Antibacterial Activity and Phytochemical Analysis of the Crude Extracts of Endophytic Fungus, Alternaria sp. from the Medicinal Plant Euphorbia hirta (L)

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Abstract
Endophytic fungi are a noble and consistent source of unique natural amalgams with a high level of biodiversity and may also yield several compounds of pharmaceutical significance, which is currently attracting scientific surveys worldwide. Every plant in the world is reservoir of much number of endophytes. In nature, plants seem to be in a close interface with endophytic fungi. Euphorbia hirta (L) is known to possess medicinal properties. Only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive secondary metabolites. There is a need to understand the biodiversity of endophytic fungi and their potential of producing novel compounds of medicinal importance. Our study revealed potential antimicrobial activity and phytochemical analysis of endophytic fungi isolated from fresh leaves of Euphorbia hirta. A total of six endophytic fungi were isolated on PDA medium and cultural characteristics of the obtained isolates were studied. The crude extracts of six endophytic fungi obtained were screened for antibacterial activity against E. coli, Pseudomonas sp1, Pseudomonas sp2, S.typhi, Bacillus sp, and S.aureus by using three different solvents such as methanol, ethyl acetate and chloroform by disc diffusion method. Amongst all the endophytic fungi, VDPS-3 crude extract from ethyl acetate showed encouraging results with good zone of inhibition compared to the other two solvents, so further it was subjected for the phytochemical analysis and this was identified as Alternaria sp. Phytochemical analysis of VDPS-3 revealed that the culture filtrate contains alkaloids, terpenoids, cardiac glycoside and tannins with a rich source of wide range of bioactive compounds which evaluates not only their clinical potential but also their use in various other industries.

Key words: Endophytic Fungi, Alternaria sp., Phytochemical analysis, Antibacterial activity.

1. Introduction
The word endophyte means “in the plant” (endo-within, phyt-on-plant). The term “Endophyte” originally coined by German scientist Heinrich Anton De Bray in 1884, who recognized that fungi and bacteria could dwell within plant tissues without causing any apparent harm to the host plant. Endophytes are organisms that live within plant tissues for all or part of their life cycle and cause no apparent infection [1]. As endophytes are ubiquitous within the plant kingdom, many evidences have demonstrated that the fungal endophytes are of hyper diverse and abundant groups [2, 3, and 4]. Endophytic fungi that reside in the above ground tissues of plant have been considered as source of novel biologically active secondary metabolites [5]. Over the past several decades there have been numerous published articles on endophytic fungi as a source of bioactive natural products with numerous potential applications in agricultural, medicine and food industry. Endophytic fungal communities are influenced by many factors such as geographical location, climatic patterns, physiology and specificity of colonized tissue. It is assumed that because of a long period of co-evolution and association, there is possible inter-generic exchange of genetic information between host plant and endophytes. Endophytic fungi have the ability to produce same or similar bioactive compounds as those originated from their host plant. Some endophytic fungi are rare; many of the biological active substances extracted from endophytic fungi are reported to be novel. Therefore, there is a worldwide renewed scientific effort to isolate endophytes and study their natural products, which play a vital role as
anti-bacterial, anti-viral, anti-oxidant, anti-arthritis, anti-diabetic, and as immune suppressive compound [6, 7].

Emergence and reemergence of new diseases, development of drug resistant pathogen is big challenge in fields like pharmaceutical and biomedicine [8]. Therefore, development of odor modification in antimicrobial compounds to improve bactericidal potential is a priority area of research in the modern era. This situation has forced researchers to explore different natural sources for the safe and potent agents to meet the challenges of 21st century [9].

In the present study, we have isolated and identified endophytic fungi till generic level from a medicinal plant Euphorbia hirta (L) and its extracts were separated using different solvents; methanol, ethyl acetate and chloroform and these extracts were further subjected for antibacterial activity and also phytochemical analysis.

1. Materials and Methods
1.1. Collection of Plant Material
Plant material was collected from Botanical Garden, Gulbarga University Campus, Kalaburgi, India in rainy season. The plant was identified on the basis of external morphology and characteristic features. A complete mature and healthy plant leaves were collected in sterile polythene bags and brought to the laboratory. Samples were preserved for the isolation of endophytic fungi immediately [10].

