ISOLATION AND CHARACTERIZATION OF ENDOPHYTHIC FUNGI FROM CALOTROPIS PROCERA FOR THEIR ANTIOXIDANT ACTIVITY

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ABSTRACT

Objective: To investigate the antioxidant activity of methanolic extract of endophytic fungi isolated from Calotropis procera plant.

Methods: The endophytic fungi were isolated from explants of leaf, stem, and flower of C. procera on potato dextrose broth (PDB) medium. The fungal isolates were mass cultured in PDB. The methanolic extracts of these fungi were prepared and evaluated for the antioxidant activity by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay and reducing power assay. The extracts were characterized for the presence of phenolics and flavonoids.

Results: A total of 8 endophytic fungi were isolated from C. procera explants. Out of these 8 fungal isolates, the crude extracts of two fungi, i.e. CPIMR-2 and CPI-1, showed positive antioxidant activity. From the morphological characteristics, the isolates CPIMR-2 and CPI-1 were identified as Penicillium spp. and Aspergillus spp., respectively. Both the isolates were found to possess antioxidant potential with 81.16% and 80.97% inhibition in the DPPH radical scavenging assay. The phytochemical screening of the methanolic extracts showed the presence of phenolics and flavonoids. The total phenolic content in Penicillium species was found to be 130.50 µg/mg and 94.91 µg/mg of extracts, respectively. The total flavonoid content was found to be 9.16 µg/mg and 12.13 µg/mg of extracts in Penicillium species and Aspergillus species, respectively.

Conclusions: Endophytic fungi were found to be present in medicinal value plant C. procera. The probable bioactive component for antioxidant activity possessed by the fungi would be the flavonoids and phenolics. These metabolite produced by endophytic fungi from C. procera need to be explored further as potential source of novel antioxidant compound.

Keywords: Endophytes, Calotropis procera, Antioxidant, Radical scavenging, Reducing power, Flavonoids.

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INTRODUCTION

Antioxidants play a very important role in preventing cell damage. The diets containing antioxidants are recommended for health improvement as naturally derived antioxidant could delay or provide protection to living organisms from damage. Reactive oxygen species (ROS), e.g., O²⁻ and OH⁻, are byproducts of normal metabolism and are found in all types of organisms from microbes to higher plants and animals. It has been established that ROS and free radical mediated reactions can cause oxidative damage to biomolecules (e.g., lipids, proteins, and DNA), eventually contributing to aging, cancer, atherosclerosis, coronary heart ailment, diabetes, Alzheimer’s disease, and other neurodegenerative disorders, etc. [1,2]. Antioxidants are thought to be highly effective in the management of ROS-mediated tissue impairments.

Medicinal plants contain a wide variety of radical scavenging molecules such as phenolic compounds (e.g., phenolic acids, flavonoids, quinones, coumarins, lignans, lignin, stilbenes, and tannins), nitrogen compounds (e.g., alkaloids and amines), vitamins, terpenoids, and other endogenous metabolites [3-5]. In addition to plants, microorganisms have been regarded as an important source of novel metabolite with promising antibacterial, antifungal, and antiviral activity. Fungi are one of the major sources of natural bioactive compounds [6]. Endophytes are recognized as rich sources of bioactive metabolites of multifold importance in medicine, agriculture, and industries [7]. Many endophytic fungi have been reported to produce novel antibacterial, antifungal, antiviral, anti-inflammatory, antitumor, and other compounds belonging to the alkaloids, steroid, flavonoid, and terpenoids derivatives and other structure types [8].

Discovery of pestacin and isopestacin as antioxidant compounds from Pestalotiopsis microspora residing in Terminalia morobenis led to the exploration of antioxidant potential [9]. A new isobenzofuranone derivative 4,6-dihydroxy-5-methoxy-7-methylphthalide with antioxidant activity was obtained in Cephalosporium sp. from Sinarundaria nitida [10]. Graphisactone A was isolated from Cephalosporium sp. as endophyte of Trachelospermum jasminoides [11]. The natural antioxidant cajaninstilbene acid, 3-hydroxy-4-prenyl-5-methoxy-stibliline-2-carboxylic acid has been reported from Fusarium as endophyte of pigeon pea. Cajanus cajan [12]. Xylaria species isolated from Ginkgo biloba and Chaetomium species isolated from Nerium oleander are some of the potential endophytes reported with a strong antioxidant activity due to their ability to produce phenolic and flavonoid [13,14].

