PHYTOCHEMICAL, ANTIOXIDANT AND ANTIMICROBIAL STUDIES OF THE CRUDE EXTRACT OF Curvularia sp. ISOLATED FROM THE LEAVES OF THE MEDICINAL PLANT Pilostigma thonningii (Fabaceae)

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ABSTRACT
Recently, there is biotechnological interest in secondary metabolites of endophytes since they have been shown to be capable of producing substances of pharmacological importance. Pilostigmathonningii is a tree whose parts are widely employed in Nigeria for diverse medicinal applications. The aim of this study is to investigate the phytochemical composition, the antiradical and in vitro antimicrobial potentials of the crude ethyl acetate extract of Curvularia sp isolated from the leaves of the plant. The endophytic fungus was isolated, purified, and identified using conventional methods. While standard procedures was employed in the determination of the phytochemical constituents, quantitative estimation of the total phenol and flavonoid were done by spectrophotometric method using gallic acid and quercetin as standards respectively. While the antioxidant activity was determined by DPPH scavenging assay, the agar well diffusion method was employed to screen for its antimicrobial capabilities. Results showed the presence of important phytoconstituents notably alkaloid, flavonoid, phenolics, tannin, saponins among others. While the antioxidant assay showed the total phenolic and flavonoid contents to be 212.55 ± 1.2 mgGAE/g extract and 143.16 ± 0.8 mgQE/g extract respectively, a 74.45 ± 0.11% free radical scavenging activity was observed compared to ascobic acid standard that exhibited a 87.92 ± 0.17% inhibition. At the highest investigated concentration of 1 mg/mL, the extract produced antibacterial activity only against Escherichia coli and Pseudomonas aeruginosa. The results showed that the extract exhibits marked antioxidant and selected antibacterial effects thus justifying the assertion that endophytic bioactive compounds have promising applications in the field of medicine.

Keywords: Antioxidant, Antimicrobial, Curvularia sp, Phytochemical, Secondary Metabolites

INTRODUCTION
Free radicals are chemically aggressive molecules that have been implicated for damages to different macromolecules in the body such as DNA, proteins and lipids (Harman, 1981). They contribute to numerous disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen and Salonen, 1999; Cook and Samman, 1996). Cells are equipped with natural antioxidant systems like α-tocopherol, β-carotene, ascorbic acid as well as synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butyldihydroquinone (TBHQ) whose mechanisms are primarily to scavenge these free radicals to maintain the redox homeostasis of cells (Farrukh et al., 2006). The increase in global chemotherapeutic failure could be attributed to the antimicrobial toxicity and undesirable side effects of the present antibiotics employed to curb the present and emerging infections (Ogbiko et al., 2020). As a result of the unbearable side effects, carcinogenicity, high cost and inaccessibility, there is an inspired search for more effective antioxidant from natural source, especially from medicinal plants known to be biodegradable, less toxic, affordable and readily accessible (Deepa and Renuka, 2014; Ezeja et al., 2015). Natural products have long been recognized as specialized product structures possessing high chemical diversity, biochemical specificity and other molecular properties that make them favorable as lead structures for drug discovery (Okoye and Osadebe, 2010). Pilostigmathonningii, Schum (Milne-Redhead) of the family Fabaceae commonly known as monkey's bread or camel's foot tree and locally known as “kalgo” (Hausa), “kalur” (Kanuri) and “impilatiki” (Higgi) have long been used in the management and treatment of inflammation, bilharzia, eye diseases, catarrh malaria fever among others (Kwaji et al., 2010). Endophytic fungal populations from Nigerian medicinal plants have been shown to possess enormous potentials as sources of biologically active compounds of pharmaceutical and industrial importance (Chika et al., 2018). It is estimated that there might be as many as one million different endophytic fungal species, however, only a handful of them have been described, which implies that investigating the metabolites of endophytes for pharmacological activities will enhance the chance of finding novel bioactive compounds (Okoye et al., 2015). There is the need to explore medicinal plants endophytic population for biologically important molecules, hence our study seeks to phytochemically profile and ascertain the in vitro antioxidant and antimicrobial potential of the secondary metabolites of an endophytic fungus isolated from the leaves of P. thonningii growing in Sokoto State, North-West Nigeria.
MATERIALS AND METHODS