1.2. Isolation of Endophytic Fungi
Methods used for isolating endophytic fungi were described previously with minor modification [11, 12]. The plant sample was washed with running water. The leaves were cut into 1cm; the segments were sectioned vertically into small pieces. These pieces were surface sterilized by sequentially rinsing in 70% ethanol (C2H5OH) for 30 sec, 0.01% mercuric chloride (HgCl2) for 5 min, and 0.5% sodium hypochlorite (NaOCl) solution for 2-3 min, then finally rinsed with sterile distilled water and allowed to dry under sterile conditions. The cut surface of the segments were placed on petridish containing Potato Dextrose Agar (PDA) supplemented with Streptomycin sulfate (250 mg/ml), incubated at 28°C for 6-8 days and monitored every day for the growth of endophytic fungal colony from leaf segment [10,13]. The fungal isolate were identified based on its morphological and reproductive characters using standard identification manual.

1.3. Extraction of Secondary Metabolites from Endophytic Fungi
The fungi grown out from tissues were brought into pure culture on PDA plates and incubated for 72h at 28°C.Obtained fresh mycelia were transferred into 250ml flask containing 100ml Potato Dextrose Broth and kept under static condition for 4-5 days at 28°C [11]. The fungal mycelia were separated by filtering through filter paper and then repeatedly washed with distilled water to remove the media components from the fungal biomass. The biomass obtained was re-suspended into solvents such as methanol, ethyl acetate and chloroform and kept under shaking condition at 28°C for 10 days. Then the solvent phase was separated from the flask containing biomass and collected into a watch glass and air dried in sterile condition. The dried extracts were re-dissolved in solvents and stored at 4°C for further use [14, 15].

1.4. Antibacterial Activity of the Crude Extracts from Endophytic Fungi
Antibacterial activity was analysed using crude extracts of endophytic fungi isolated from Euphorbia hirta (L) using solvents such as methanol, ethyl acetate and chloroform against bacterial pathogens such as E.coli, Salmonella typhi, Bacillus sp, Pseudomonas strains and Staphylococcus aureus. The test bacterial strains were grown in nutrient broth for 4-5 h at 37°C. The lawn of bacterial pathogens was prepared on nutrient agar plate using sterile swabs. Sterile filter paper discs were impregnated with 10µl of crude extracts and placed on the surface of the nutrient agar medium and incubated at 37°C for 24 h. The plates were examined for the zone of inhibition which appeared as clear area around the discs and zone diameter was measured [14].

1.5. Phytochemical Screening
The endophytic fungal extract exhibiting broad spectrum antibacterial activity was further subjected for phytochemical analysis which serves as a major resource for information on analytical and instrumental methodology in the plant sciences. This determines the presence of bioactive compounds in the extract. Phytochemical analysis was carried out according to Senthilmurugan et al., [16] with slight modifications.

1.5.1. Test for Flavonoids
Test-tube containing 1-2 ml of fungal crude extract, 5-10 drops of dilute HCl, a piece of Magnesium strips were added and the solution was boiled for few minutes . A reddish pink or dirty brown coloration of solution indicates the presence of flavonoids in the extract [16, 17].

1.5.2. Test for Alkaloids
The fungal crude extract was evaporated to dryness in boiling water bath. The residue was dissolved in 2N HCl. The mixture was treated with equal amount of Wagner’s reagent. The reaction shows the appearance of brown precipitate, indicates presences of alkaloids [18].

1.5.3. Test for Terpenoids
Fungal crude extract of 1ml was mixed in 1ml of chloroform. 1-2 drops of concentrated H2SO4 was then added to form a layer. A cherry red color or reddish-brown precipitate at the interface indicates the presence of terpenoids [16].

1.5.4. Test for Cardiac glycosides
The presence of cardiac glycosides was analyzed by taking fungal crude extract treated with 1ml of FeCl3 reagent (mixture of 1 volume of 5% FeCl3 solution and 99 volumes of glacial acetic acid). To this solution few drops of concentrated H2SO4 was added. Appearance of greenish blue color within a few minutes indicates the presence of cardiac glycosides [16, 19].

