International Journal of Biological Research, 5 (1) (2017) 15-21

International Journal of Biological Research

Website: www.sciencepubco.com/index.php/IJBR
doi: 10.14419/ijbr.v5i1.6069
Research paper

Bioprospective potentials of endophytic fungi penicillium SPP isolated from leaves of azadirachta indica (A. JUSS)

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Abstract

The focus of this research work was to isolates and identify endophytic fungi from young leaves and stems (twig) of Azadirachta indica, family (Meliaceae) for the production and assay of the produced secondary metabolites. Altogether 126 segments were used, of which 63 segments each from leaves and stems (twig) tissues were screened, using modified surface sterilization techniques. A total of 12 species of endophytic fungi were purely isolated, Penicillium spp was randomly selected for the extraction and evaluation of its secondary metabolites. The eluent collected from column chromatography mixture of ethylacetate and n- hexane (50:50v/v) was phytochemically screened, and the results showed the presence of saponins, flavonoids and phenols. Based on disc diffusion method of sensitivity, the eluent possessed some degree of antibacterial and antifungal activities. In addition, in-vitro antioxidant potentiality of the eluent was also evaluated using 2, 2- Diphenyl-1-picrylhydrazyl radical (DPPH, Sigma Aldrich). Statistical package for social science (SPSS 3/93) software was used to analyze the results using one way Analysis of Variance (ANOVA), which revealed no significant difference, on the effect of concentrations of eluent on test organisms but the eluent showed significant difference on scavenging free radicals at a critical value ($p > 0.05$).

Keywords: *Antimicrobial Screenin; Antioxidant, Endophytic Fungi; Secondary Metabolites; Surface Sterilization.*

1. Introduction

Endophytic fungi are symbiotic microbes that reside in the internal tissue of plants for all part of their life cycle without causing any immediate overt negative effect (Strobel, 2002). Approximately about 300,000 higher plants species are existing on the earth, and each individual plant host one or more endophytic fungi. Many recent studies have revealed the ubiquity of these fungi, with an estimate of at least 1 million species residing in plants (Dreyfuss and Chapela, 1994). A variety of symbiotic relationships exist between fungal endophytes and their host plants ranging from mutualisms (Schardl and Philips, 1997), to commensalisms. Beneficial effects for host plants include increased drought tolerance (Arechavaleta et al., 1989), deterrence of insect herbivores (Breen, 1994), protection against nematodes, fecundity, general fitness, resistance against bacterial and fungal pathogens (Clarke et al., 2006). In return the plant host is able to provide shelter and supplies the necessary nutrients and other compounds required by the endophytes to complete their life circle (Breen, 1994; Clarke et al., 2006). Azadirachta indica (L.) (Neem) is an evergreen deciduous tree up to 25m high bearing a dense wide-spreading crown with panicles of white flowers and yellow fruits when ripe, leaves imparipinnate, alternate or opposite, 6-10cm long, 1-3cm wide and dark green in colour. In Nigeria, like in other parts of the world, different parts of the "Neem" plant are used for the treatment of various diseases such as, malaria, intestinal worms, piles, diabetes, respiratory disorders, constipation, treatment of rheumatism, and chronic syphilitic sores (Okujaku et al., 2004). A review on A. indica fungal endophytes showed that Javanicin, which is an antibacterial agent was identified from an endophytic fungus of A. indica, Chloridium spp, (Kharwar et al., 2009). Another endophytic fungus (Eupenicillium parvum) was also isolated from A. indica which produces azadirachtin A and B compounds (Kusari et al., 2012).Therefore the focus of this work was to isolate and identify endophytic fungi from A. indica stems (twig) and young leaves for the production and preliminary pharmacological screening of the secondary metabolites produced by the selected isolate.