Plant material collection, authentication and preparation
Fresh and healthy leaves of P. thonningii were collected from Tambuwal local government area of Sokoto State in April, 2020. Taxonomic identification was done by Mallam Abdulaziz Salihu of Botany Unit, Department of Biological Sciences, Faculty of Science, Usmanu Danfodiyo University Sokoto. Herbarium specimen was prepared, deposited and voucher number UDUH/ANS/0137 issued. The fresh leaves were prepared and transported to the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University Awka, Anambra State for the endophytic fungus isolation and purification.

Isolation of endophytic fungus
Isolation of the endophytic fungus from the plant leaves, solid-state fermentation of the fungus and extraction of the fungal metabolites were carried out using methods previously described by Akpotu et al., 2017a and Akpotuet al., 2017b. Selected healthy plant leaves were washed thoroughly in running tap water and processed as follows: the leaves were cut into 1 cm fragments and 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. The leaf fragments were placed on Petri plates containing malt extract agar (MEA) supplemented with chloramphenicol. The plates were then incubated at 28°C temperature and fungal growth from the leaf fragments was monitored. Hyphal tips from distinct colonies emerging from leaf segments were sub-cultured onto fresh MEA plates to obtain pure colonies.

Fermentation and extraction of endophyte secondary metabolites
Solid state fermentation of the endophytic fungus was carried out in 1L Erlenmeyer flask containing autoclaved rice medium (100 g of rice and 200 mL of distilled water). The flask was inoculated with 3 mm diameter agar blocks containing the fungi and incubated at 28°C for 21 days. At the completion of fermentation, the secondary metabolites were extracted in ethyl acetate and then concentrated under vacuum at 40°C using a rotary evaporator.

Phytochemical screening
The phytochemical screening was conducted on the crude secondary metabolite in accordance to standard procedures (Khandewal, 2000).

Determination of total phenolic content
The total phenolic content in the extract was determined according to the Folin-Ciocalteu procedure outlined by Kim et al., 2003 with slight modifications. The extract solution (0.5 mL) with a concentration of 1000 μg/mL was added to 4.5 mL of deionized distilled water and 0.5 mL of Folin-Ciocalteu’s reagent previously diluted with deionized water (1:10 v/v). The solution maintained at room temperature for 5 minutes was followed by the addition of 5 mL of 7% sodium carbonate and 2 mL of deionized distilled water and mixed thoroughly. The samples were incubated for 90 minutes at 23°C with the absorbance measured with a UV-VIS spectrophotometer at 765 nm. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. Gallic acid was used as the positive control. The standard curve was prepared by gallic acid in five different concentrations (50, 100, 150, 250 and 500 mg/L).

Determination of total flavonoid content
The total flavonoid content in the plant extracts was determined by the Aluminum Chloride method as outlined by Ebrahimzadeh et al., 2008. 0.5 ml of the extract (5 g/L) was mixed with 1.5 mL of methanol and then 0.1 ml of 10% aluminum chloride was added followed by 0.1 ml of potassium acetate and 2.8 ml of distilled water. The mixture was incubated at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 415 nm. The results were expressed as milligrams quercetin equivalents (QE) per gram of extract (mg QE/g dry extract). Quercetin was used as positive control and the standard curve was prepared by quercetin in different concentrations (12.5, 25, 50, 80, and 100 mg/L).

