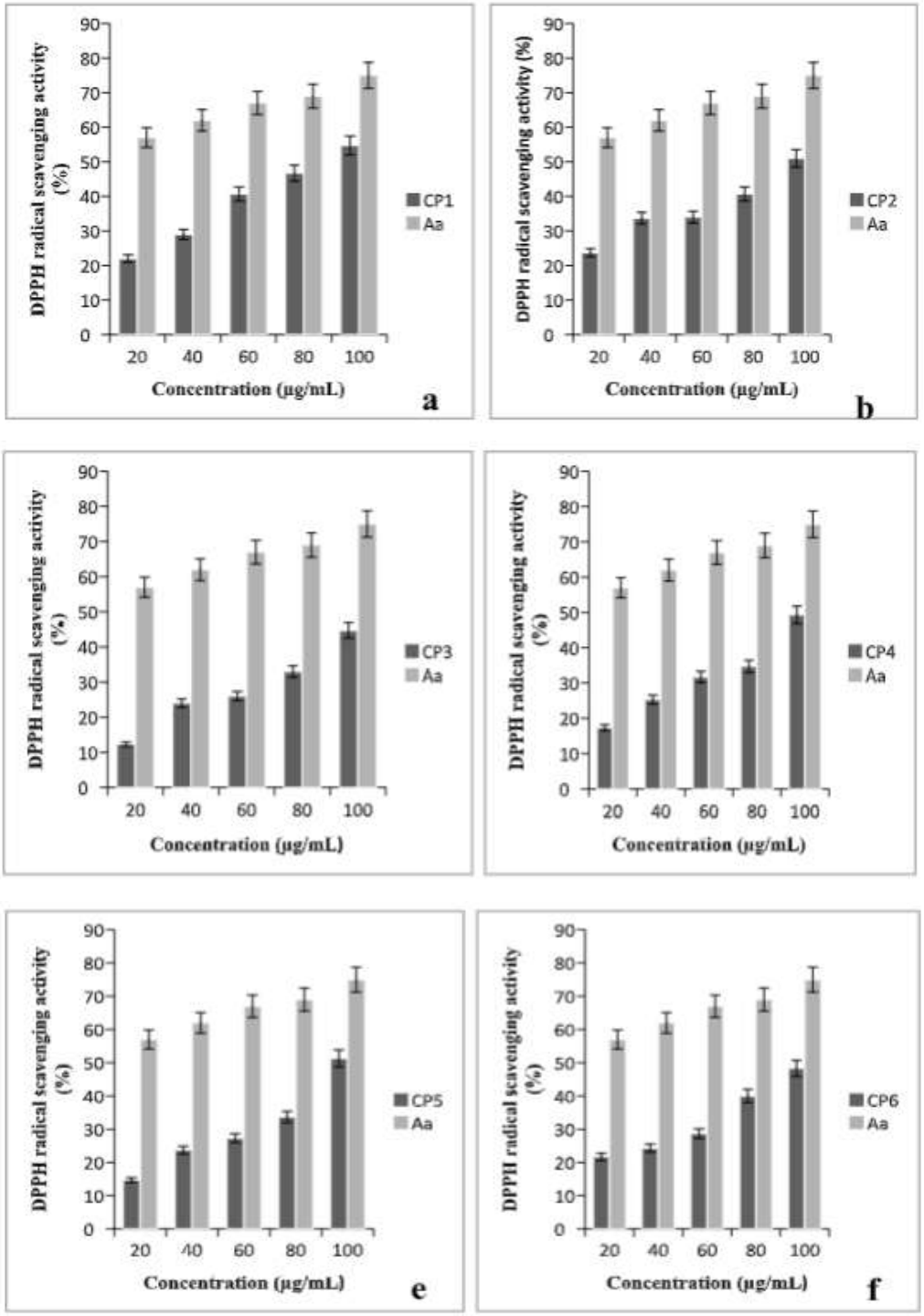
Table2. *The wavelength for maximum absorbance λ_{max} and retention time of the phytochemical compounds detected by HPLC-DADS for all the fractions of methanol extract*

Compound	**Class of phytochemical**	**Molecular Formulae**	**Molecular Weight**	**Rt (min)**	**λ_{max} (nm)**	**Biological activity**
Beauvericin	Depsipeptide	$C_{45}H_{57}N_3O_9$	783.96 g/mol	1.98	214.6, 267.9	Antibacterial, (Xu *et al*., 2010; Antimalaria, antituberculosis, cytotoxicity, (Isaka *et al*., 2011); anti-hepatoma, (Wang *et al*., 2014)
p-methoxycoumarin	Terpene lactones	$C_{10}H_8O_3$	176.17 g/mol	21.75	227.0, 310.0	Antioxidant, anti-inflammatory, antimicrobial, anticancer, (Mohammad Asif, 2015)
Pavetannin A2	Flavonoids	$C_{30}H_{24}O_{12}$	576.51 g/mol	2.02	212.1	Antioxidant, (Ramasamy *et al*., 2013)
Indole carboxaldehyde	alkaloid	C_9H_7NO	145.05 g/mol	23.24	206.4, 243.1, 298.6	

3.3. DPPH Scavenging Activity

The ability of an extract to scavenge free radicals (DPPH) has been widely adopted in measuring the antioxidant potentials of plant and microbial extracts as well as chemically synthesized samples. This is attributed to its stable nature (Nabil, *et al*., 2014; Adél, *et al.*, 2019).