2. Materials and methods

The study area of this research work was Sheda Science and Technology Complex (SHESTCO) located at Kwali Area Council South Western part of the Federal Capital Territory of Nigeria, which lies between latitude 8.9 degrees south and longitude 78 degrees east. With a total landmass of about 1,700.400 square kilometers. The plant was authenticated by Mr. O. Segun and verified by Professor O. Olorode (Botanist/ Taxonomist). Department of Biological Sciences, University of Abuja. FCT Abuja, Nigeria.

2.1. Sterilization, equipments and routine laboratory operations.

Bacteriological media, Mycological media, autoclavable plates, polyethene bags were sterilized by autoclaving at 121^0c for 20 minutes. Glass wares were sterilized by dry heat in an oven (Memmert Electric Co., Ltd. Japan) at 180^oc for one hour. Wire loops, forceps were sterilized by flaming. Elution was sterilized by filtration through 0.22µm pore size membrane filter in a sterile glass filtration unit using negative pressure generated by a vacuum pump. Bacteriological and mycological media were purchased from (Sigma – Aldrich) and prepared in accordance with manufacturer's

instructions. All aseptic operations were carried out in a safety cabinet (Labconco, Missouri, USA) that had been left running with the ultraviolet rays for at least ten minutes before use, and analytical weighing balance (OHAUS Cop., USA) which is routinely calibrated.

2.2. Sample collection and processing

Healthy and mature plants (showing no visual disease symptom) were randomly collected for this work. Stems (twigs) and young leaves from different parts of the tree plant were collected. The samples were taken to the laboratory in a sterile polyethene bag and processed within 2 to 3 hours. Fresh plant materials were used for the isolation work in order to reduce the chances of contamination.

2.3. Mycological media used for the isolation of endophytic fungi

The main mycological media used for the isolation of endophytic fungi in this research work was Potato Dextrose Agar. (Sigma -Aldrich). Freshly prepared media supplemented with antibiotic Chloramphenicol (Laborate pham. Inc) was used.

2.4. Isolation procedure

The samples were rinsed gently in running tap water to remove dust, debris and epiphytic microbes. After proper washing, stems and leaves were chopped into smaller pieces, and processed under aseptic conditions. Highly sterile conditions were maintained for the isolation of fungal endophytes. All the work was carried out in the laminar flow hood. The stems were cut into 0.5-1cm and the leaves were also cut with or without midrib into 0.5-1cm length. The protocol for isolation was adapted from the methods used by (Rungjindamai et al., 2008; Oses et al., 2008; Theantana and Lumyong, 2009), with slight modifications. The surface sterilization was carried out using sodium hypochlorite (3%NaOCl v/v) and (70% v/v) ethanol. Each set of plant materials were treated with 70% ethanol for 1 minute followed by immersion in sodium hypochlorite for 3 minutes and rinsed with sterile water. This was followed by immersion in 70% ethanol for 30 seconds and finally, rinsed with sterile water three times. The treated samples were later dried on a sterile tissue paper. The efficiency of the surface sterilization procedure was ascertained by culturing the final rinsed water (aliquot) followed by subsequent observation. If any growth is observed in the aliquot, the set of plant materials were discarded, otherwise, the set is good for culturing. In each Petri dish, 3-7 segments of the treated samples were placed on a solid medium PDA supplemented with Chloramphenicol (Laborate phamac.inc.), in order to retard the growth of bacteria. The plates were incubated at 25℃ ± 2 for 1-4 weeks.

2.5. Subculturing

Subculturing was done by transferring hyphal tips from the master plates to Potato Dextrose Agar (PDA) plates without addition of antibiotics for proper propagation, and also to obtain monoclonal (pure) cultures for identifications.