Screening of the crude extract for free radical scavenging activity
The ability of plant extracts to scavenge 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radicals was estimated as previously described by Jain et al., 2008. The extracts (3 mL) with five different concentrations (12.50, 25.00, 50.00, 100.00, and 200.00 μg/ml) were mixed with 1 mL of a 0.1 mM methanol solution of DPPH. Each of the mixtures was shaken vigorously, and then incubated in the dark at room temperature for 30 min. The UV absorbance was measured at 517 nm using a UV-VIS spectrophotometer. 2 mL of MeOH solvent mixed with 2 mL of DPPH solution served as blank (negative control) while ascorbic acid at the same concentrations was used as the positive control. The DPPH free radical scavenging effect of the samples was calculated using the following formula:

\[ \text{DPPH radical inhibition} \% = \frac{1 - \left( \frac{A_{\text{test}}}{A_{\text{control}}} \right) \times 100}{A_{\text{control}}} \times 100 \]

Where Acontrol is the absorbance of the control and Atest is the absorbance of the tested extracts. The antioxidant activity of the sample was also expressed in terms of IC50 value, which represents the concentration of the sample required to inhibit 50% formation of DPPH radical.

Antimicrobial assay
Antibacterial and antifungal screening of 1 mg/mL concentration of the crude extract of the fungus was prepared using standard procedures and agar well diffusion method previously described by Akpotu et al., 2017(a) and (b). It was employed to ascertain its antimicrobial activity. The extract was challenged with confirmed laboratory strains of Staphylococcus aureus; Bacillus subtilis; E. coli; Pseudomonas aeruginosa; Salmonella species; Klebsiella pneumoniae, Candida albicans, and Aspergillus niger procured from the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences Nnamdi Azikiwe University Awka Anambra State. The inhibition zone diameters (IZDs) were measured and recorded. The procedure was conducted in triplicate, and the mean IZDs calculated.

Statistical analysis
In the antioxidant assays, UV absorbance was measured in triplicates with the average calculated and recorded. They are expressed as mean ± standard error of mean (SEM) with statistical comparisons made using the student t-test. Confidence limit was set at p < 0.05.

RESULTS AND DISCUSSION

Phytochemical screening
Preliminary phytochemical analysis showed the presence of major classes of secondary metabolites as presented in Table 1.
Table 1: Phytochemical screening of the crude EtOAc extract of Curvularia sp isolated from the leaves of P. thonningii

| Phytochemical      | Inference |
|--------------------|-----------|
| Alkaloids          | +         |
| Saponins           | +         |
| Flavonoids         | +         |
| Phenolics          | +         |
| Tannins            | +         |
| Carbohydrate       | +         |
| Reducing sugars    | +         |
| Terpenoids         | -         |
| Cardiac glycosides | -         |

Key: (+) = present; (-) = absence of phytoconstituents

Preliminary phytochemical screening of medicinal plants to reveal their phytoconstituents is a valuable step in the detection of the bioactive principles often explored in drug discovery and development (Jain et al., 2008). The crude ethyl acetate extract of the endophyte isolated from P. thonningii revealed the presence of various phytochemicals of pharmacological relevance.

Table 2: Total phenolics and flavonoids content of the crude EtOAc extract of Curvularia sp endophyte isolated from P. thonningiileaves

| Total phenolic content* | 212.55 ± 1.2 |
| Total flavonoid content** | 143.16 ± 0.8 |

*mg gallic acid equivalent (mgGAE)/g extract; **mg Quercetin equivalent (mgQE/g extract)

Values are means of three biological replicates.

Figure 1: DPPH radical scavenging activity of crude ethyl acetate extract of the endophytic fungal and the synthetic antioxidant (ascorbic acid) at different concentrations.

The results presented in Table 3 show the in vitro susceptibility of the microbial isolates of the crude extract of the endophytic fungus in comparison to ciprofloxacin (5 μg/mL) and miconazole (50 μg/mL) standards respectively. The ethyl acetate crude extract of the endophytic fungus at 1.00 mg/mL showed activity only when tested against Escherichia coli and Pseudomonas aeruginosa with no inhibition noticed for all other microbes. There were significant differences between the IZDs of the standard antibiotics and the extract for the sensitive microbes.