The antioxidant activities demonstrated by the fungal extracts in this study was observed to be concentration dependent (**CP1:** 54.7 ± 3.2, 46.7 ± 1.5, 40.7 ± 2.1, 29.0 ± 5.6, and 22.0 ± 2.0; **CP5**: 51.3 ± 3.5, 33.7 ± 5.5, 27.3 ± 1.5, 23.7 ± 2.9, and 14.7 ± 2.9) at 100, 80, 60, 40, 20 µg/mL respectively. **Figures 3 (a-g)**. Although the activities demonstrated by these extracts can be said to be comparable to one another, but moderate when compared with the activity (AA: 75.0 ± 2.6; 69.0 ± 1.0, 67.0 ± 1.0, 62.0 ± 2.0, and 57.0 ± 0.0) of the standard ascorbic acid at 100, 80, 60, 40, 20 µg/mL. The DPPH radical scavenging activity of the fungal extracts was calculated and presented in **Figures 3(a-g).**



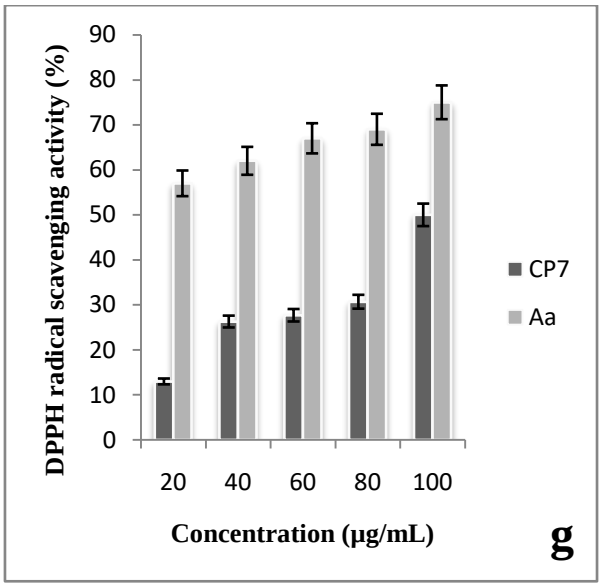


Figure3 (a-g). *Result of the Percentage inhibition (%) Produced by the endophytic fungi extracts and the standard Ascorbic acid, (n = 3) at p < 0.05.*