2.6. Percentage (%) frequency of colonization

The colonization frequency (CF) was calculated as described by (Suryanarayanan et al., 2003) as follows:-

$$C.F = \frac{\text{No.of segment colonized by fungi}}{\text{Total number of segment observed.}} \times 100$$

2.7. Identification of selected fungal species

The endophytic fungi was identified on the basis of their morphological and cultural characteristics. Isolates were identified on the basis of their growth on PDA media. Both the macroscopic and microscopic identifications were carried out followed by other biochemical test such as Germ tubes test, Dalamau test, and Urease test and verified using mycological Atlas (Harold, 1998; Brooth, 1997; Barnett and Hunter, 1987; St-Germain and Summer, 1996).

2.8. Macroscopic identifications

The morphology of the isolates from both upper and reverse side of the cultured was identified based on the following features, such as colour (Upper and reverse) texture, and colonial topography.

2.9. Microscopic identification

The microscopic morphological features was examined by one step staining technique using methylene blue. The definitive microscopic identification is based on the morphology of the spores and hyphae. Spores, such as (sprongiospore, chlamydiospores, microconidia and macro-conidia), characteristic shape such as (fusiform, ovoid, cylindrical, porospore and oblong). Arrangement of the spores on the hyphae such as (singly, in chains, cluster, terminal, and flower-like). Hyphae: The size and shape of the hyphae are also very relevant which showed the characteristics such as (spiral and pectinate). Capsule staining using Indian ink, and Dalamau plate techniques was also applied for microscopic identification.

2.10. Fungal cultivation

The endophytic fungi were cultivated on Potato Dextrose Broth using procedure by (Raviraja et al., 2006). This involves placing agar blocks of actively growing pure cultured in 250ml Erlenmeyer flask containing 100ml of the medium. The flask was incubated inside incubator shaker (annova 44 UK) at 25±2 °C for 1 week with periodical shaking at 150 rpm. After the incubation period, the cultured was taken out and filtered through sterile cheesecloth to remove the mycelia mats.

2.11. Extraction of metabolites

The fungal metabolites from the identified isolate Penicillium spp., was extracted using ethyl acetate as a solvent of extraction. Equal volume of the filtrate and solvents (50:50v/v) was taken and shaken vigorously for 10 min. Using separating funnel the mixtures was separated. The solvents of extraction was evaporated and the resultant components was dried in a water bath (Buchi waterbath B-480) at 40^0c to yield the crude extract.

2.12. Column chromatography

The column was packed using the dry pack method. A piece of cotton wool was inserted into a clean dry glass column followed by 20g absorbent silica gel of mesh size (120-150 mm), 2g of the extract (aliquot) was loaded with mobile phase mixture of Ethylacetate and n-hexane 50:50v/v a single fraction was obtained and concentrated in a water bath (Buchi waterbath B-480) at 40^0c.

2.13. Preliminary phytochemical screening of the eluent

The qualitative phytochemicals screening of the eluent was carried out, for flavonoids, glycosides, alkaloids, steroids, saponins (frothing test), phenols ferric chloride test ($Fecl_3$), and Test for tannins:

2.14. Test culture (microorganisms)

The test organisms (Bacteria) was isolated from different clinical specimen from patients attending Murtala Muhammad Special Hospital Kano, Kano State, and identified using standard methods by (Cheesbrough, 2000). They include, Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Klebsiella pneumonia, Salmonella para typhi. While the pathogenic fungi (Candida spp) were isolated and characterized in Nasarawa Special Hospital Kano, Kano State

using fungichrome test kits. Specimens were collected from patients all symptomatic with vaginitis. They include Candida albican, C. glabrata, C. krusei, C. tropicalis and Tricosporon spp. The bacterial isolates were maintained in a freshly prepared nutrient agar slant while the Candida species were maintained in a potato dextrose agar slant and kept in a refrigerator at 4℃ until required for use.