Table 3: Result of antimicrobial activities (inhibition zone diameter) of the crude extract of Curvularia sp isolated from the leaves of P. thonningii

| Drugs | Microorganisms/ IZDs (mm) at 1.00 mg/mL |
|-------|----------------------------------------|
|       | SA  | BS  | EC  | PA  | SS  | KP  | CA  | AN  |
| CECS  | 0   | 0   | 2±0.7   | 18±0.7    | 0   | 0   | 0   | 0   |
| CIP 5μg/mL | 15±0.7 | 17±0 | 18±0.7    | 11±0   | 15±0.7 | 15±0.7 | ND  | ND  |
| MICO 50 μg/mL | ND | ND | ND | ND | ND | 14±0 | 11±0.4 |
| DMSO (NC) | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   |

Natural antioxidants have been established to be pivotal in the management of disorders associated with oxidative stress (Yadav et al., 2014). The result of the DPPH assay of the CECS showed an increase in the radical scavenging ability in a dose dependent manner although lower than the synthetic antioxidant. This could be strongly linked to the phenolic and flavonoid contents (Table 2). Polyphenolic compounds like phenols, flavonoids, phenolic acids, stilbenes, tannins, lignins among others are known to scavenge radicals such as singlet oxygen, superoxide, and hydroxyl because of their phenolic hydroxyl groups that allow them to act as antioxidants (Gandía-Herrero et al., 2009; Vijay and Vimukta, 2014). This agreed to the report of several researchers who stated that plants with high contents of phenolics and flavonoids had high antioxidant activities (Maisuthisakul et al., 2007; Maisuthisakul et al., 2008; Zhang and Lin, 2008; Zhang et al., 2015).

Antimicrobial Activity
Key: SA (Staphylococcus aureus); BS (Bacillus subtilis); EC (Escherichia coli); PA (Pseudomonas aeruginosa); SS (Salmonella species); KP (Klebsiellapneumonia); CA (Candida albicans); AN (Aspergillus niger); CECS = Crude Extract of Curvulariaasper; CIP = Ciprofloxacin; MICO = Miconazol; DMSO = Dimethyl sulphoxide; NC = Negative Control; ND = Not Determined. Values are presented as mean ± standard error of mean of three determinations; Values with different superscript in the same column are significantly different at p < 0.05.

The antibacterial activity test as depicted by the inhibition zone diameter was seen to be concentration-dependent. The reported antimicrobial activity for the extract and fractions may be due to the presence of the detected phytochemicals (Table 1) especially phenols, flavonoids, tannins and saponins that were all detected in the extract have been shown to have antibacterial, anthelmintic, and antineoplastic activities (Akinpelu et al., 2011). While saponins are surface-active agents which alter the permeability of the cell wall of organisms thus facilitating the entry of toxic materials or agents which alter the permeability of the cell wall of (Daniyan et al., 2010), tannins were reported to exhibit antibacterial, antiviral and antitumour activities (Evans, 2002; Kolodziej and Kiderlen, 2005) and alkaloids are reported to possess antibacterial, anti-arrhythmics effect, anti-inflammatory and anti-asthmatic activity (Daniyan and Abalaka, 2012). The superior antioxidant and antimicrobial activity of the standard drugs compared to the endophytic fungus crude extract could be attributed to them containing more purified compounds than the extract (Ogbiko et al., 2018).

CONCLUSION

The study demonstrated that Curvulariaasper – an isolated endophytic fungus of Pilostigma thonningii leaf produce secondary metabolites with biological properties. These endophytic fungi can be a good source of novel therapeutic compounds, which may play a vital role in the development of antioxidant and antibacterial drugs for the effective treatment of free radical implicated disorders and selected microbial actions. Studies are still ongoing by the authors to isolate and characterize the particular compound(s) that is/are involved in the antioxidant and antimicrobial activities, and ascertain their potency in vivo.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest with the contents of this article.

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