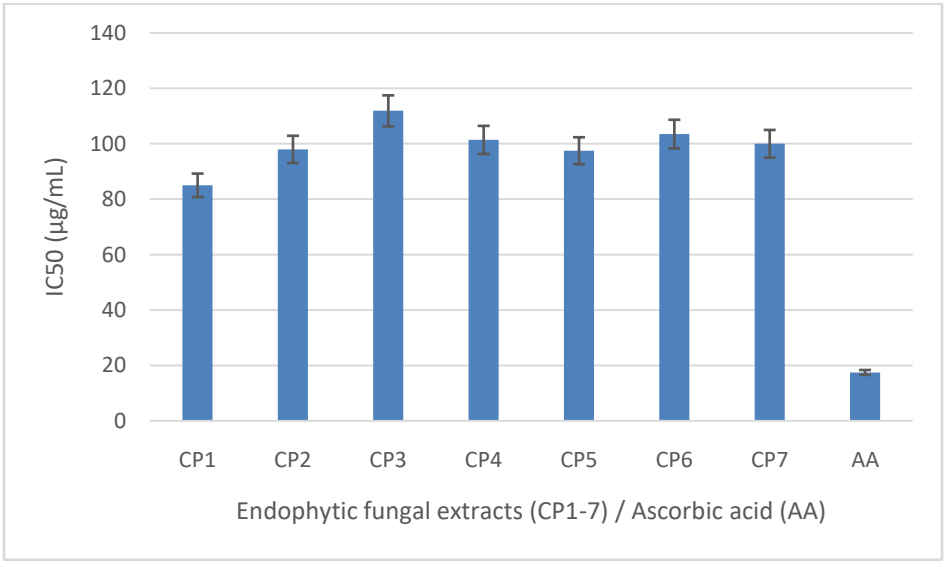


Figure4. *Results of IC50*

4. DISCUSSION

The free radical scavenging activity (RSA) of the endophytic fungal extracts at different concentrations (20, 40, 60, 80 and 100 μg/mL) was carried out in the presence of a freshly prepared solution of stable free radical DPPH (0.1 mol/L) and compared with ascorbic acid as standard. DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine; this gives rise to the deep violet colour (Mensor *et al*., 2001). We evaluated the scavenging effects of the endophytic fungal crude extracts on the DPPH radical. The activities of the fungal extracts were compared with that of the standard ascorbic acid. The results suggested that Cp1, Cp5, Cp2 and Cp7 fungal extracts exhibited moderate radical scavenging activities at 100 µg/mL, demonstrating an inhibition that was observed to be concentration dependent Figure 3 (a-g). At a maximum concentration of 100 µg/mL, fungal extracts Cp1, Cp5, Cp2 and Cp7 exhibited higher radical scavenging activities 54.7 ± 3.2%, 51.3 ± 3.5%, 51.0± 2.0%, and 50.0± 3.6%, IC_{50} 85 ± 1.5, 97.5 ± 3.5, 98 ± 2.0 and 100 ± 3.6 respectively, [figure-3(a-g); figure-4], than other fungal extracts. These were the most effective fungal extracts whose antioxidant activities were observed to be moderate when compared to that of the standard ascorbic acid 75.0 ± 2.6%. However, Cp3 and Cp4 did not inhibit radical scavenging capacity. The radical scavenging activities by the active fungal extracts may be due to the presence of bioactive compounds as detected by HPLC analysis.

The HPLC analysis of the fungal extract revealed the presence of important bioactive compounds with various biological activities including antimicrobial, cytotoxic, antiapoptotic, antimalarial, anti-hepatoma, (Xu *et al.*, 2010; Isako *et al.*, 2011; Wang *et al.*, 2014). Therefore, the antioxidant activities demonstrated by the endophytic fungal extracts of *C. papaya* may be attributed to the combined effects of these bioactive compounds belonging to various classes (polyketides, phenols, alkaloids, terpenes, isoprenoids) detected in the extracts such as beauvericin, p-methoxycoumarin,

pavetannin A2 and indol-3-carboxaldehyde detected in Cp1, Cp2, Cp5 and Cp7 respectively. A comparison of the radical scavenging activities exhibited by the active fungal extracts and ascorbic acid in part is because the crude extracts may contain other classes of compounds that may be causing the antagonistic effect. However, isolation and testing of these bioactive compounds may show better activities. In this work, phenolic compounds (such as beauvericin, p-methoxycoumarin, pavetannin A2 and indol-3-carboxaldehyde) were detected in the active fungal extracts. Phenolic compounds are known for their good antioxidant capacities and also radical scavenging potentials is linked to both their phenolic rings and hydroxyl groups that combine to exhibit the antioxidative effect, and this is in accordance with previous studies Bahri-Sahloul *et al*., (2009); Djeridane *et al.*, (2006), who demonstrated a significant correlation between phenolic composition and antioxidant activity. Djeridane *et al*., (2006) demonstrated also that coefficient correlation is respectively equal to 0.79 and 0.78 between antioxidant activity and total phenolic and flavonoids composition of Algerian plant extracts. Iwalokun *et al.,* (2006) attributed the antioxidant activity to the presence of phenolic acids. Jung *et al*., (2002) reported that 4-hydroxyphenylacetic acid possesses radical scavenging abilities.

Indol-3-carboxaldehyde an indole alkaloid detected in Cp7 fungal extract indicates that the antioxidant potential demonstrated by the extracts may be due to the presence of both indole alkaloid and phenolic compound present in the extracts. These phytochemicals have been established to have very good antioxidant potentials (Adrianna *et al*., 2009; Ebenezer *et al*., 2011; Romasi *et al.*, 2011). Although no report on the antioxidant activities of beauvericin and indol-3-carboxaldehyde exist rather antimicrobial activities nevertheless, several authors associated antimicrobial activity with the antioxidant potential.

Finally, this study showed that the antioxidant activity of the extracts of the endophytic fungi isolated from*C. papaya* leaves was higher at the highest concentration (100 µg/mL) with Cp1demonstrating the highest antioxidant activity, but lower than the standard ascorbic acid and thus cannot be compared favorably with the standard antioxidant ascorbic acid. Thus further isolation and purification using advanced chromatographic separation techniques of the fungal crude extract may yield pure lead compounds that can be used in the development of antioxidant drugs.

5. CONCLUSION

Going by the vast amount of data available on bio-prospecting microorganism especially endophytic fungi for newer antioxidant agents, this work further validates the fact that these arereliable source of bioactive (lead compounds) molecules with greater intrinsic chemodiversity capable of being expressed by a single strain of an organism with a good prospect for discovery and development of newer effective drugs. Also, this is the first report on the detection ofbeauvericin biosynthesized by an endophytic fungus isolated from *C. papaya* leaves.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.

ACKNOWLEDGMENTS

We sincerely appreciate Institut für Pharmazeutische Biologie und Biotechnologie, Heinrinch-Heine-Universität, Düsseldorf, Gernany were the HPLC analysis was carried out.

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Citation: *U.M. Okezie, et al., "Antioxidant Activities of Extracts of Endophytic Fungi Isolated from Healthy Leaves of Carica Papaya. International Journal of Research Studies in Biosciences". 2020; 8(8): 48-56. DOI:https://doi.org/10.20431/2349-0365.0808005.*