2.14. Antimicrobial activity test of the eluent

Agar diffusion method designed by European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2012) was employed. The freshly prepared Mueller-Hinton agar (Sigma- Aldrich) and Potato Dextrose Agar (Sigma- Aldrich) plates were dried in a dryer for about 15 minutes to remove surface moisture. The plates were aseptically inoculated uniformly with test organism by streaking methods. After diluting the extract with ethyl acetate, the discs were prepared according to EUCAST (2012). Using double serial dilutions to prepared three set of disk of sterile impregnated Whatman No.1 filter paper, with potency of 60, 30 and 15µg/disk respectively, Chloramphenicol (Laborate pham. Inc) and Griseofulvin (Medrel Pham.) are used as positive control for antibacterial and antifungal respectively. While DMSO + Distilled Water served as Negative control. With the aid of a sterile forceps, the impregnated paper discs (10mm) were arranged and pressed firmly onto the inoculated agar surface to ensure even contact. Each disc was sufficiently spaced out and kept at least 15 mm from the edge of the plate and 25mm from disc to disc to prevent overlapping of zones. The experiments were duplicated and the zones of inhibition (mm) expressed as the mean standard deviation.

2.15. Standardization of inoculum

Using inoculating loop, enough material from an overnight culture of the test organisms were transferred into a test tube containing about 2ml of Mueller- Hinton broth (Sigma -Aldrich) for bacteria and Potato dextrose broth for non-filamentous fungi (Candida sp.), until the turbidity of the suspension matched the turbidity standard 0.5 McFarland (EUCAST, 2012; NCCLS, 2000).

2.16. Antioxidant activity

The in vitro radical scavenging activity of the eluent against 2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH Sigma-Aldrich) were determined by UV-Visible spectrophotometer at 517 nm. Radical scavenging activity was measured by slightly modifying the method previously described by Ahmad (Ahmad et al., 1999). The following concentrations of the eluent was prepared in a duplicate, 0.5, 0.25, 0.125, 0.0625, and 0.03125, in methanol (Analar grade). Buthylated Hydroxyl Anisole (BHA) was prepared and compared as antioxidant standard. 1ml of eluent was placed in each 5 test tubes, and 3 ml of methanol was added each followed by 0.5 ml of 0.1mM DPPH in methanol. The solutions were allowed to stand for 30minutes, while the absorbance was determined on a UV-Visible spectrophotometer at 517nm. A blank solution was prepared containing the same amount of methanol and DPPH. The radical scavenging activity was calculated using the following formula:

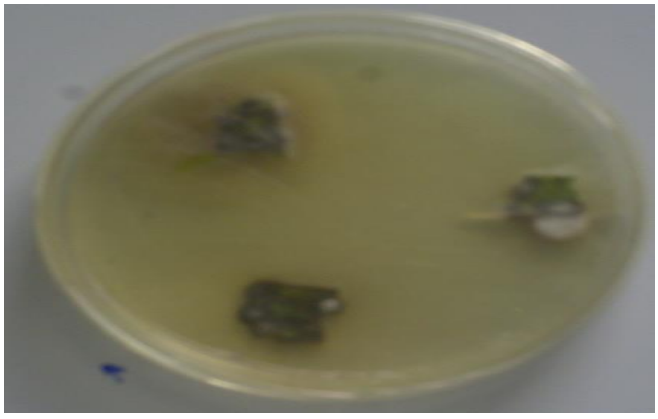
$$\% \text{ Inhibition} = \frac{Ab - Aa}{Aa} \times \frac{100}{1}$$

A_b = Absorbance of blank
A_a = Absorbance in the presence of extract.