1.5.5. Test for Steroids
Fungal crude extract was added with 1ml of chloroform solution. The extract was treated with acetic anhydride and a few drops of concentrated H2SO4 were added down the sides of the test tube. A blue green ring indicates the presence of steroids [16, 19].

1.5.6. Test for Saponins
The presence of saponins was determined by frothing test.
The crude dry powder of fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 min. No froth indicates absence of saponins and stable forth more than 1.5cm indicated the presence of saponins [18, 19].

1.5.7. **Test for Phenols**

The fungal crude extract was dissolved in 5ml of distilled water. To this few drops of neutral 5% FeCl₃ solution was added. A dark green indicates the presence of phenolic compounds [16, 19].

1.5.8. **Test for Tannins**

The fungal crude extract was treated with alcoholic FeCl₃ reagent. A bluish black color, which disappears on addition of a little dilute H₂SO₄ followed by the formation of yellowish brown precipitate, indicates the presence of tannins [16, 20].

**Table-1:** Cultural characteristics of the endophytic fungi isolated from sterilized leaf segments of *Euphorbia hirta*

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Colony Code</th>
<th>Media Type</th>
<th>Size</th>
<th>Texture</th>
<th>Surface color</th>
<th>Pigmentation</th>
<th>Topography</th>
<th>Sporulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>VDPS-1</td>
<td>PDA</td>
<td>Large</td>
<td>Velvety</td>
<td>Grayish black</td>
<td>Black</td>
<td>Concentric</td>
<td>Heavy</td>
</tr>
<tr>
<td>2.</td>
<td>VDPS-2</td>
<td>PDA</td>
<td>Large</td>
<td>Thick velvety</td>
<td>Creamish brown</td>
<td>Black</td>
<td>Concentric</td>
<td>Heavy</td>
</tr>
<tr>
<td>3.</td>
<td>VDPS-3</td>
<td>PDA</td>
<td>Medium</td>
<td>Velvety</td>
<td>Creamish white</td>
<td>No Pigment</td>
<td>Raised</td>
<td>Medium</td>
</tr>
<tr>
<td>4.</td>
<td>VDPS-4</td>
<td>PDA</td>
<td>Medium</td>
<td>Cottony</td>
<td>Creamish yellow</td>
<td>Light yellow</td>
<td>Flat</td>
<td>Poor</td>
</tr>
<tr>
<td>5.</td>
<td>VDPS-5</td>
<td>PDA</td>
<td>Medium</td>
<td>Velvety</td>
<td>Centre light brown with peripheral Creamish white</td>
<td>No Pigment</td>
<td>Concentric</td>
<td>Medium</td>
</tr>
<tr>
<td>6.</td>
<td>VDPS-6</td>
<td>PDA</td>
<td>Small</td>
<td>Solid velvety</td>
<td>Centre brown with orange margin</td>
<td>Orange</td>
<td>Hilly</td>
<td>Poor</td>
</tr>
</tbody>
</table>

**Table-2:** Crude extracts of endophytic fungi and their antibacterial activity against pathogenic bacteria

<table>
<thead>
<tr>
<th>Bacterial Pathogens</th>
<th>Methanol extract</th>
<th>Ethyl acetate extract</th>
<th>Chloroform extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VDPS 1</td>
<td>VDPS 2</td>
<td>VDPS 3</td>
</tr>
<tr>
<td><em>E.coli</em></td>
<td>04</td>
<td>04</td>
<td>08</td>
</tr>
<tr>
<td><em>Pseudomonas sp-1</em></td>
<td>02</td>
<td>10</td>
<td>05</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>03</td>
<td>-</td>
<td>06</td>
</tr>
<tr>
<td><em>Bacillus sp</em></td>
<td>02</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td><em>S.aureus</em></td>
<td>06</td>
<td>-</td>
<td>11</td>
</tr>
</tbody>
</table>

**Fig.1:** Isolation of Endophytic fungi from sterilized leaf segments of *Euphorbia hirta* plant

The crude dry powder of fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 min. No froth indicates absence of saponins and stable forth more than 1.5cm indicated the presence of saponins [18, 19].