There is great need to isolate, characterize, and develop new natural compounds present in medicinal plant which are used as a traditional (Ayurvedic) medicine to treat diseases. Endophytes are reported to produce natural products similar as to that of the host plant. Calotropis procera is one such medicinally important plant which is not much explored for the endophytes and its bioactivities. The present investigation was performed to identify the endophytic fungi of C. procera for their antioxidant potential and characterization of the active biomolecules.

MATERIALS AND METHODS

Plant material
C. procera plant samples, viz., leaf, stem, and flower parts were collected for isolation of fungi from Udaipur Rajasthan, India. Most samples
were collected during summer (temperature 35°C±2°C), the plant was authenticated by the Department of Biotechnology B.N.P.G. College, Udaipur, Rajasthan. Plant material was collected in a sterile container and carried to the laboratory. The material was used within few hours of collection. Fresh plant parts were used for isolation of plant associated fungi.

Isolation of plant associated fungi
Isolation of fungi was carried out using a modified method described by Schulz et al. [15]. The collected cut section of leaf, flower, and stem of C. procera was washed under running tap water for 15 minutes to remove the entire soil particle, and the plant samples were treated with detergent and then washed with sterile distilled water. Further surface sterilization of plant material was performed. The samples were transferred to a sterile conical flask and treated with 70% alcohol for 1 minute followed by 5% sodium hypochlorite treatment for 15 minute. The samples were finally rinsed with distilled water to eliminate the traces of any chemical left on the samples. After sterilization each plant sample was cut into small segments of 5×5 mm size and placed on freshly prepared potato dextrose agar (PDA) plates supplemented with streptomycin (500 mg/L). The plates were incubated at 27°C until fungal growth appeared. The fungal colonies appeared on the plates were transferred to fresh PDA plates, further purified and maintained on the PDA by regular sub culturing.

Morphological characterization
The fungal isolates were characterized based on colonial characterization and microscopic investigation, for colonial characterization, parameters like color of the colony, filamentous, and mat type growth was considered. The slides of both old and fresh fungal cultures were prepared using lactophenol cotton blue stain [16] and observed under microscope (Olympus CH20) at ×40 and ×100 magnification. In a microscopic investigation of the slides the structure, shape and patterns of mycelia, reproductive and nonreproductive structures, fruiting bodies, conidia, conidiophores were observed.

Preparation of fungal crude extract
Extraction of fungal secondary metabolite was performed according to the method described by Lin et al. [17] and Choudhary et al. [18]. Mycelia from 5 days old actively growing fungal culture were inoculated in 100 ml potato dextrose broth in 250 ml Erlenmeyer flasks. After 15 days of incubation at 28°C±2°C in a rotary shaker (100 RPM) culture broths were filtered with cheese cloth. The separated mycelia were soaked in methanol for 30 minutes and then crushed in homogenizer. The suspension was filtered with Whatman filter paper no.1. Filtrates were evaporated at 60°C till the residue was obtained. The residue was used as crude extract.

Radical scavenging activity by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay
Various concentrations of fungal extracts (20-100 μg/ml, 2.5 ml) were mixed with a methanolic solution of DPPH radicals (0.1 mm, 0.5 ml). The mixture was shaken vigorously and left to stand in the dark for 30 minutes. The reduction in the DPPH radical concentration was measured after the appearance of the mycelia ranging from 48 to 96 hrs. The fungal isolates were nomenclatures based on the name of the plant and the plant part from which it was obtained (Table 1).
Table 1: Fungal endophytes obtained from different plant parts and their characteristics

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Explant</th>
<th>No. of fungal isolates</th>
<th>Isolates</th>
<th>Colonial characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Leaf Midrib</td>
<td>3</td>
<td>CPIMR-1</td>
<td>White filamentous, filiform</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CPIMR-2</td>
<td>Fast growing colonies, phialide, metulae, gray color</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CPIMR-3</td>
<td>White filamentous, filiform</td>
</tr>
<tr>
<td>2</td>
<td>Leaf</td>
<td>2</td>
<td>CPIL-1</td>
<td>Fast growing colonies, yellow-brown, phialides, metulae</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CPIL-2</td>
<td>Dark brown, irregular, undulate</td>
</tr>
<tr>
<td>3</td>
<td>Stem</td>
<td>1</td>
<td>CPIS-1</td>
<td>White color, irregular and undulate</td>
</tr>
<tr>
<td>4</td>
<td>Flower</td>
<td>2</td>
<td>CPIF-1</td>
<td>White, circular, entire</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CPIF-2</td>
<td>White color, filamentous and filiform</td>
</tr>
</tbody>
</table>