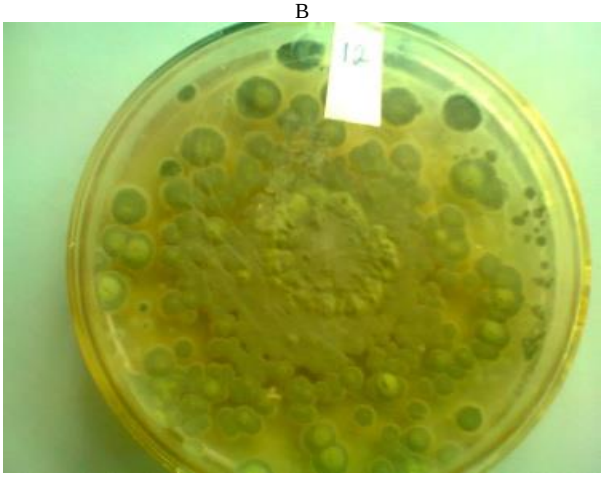
3. Results

A total of Two hundred and fifty two samples segments were collected. One hundred and twenty six from each healthy stems (twig) and young leaves was screened for their endophytic mycoflora. In each isolation plates three to six segments were cultured, and the emergence of fungal mycelium was observed after 3-7days of cultured (Figure 1A).
Twelve (12) species of endophytic fungi were isolated, seven species from stems (twig) and five from leaves (young) two species one from each stems and leaves was recorded unidentified, the Cryptococcus spp isolated from the stems (twigs) proved to be the most dominant species, with high percentage frequency of colonization 14.9%, while Cladosporium spp dominated the leaves with frequency of colonization 11%, (Figure 2).

A



B



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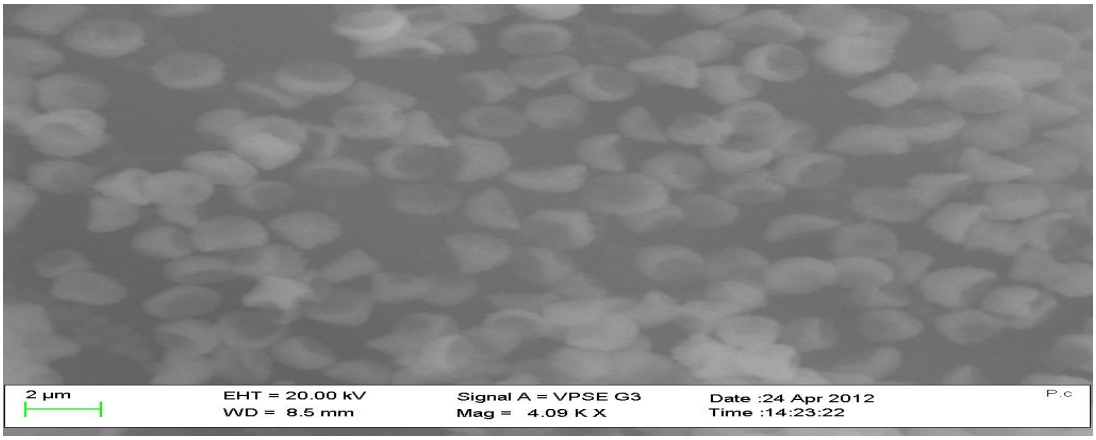

D

Fig. 1: A. Emerged of Endophytes from A. Indica Leaves Cultured on PDA; B. Pure Cultured of Penicillium Spp on PDA Plate. C. Spores Structure of Penicillium Species Using Scan Electric Microscope (Mag. 4.09 Kx). D. The Microscopic Structure of Penicillium Spp. Mycelium under Carl Zeiss Microscope Mgx400.