2. **Results and Discussion**

2.1. **Isolation of Endophytic Fungi**

Endophytic fungi were isolated from the fresh leaves of *Euphorbia hirta* a medicinal plant. A total of six endophytic fungi were isolated on PDA medium and coded as VDPS-1, VDPS-2, VDPS-3, VDPS-4, VDPS-5, and VDPS-6 (Fig-1). Cultural characteristics of the obtained isolates were also studied and are presented in (Table-1).

2.2. **Antibacterial Activity of the crude extract screened from only ethyl acetate**

The crude extracts of six endophytic fungi obtained using three solvents such as methanol; ethyl acetate and chloroform were subjected for antibacterial activity using disc diffusion method (Table-2). The results of the antibacterial activity of the crude extract screened from the
solvent methanol against the bacterial pathogens revealed that VDPS-5 showed maximum zone of inhibition of 13mm and 11mm against Bacillus sp and Pseudomonas sp-1, while VDPS-6 showed 12mm zone of inhibition against Salmonella typhi. The crude extract of VDPS-3 showed 11mm and 10mm zone of inhibition against Staphylococcus aureus and Bacillus sp respectively, while VDPS-2 showed 10mm zone of inhibition with Pseudomonas sp-2. The crude extract from other isolates lied in between. The chloroform extract of six fungal endophytic cultural isolates revealed that 11mm each zone of inhibition was obtained with crude extract of VDPS-3 and VDPS-6 against Pseudomonas sp-1 and Bacillus sp, while 10mm each zone of inhibition was observed with VDPS-5 and VDPS-2 against Pseudomonas sp-2 and E. coli. The zone of inhibition with other isolates was in between.

The antibacterial activity of the endophytic fungal crude extract from ethyl acetate showed very good results compared to the other two solvents. VDPS-3 showed the maximum zone of inhibition of 22mm against Pseudomonas sp-1, 14mm each with Bacillus sp and S aureus, 11mm and 10mm with Pseudomonas sp-2 and E. coli. Next to it was VDPS-4 which showed maximum zone of inhibition of 19mm with E. coli and 11mm with Bacillus sp. VDPS-2 and VDPS-5 showed 12mm each zone of inhibition with Pseudomonas sp-2 (Fig-2).

| Table-3: Phytochemical analysis of Alternaria sp. (VDPS-3) |
|----------------|-----------------|
| **Phytochemical** | **Results** |
| Analysis of VDPS-3 |          |
| Flavonoids         | Negative        |
| Alkaloids          | Positive        |
| Terpenoids         | Positive        |
| Cardiac glycosides | Positive        |
| Steroids           | Negative        |
| Saponins           | Negative        |
| Phenols            | Negative        |
| Tannins            | Positive        |

Looking into the results of antibacterial activity of endophytic fungal crude extracts by using solvent such as methanol, ethyl acetate and chloroform. It is deduced that ethyl acetate acted as a good solvent in extracting the compounds which showed good zone of inhibition. Further, amongst the isolates the crude extract of VDPS-3 extracted using ethyl acetate was only taken further for phytochemical analysis and this was identified as Alternaria sp (Fig-3).

Fig. 2. Zone of inhibition of ethyl acetate crude extract of VDPS-3 only

Fig. 3: (A) Ethnobotanical medicinal plant, Euphorbia hirta. B) Endophytic fungi grown from sterilized leaf segment on PDA media. C) Microscopic image of endophytic fungi, Alternaria sp.

2.3. Phytochemical Analysis

Phytochemical analysis of VDPS-3 was done. Of the three solvent used, the solvent ethyl acetate extract showed encouraging results against pathogenic organisms. Therefore, the phytochemical analysis of VDPS-3 was done. The results of the Phytochemical analysis revealed that the culture filtrate contain alkaloids, terpenoids, cardiac glycoside and tannins (Fig-4 and Table-3).

The medicinal plant chosen was Euphorbia hirta (snake root) which is a pantropical weed. This plant is selected for the work due to the fact that the entire plant parts have medicinal value such as antibacterial, antiviral, anti-inflammatory [21].

Tannins are phenolic compounds found in variety of plants. They are reported to have close interaction with proteins in the body exhibiting antimicrobial effects [22]. Tannins are reported to be effective against a wide range of bacterial strains. They have ability to inactive microbial adhesion, enzymes, cell envelop, transport protein and also have ability to complex polysaccharides.