The colonial characteristics of the isolates showed velvety, furrowed, and mottled type of growth with gray and yellow-brown colored colony on PDA medium. The microscopic observation of 7 days old mycelium stained with lactophenol cotton blue showed the presence of conidiophore, spores, metulae, and phialides structures in isolates. CPIMR-2 and CPIL-1 were observed with spore forming mycelium (Fig. 1). From this pattern, the fungal isolates CPIMR-2 and CPIL-1 were identified as *Penicillium* species and *Aspergillus* species, respectively.

Various medicinal plants were reported in the presence of endophytes in different plant parts where *Calotropis* is one of the less explored plants for their fungal endophytes. So far two species of *Calotropis*, *C. procera*, and *Calotropis gigantea* were studied for the endophytic fungal biodiversity and were reported with endophytes in leaf midrib, leaf lateral, stem, and flower parts [26,27]. In this study, fungal isolates were obtained from flower and leaf explants. Two of the fungal isolates that we obtained from the leaf were identified to be *Penicillium* species and *Aspergillus* species. Similar endophytes were reported by Khan *et al.* (2007) in *C. procera* that include *Aspergillus* and *Penicillium* species along with *Candida* and *Phoma* species from stem part [26]. *Aspergillus* and *Penicillium* species were also reported in other medicinal plants, viz., *Hibiscus tilaicus*, * Catharanthus roseus*, *Salvadora oleoides*, and *Biophytum sensitivum* [28-30].

**Radical scavenging activity by DPPH assay**

Out of total 8 fungi, the extracts of *Penicillium* species (CPIMR-2) and *Aspergillus* species (CPIL-1) showed positive results for antioxidant activity by DPPH radical scavenging assay. A decrease in the oxidation activity of DPPH radical was observed in both the fungal extracts due to their radical scavenging ability. A maximum inhibition of DPPH oxidation was found to be 81.00±0.71% in the methanolic extract of CPIMR-2 at a concentration of 60 µg/ml and 80.97±0.78% in CPIL-1 at a concentration of 80 µg/ml which was considered to be significant as compared to the percent inhibition values obtained with AA as a standard antioxidant (Fig. 2).

In our study, the methanolic extract of two endophytic isolates CPIMR-2, i.e., *Penicillium* species and CPIL-1, i.e., *Aspergillus* species has showed an increasing radical scavenging effect with increased concentration of crude extract. The antioxidant activity was encouraging (80.27% and 79.62%) even at 60-80 µg/ml in comparison to the activity reported in the extract of endophytic fungi *Aspergillus* species and *Penicillium* species isolated from *Hugonia mystax* which showed 88.1% and 36.5% at 100 µg/ml concentration [31]. A 72% inhibition of oxidation was reported in *Aspergillus fumigatus* isolated from *Gracina species* [32]. Similarly, 70% inhibition at 200 µg/ml concentration was found to be reported in crude extracts of *Penicillium* species from *Centella asiatica*, whereas, 53.12% inhibition was reported in extract of fungi isolated from *Ocimum sanctum* at 100 µg/ml [33-34]. In comparison to these values the potential of crude extracts of CPIMR-2 and CPIL-1 for quenching the free radical (DPPH) can be considered to be potential resources for antioxidant actives.

**Reducing power**

The reductive ability of sample extracts was determined by measuring its ability to transform Fe³⁺ to Fe²⁺. The color change of the test solution from yellow to various shades of green and blue were observed depending on concentration and the reducing power of the compounds present in fungal extracts. Fungal extracts of CPIMR-2 (*Penicillium* sp.) and CPIL-1 (*Aspergillus* sp.) were observed positive for reducing power, similar to that of DPPH assay. The reducing power of the extracts and standard AA increased with an increase in the concentration as appeared form the absorbance (OD) values. The fungal extracts showed less reducing power as compared to that of AA, which was analyzed as a positive control (Fig. 3).