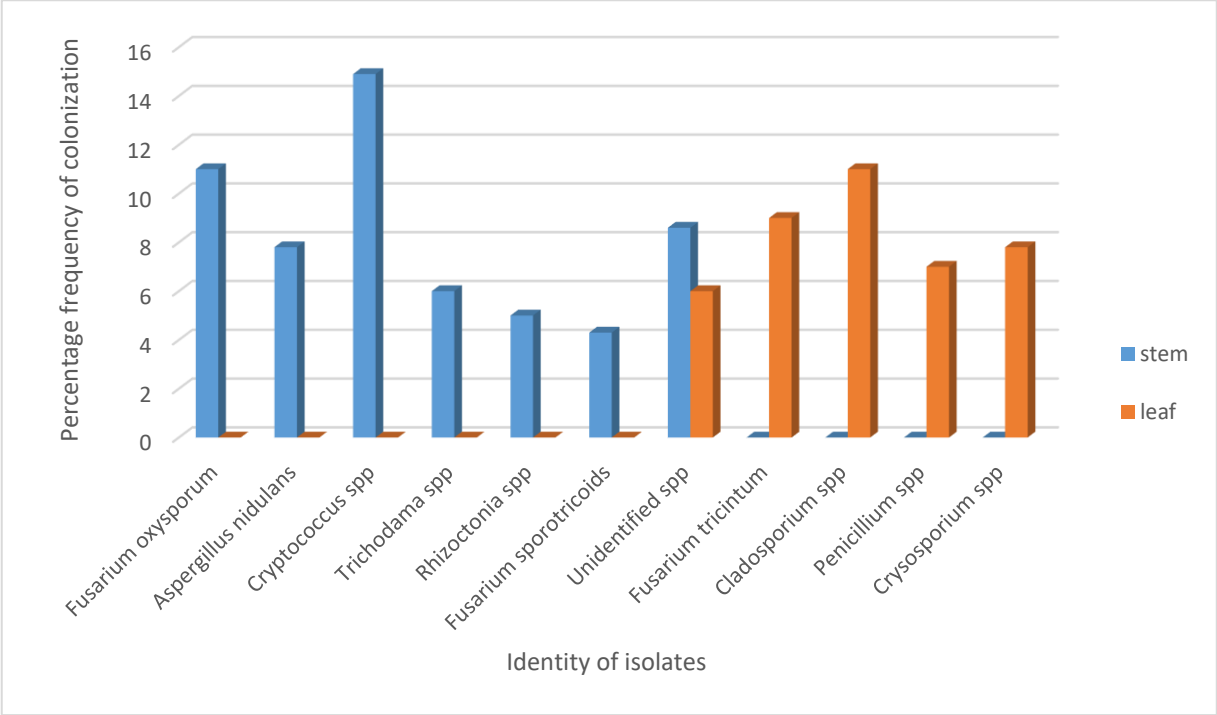

Fig. 2: Percentage (%) Frequency of Colonization of Fungal Isolates.

Taxonomically nine (9) of the identity isolates are belong to the class Ascomycetes, one (1) species Rhizoctonia spp, is belong to Basidiomycetes and two (2) species remains unidentified, from the identified isolates Penicillium species was randomly selected. The pure culture of the selected Penicillium spp, was maintained on PDA media free from antibiotic for proper growth and identification as showed in (Figure 1B). The structures of the spores were identified using scan electron microscope Carl Zeiss, SEM Evo/ MA10 Germany (Figure1C), and the mycelium was wet mounted using methylene blue and viewed under Carl Zeiss Microscope Mag. X400 (Figure 1D). The eluent of the selected Penicillium spp was subjected into phytochemical, antimicrobial and antioxidant activity. The preliminary screening of the eluent showed the pres-

ence of saponins, flavonoids and phenols which are reported to possess some degree of antimicrobial and antioxidant activities (Table 1).

Table 1: Phytochemical Screening of Eluent.

S/no.	(50:50v/v) Ethylacetate/n-hexane Phytochemicals	Penicillium sp.
1	Steroids	-
2	Tannins	-
3	Saponins	+
4	Flavonoids	+
5	Phenols	+
6	Glycosides	-
7	Alkaloids	-

The eluent showed low antibacterial activity compared to that of positive control (Chloramphenicol) which showed activity ranges from 27±00 – 22.5±0.5mm at 60µg/disc, while the highest zone of inhibition of the extract was showed on S. aureus 12.5±0.5mm, K. pneumonia and P. mirabilis showed 8.5±0.5mm each, while Salmonella para-typhi and E. coli showed 10.5±0.5 and 9.5±0.5mm respectively, at 60µg/disc (Table 2).