Alkaloids are the group of naturally occurring chemical compounds that contain mostly basic nitrogen atoms. Most of the alkaloids are amphoteric in nature. Many of alkaloids are usually used as salts in medicine. Alkaloids like Ajmaline is used as anti arrhythmic drug. Tuboeruranie as the muscle relaxant, Vinblastine as antitumor, Yohimbine as the stimulant in many medicinal therapies. Most of the alkaloids affect the nervous system, particularly the action of the chemical transmitters (example: acetylcholine, epinephrine). Many alkaloids serve as models for the chemical synthesis of analogues with better properties. Important examples are Hyoscymaine and Scopolamine as a model for synthetic parapamatholyptic agents [23].

Cardiac glycosides are organic compounds containing a glycoside (sugar) that act on the contractile force of the cardiac muscle. These glycosides are found as secondary metabolites in several plants but also in some insects such as butterflies. Purified extract or synthetic analogues of few have been adapted for the treatment of congestive heart failure and cardiac arrhythmia. Therapeutic uses of cardiac glycosides primarily involve the treatment of cardiac failure. There utility results from
Terpenoids are generally the secondary metabolites occurring in the plants. Terpenoids are the largest group of natural products which have proved increased interest in researcher for their commercial use [25].

Our results revealed the presence of alkaloids, terpenoids, cardiac glycosides and tannins. Large number of investigations has unraveled the unique properties of these compounds by evaluating not only their clinical potential but also their use in food, feed and also other industrial uses. These compounds are suggested to be novel candidates in chemo preventive and chemotherapeutic strategy to combat various diseases.

A total six Endophytic fungi were isolated on PDA medium from the fresh leaves of Euphorbia hirta. Sadanand et al. [26] reported the presence of phytochemicals in endophytic fungi is an indicator which will be a potential source of precursors in the development of synthetic drugs. Many valuable bioactive compounds antimicrobial, insecticidal, cytotoxic anticancer activities have been successfully discovered from these Endophytic fungi. These bioactive compounds can be classified as alkaloids, terpenoids, steroids, quinines, lignin, phenols and lactones [27].

The antibacterial activity of the Endophytic crude extract of Alternaria sp. from ethyl acetate showed maximum zone of inhibition 22mm against Pseudomonas sp by VDPS-3. Chandrappa et al., [19] also reported that the ethyl acetate extract of Aspergillus niger showed maximum zone against Shigella flexner followed by Pseudomonas aeroginosa and Bacillus subtilis whereas no inhibition against Klebsiella pneumonia.

Ravindra Prasad Aharwal et al. [28] revealed that out of 12 endophytic fungi, 2 fungal strains Curvularia pallescens and Alternaria alternate showed potent antibacterial activity. Alternaria alternate showed 25mm and 25mm, Klebsiella pneumonia, S. epidermis, B. subtilis and E.coli, and Curvularia pallescens showed 27mm, 25mm, and 24mm zone against B. subtilis, Klebsiella pneumonia, S. epidermis and E.coli respectively. Drechslera nodulosa showed minimum activity 12mm against Pseudomonas aeroginosa.

Other endophytic fungal extract which showed low antimicrobial activity in the bioassay may have active compounds but probably in smaller amounts and or the screened crude extracts could yield more potent compounds once they had undergone some purification [29].

The Phytochemical analysis of VDPS-3 revealed that the culture filtrate contain alkaloids, terpenoids, cardiac glycosides and tannins. The results of antibacterial activity of these phytochemical compounds may be due to the potential components present in the phytochemicals in the Endophytic fungi. Our results are confirmed with the finding of Cushine and Lamb, [30], Saravanaakumar et al., [31], Doss et al., [32] and Maneemagalai et al., [33].

**Conclusion**

Endophytes are gaining importance because of their enormous potential to produce novel bioactive compounds of agricultural and medicinal importance. The current study has presented the endophytic fungi from Euphorbia hirta (L). Further, studies of purification and characterization of these fungal extracts may lead to the discovery of novel antimicrobial compounds of pharmaceutical importance which would then be produce on a large scale and their applications may trigger the current research to accomplish environmental friendly technological development.

**REFERENCES**


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