The methanolic extracts of both the fungi, i.e., *Aspergillus* species (CPIL-1) and *Penicillium* species (CPIMR-2) showed a potent reducing...
power. Maximum reducing power was observed at a concentration of 0.1 mg/ml in both the extracts. Among the two endophytes, CPIL-1 exhibited high reducing power. Similar results for reducing power in the crude extracts of endophytes have been reported in studies by Kekuda et al. [35]. In the studies reported by Zheng et al., the aqueous extract of Tolypocladium species isolated from wild Cordyceps sinensis has shown a moderate reducing power activity [36].

Qualitative analysis of flavonoids

The results of the qualitative phytochemical analysis inferred that the methanolic extract of fungi contains flavonoids. A yellow was developed after addition of NaOH which disappeared on neutralization with dil. HCl. A change in color from yellow to colorless on addition of dil. HCl was an indication of the presence of flavonoids. In TLC analysis of the extracts, the chromatogram developed showed a single dark spot in the extract of CPIMR-2 and a fluorescent blue spot in the extract of CPIL-1 against a fluorescent green background (Fig. 4). The color of flavonoids may vary from dark to yellow, green, or blue fluorescent in UV light based on the chemical structure of flavonoid.

Total flavonoids and phenolics

The concentration of flavonoids and phenolics in the two fungal extracts of the C. procera that showed a positive test for RSA and reducing power (i.e., CPIMR-2 and CPIL-1) was determined using a spectrophotometric method. The total flavonoid content in the extracts was expressed in terms of quercetin equivalent using standard curve equation \( Y=0.0007X \), \( R^2=0.9586 \). In CPIMR-2 and CPIL-1, the flavonoid content was found to be 130.50±6.111 mg/g and 94.91±4.483 mg/g dry weight of fungal extract, respectively (Table 2).

The total phenolic content was expressed in terms of gallic acid equivalent using standard calibration curve equation \( Y=0.0177X \), \( R^2=0.8987 \). The phenolic content was found to be 9.16±0.71 mg/g and 12.13±3.96 mg/g of dry weight of extract in CPIMR-2 and CPIL-1, respectively (Table 2). The presence of similar quantities of phenolic content was also reported in methanolic extract of Aspergillus species and Penicillium species isolated from Tabebuia argentea (19.20 and 16.23 mg/GAE/g) [37].

Antioxidant property of natural products is attributed to the secondary metabolites of the plant kingdom, particularly phenolics and flavonoids, many polyphenols, tannic acid has been shown to possess antioxidant activity [39]. Various fungal species have been also reported to produce these compounds and found to possess the antioxidant potential as well [39]. The production of these secondary metabolites (i.e., phenolics and flavonoids) has been reported in different endophytic fungi, including Aspergillus nidulans and Aspergillus oryzae isolated from G. biloba [40] and Aspergillus niger and Fusarium oxysporum isolated from Crotalaria pallida [37].

In various studies, the population and biodiversity of the fungal endophytes have been found to be varying as the environmental conditions under which the host is growing, the host plant composition, as well as seasonal variation, affects the endophytes population [41]. Some of the medicinal characteristics of the host plant may also be found to be similar to that of the endophytes. C. procera is one of such medicinal plant which has been studied and reported with various medicinal potential such as anthelmintic properties that relieve stranguary, cure ulcers, cure piles, asthma, and wounds [42]. The compounds phenolic acids, flavonoids, and tannins are the most commonly found polyphenolic compounds in plant extracts [43]. The phenolics of C. procera were reported to possess the antioxidant property [44]. The isolates of C. procera CPIMR-2 and CPIL-1 were also found to possess the similar characteristics. From the findings of our studies, the compounds flavonoids and phenolics are the key bioactives responsible for the antioxidant and reducing power. These fungal isolates should be considered as the resource for production of antioxidants and its medicinal importance.

ACKNOWLEDGMENT

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Table 2: Total flavonoid and phenolic content present in the extracts of the endophytic fungal isolates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total flavonoid content in mg/g of extracts (mean±SD)</th>
<th>Total phenol content in mg/g of extracts (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPIMR-2 [Penicillium sp.]</td>
<td>130.50±6.111</td>
<td>9.16±0.71</td>
</tr>
<tr>
<td>CPIL-1 [Aspergillus sp.]</td>
<td>94.91±4.483</td>
<td>12.13±3.96</td>
</tr>
</tbody>
</table>

*Values are mean±SD of three triplicates (n=3), SD: Standard deviation