Table 2: In-Vitro Antibacterial Activity of the Eluent from Penicillium Sp

Test Bacteria	Disc potency (µg/disc)Average Zone of inhibition in (mm)				
	60	30	15	CLPC 10µg/ml	DMSO+DH_2O
Staph. aureus	12.5±0.5	8.±00	6.5±0.5	27.0±00	NA
Kleb. pneumonia	8.5±0.5	7.5±0.5	NA	25.5±0.5	NA
S. para typhi	10.5±0.5	7.5±0.5	NA	25.5±0.5	NA
P. mirabilis	8.5±0.5	7.5±0.5	7±00	22.5±0.5	NA
E. coli	9.5±0.5	7±00	NA	22.5±0.5	NA

Values ± are mean Standard deviation of the duplicate.
KEY
NA = No activity, µg =Microgram,
DMSO = Diamethylsophoxide, DH_2O =Distilled water
CLPC= Chloramphenicol.

Table 3: In-Vitro Antifungal Activity of the Eluent from Penicillium Sp

Test Fungi	Disc potency (µg/disc)Average Zone of inhibition in (mm)				
	60	30	15	Griseofulvin 10µg/ml	DMSO+DH_2O
Candida albicans	NA	NA	NA	18±00	NA
Candida glabrata	NA	NA	NA	18±00	NA
Candida krusen	7.5±05	7±00	NA	18±00	NA
Candida tropicalis	8±00	7.5±0.5	NA	18±00	NA
Trichosporon sp	9.0±00	NA	NA	20.5±0.5	NA

Values ± are mean Standard deviation of the duplicate.
KEY
NA = No activity, µg =Microgram,
DMSO = Diamethylsophoxide, DH_2O =Distilled water.

From the DPPH antioxidant curve (figure 3), the eluent possesses low antioxidant capability compared to BHA standard, meanwhile, both the standard and the extract are concentrations dependent. The eluent curve from Penicillium spp is showing promising similar result to that of BHA standard curve, especially at concentrations of 0.125mg/ml to 0.5mg/ml, (figure 3).

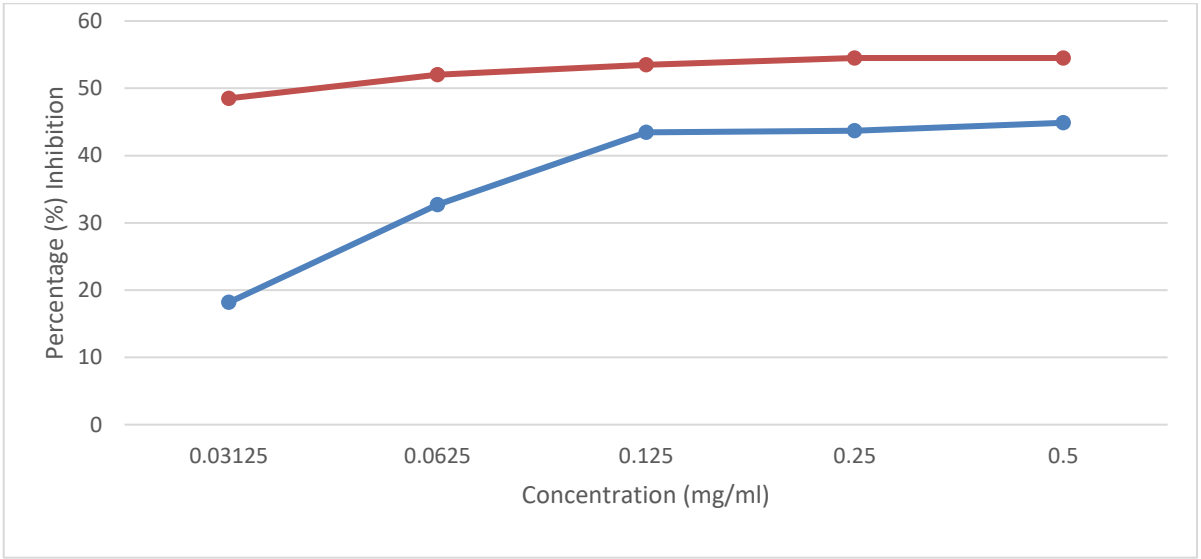

Fig. 3: Antioxidant Curve of the Eluent and BHA Standard.

Key:
BHA=Buthylated Hydroxyl Anisole curve
Eluent curve.

4. Discussion

The result was agreed with the work carried out by (Anima and Akila, 2014) on Synergistic effect of crude metabolites extracted from mould, P. citrinum and antibiotics on S. aureus (18mm), E. coli (15mm), and Proteus vulgaris (10mm). In another research work carried out by (Silval et al., 2011) on crude extract from Penicillium spp., in Brazilian mangrove plant showed zone of inhibition of (12.75mm) and (12.50mm) on S. aureus and Bacillus subtilis respectively, using disc diffusion assay at 20µl/disc. Penicillium species produce a much diversified array of active secondary metabolites, including antibacterial (Nicoletti et al., 2007; Kwon et al., 2002), antifungal substances (Nicoletti et al., 2007), immune -suppressant, cholesterol-lowering agents (Kwon et al., 2002). Thousands of Penicillium isolates have been screened for bioprospecting programs since the discovery of penicillin, and new bioactive metabolites continue to be discovered from these fungi nowadays, (Larsen et al., 2007; Ge et al., 2008; Takahashi et al., 2008), indicating their current importance as sources of high amounts of novel bioactive molecules to be used by pharmaceutical industries. The antifungal activity of the extract was only pronounced on two candida spp namely C. tropicalis and C. krusen which range between (8±00 -7±00) at 60µg/disc (Table 3).

The IC_{50} of both antioxidant standard and eluent curve was obtained at 0.19, and 0.24, respectively using linear regression. The result was agree with the work carried out by (Devi, 2014) using crude extract of Penicillium spp. which reveals high antioxidant activity with IC_{50} value, 54.72±2.19µg/ml compared to the IC_{50} value of ascorbic acid, 50.00±0.98µg/ml, which was used as antioxidant standard. The DPPH test Provides information on the reactivity of the test compounds with a stable free radical. DPPH gives strong absorption band at 517 nm in the visible region. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolourized as the colour changes from deep violet to light yellow. The degree of reduction in absorbance measurement is indicative of the radical scavenging (antioxidant) power of the extracts (Ge et al., 2008; Olajide et al., 2012). The antimicrobial screening results was analysed using Statistical package for social science (SPSS software). The Analysis of variance (ANOVA), shows no significant difference between the effects of the extract concentrations on test organisms, since the significant value (.735) is greater than critical value ($p > 0.05$). For the antifungal screening (Table 3), there is no enough data for ANOVA as it is used to compare means of samples greater than two, so the table gives rooms only for statistical t-test to compare the means of two organisms that shows sensitivity zone. Based on the analysis the significant value (.126) using a two tailed test is greater than critical value ($p > 0.05$) which reveals no significant effects of the extract on two affected test fungi.

5. Conclusion

Endophytic fungi of medicinally important host are under investigated group of microorganisms that represent untapped pool of bioactive and novel chemical compounds of biotechnological significant. This can be exploited in a variety of agriculture, pharmaceutical and industries in near future. To get more benefit from these group of microorganisms, special considerations should be given to medicinal plants as a natural host of endophytic fungi.

Acknowledgement

The authors express gratitude to Professor P. C. Onyenekwe Director (BARC SHESTCO), Head of Biological sciences Department University of Abuja and all Staff of Sheda science and technology complex (SHESTCO) for their support.

Conflict of interest statement

We hereby declare that we have no conflict of interest.

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