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BIOCHEMICAL EVIDENCES TO ESTABLISH THE POSSIBLE INTERACTION BETWEEN HOST PLANT *OROXYLUM INDICUM* AND ENDOPHYTIC FUNGUS *GEOTRICHUM PSEUDOCANDIDUM*

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ABSTRACT
Oroxylum indicum (L) Vent. is an important medicinal tree possesses numerous metabolites of medicinal value. Nowadays, research is going on to find out role of metabolic activities of endophytic fungi to support the existence of their host. In this series, present paper describes a deep study of various fractions of Plant root and fungal endophyte of root to observe similarities and differences in both type of metabolite fractions. Endophytic fungi *Geotrichum pseudocandidum* isolated and identified from root of *Oroxylum indicum* on PDA medium. Its metabolic extract showing antibacterial activities has been taken and fractionized with Chloroform, Ethyl acetate and n- butanol solvents. The compounds were analyzed by GC-MS technique. The root extracts (methanol and ethanol) from the tree *Oroxylum indicum* was also screened by the same technique and were analyzed. The main components in all fractions of root and metabolites of endophytic fungi were 3–Cyclohexene–1–ol, 4–mehtyl–1–(1–methylethyl) – (R) also known as Terpinen–4–ol and (–)–5–oxatricyclo (8.2.0.0 (4, 6) and dodecane, 12–TRI which is also known as caryphyllene oxide. The study aims to develop a link between the endophyte and its host plant through biochemical evidences.

KEYWORDS: GC-MS; *Oroxylum indicum*; Endophyte; Host- endophyte interaction.

INTRODUCTION
Endophytes are ubiquitous and have been found in all the species of plants. Many economically important grasses carry fungal endophytes some of which may enhance host growth, may improve the plants ability to tolerate abiotic stress such as drought, as well as improve their resistance to insect and mammalian herbivores (Doty, 2011). Some endophytes protect their host from insect by producing bioactive metabolites (Jalgaonwala, 2010). Recent studies suggest that endophytic fungi are not host specific (Budhiraja, 2013).

Oroxylum indicum (L.) Vent is a small to medium-sized deciduous tree with its distribution in tropical and subtropical regions. It is commonly known by various names such as Indian Trumpet, Indian Calosanthes, Midnight horror *etc*. Each and every part of *O. indicum* is considered to be useful and is used in several traditional and Ayurvedic folk medicines (Raghu *et al*., 2013). The plant has anti-cancerous (Mao 2002; Prakash *et al*., 2013); anti-inflammatory (Doshi *et al*., 2012); anti-oxidant (Kalaivani and Mathew, 2009; Moirangthem *et al*., 2013); anti-microbial (Radhika *et al*., 2011) and anti-arthritic properties (Karnati *et al*., 2013). The plant contains flavonoids like chrysin, oroxylin, and baicalein as active principal components (Choudhury *et al*., 2011; Raghu *et al*., 2013). Besides, a number of other compounds have been reported from different parts of this plant (Deka *et al*., 2013). The contribution of endophytes to their host plant in showing various beneficial properties cannot be overruled as are evident from literature already cited above. *O. indicum* is not only a potent medicinal plant with a diverse array of useful phytochemicals but also harbor diverse endophytes (Gu *et al*., 2006; Gupta *et al*., 2014; Gokhale *et al*., 2017).

The link between endophyte and the plant can be established by thorough studies of the metabolites, of the plant and the endophyte both, and thereby the characters expressed by both the entities as individuals and as an interactive unit. In the present study, an attempt was made to study the fungal endophyte *Geotrichum pseudo candidum* associated with roots of *O. indicum* and their metabolite interaction.

To make the study simple and easier the crude extract of the plant and endophyte was fractionated into five fractions. In the five fractions, four compounds were common in the metabolites of root and fungal extract.

MATERIALS AND METHODS
Collection of plant samples
Plant material (roots) were collected from healthy plants growing in the reserve forest area in and around Jabalpur, M.P, India .The samples were collected in sterile polypropylene bags and were processed within 24 hours of collection for isolation of endophytic fungi.

Surface sterilization of plant materials
The collected plant materials were thoroughly washed under running tap water; air dried and then cut into approximately 1 cm long pieces with the help of sterile surgical blade. Surface sterilization was performed aseptically by following the method of Ahmed *et al*. (2012). Plant materials were sequentially immersed in 70% ethanol (C2H5OH) for 30 sec followed by 5 min in

0.01% mercuric chloride ($HgCl_2$), 2-3 min in 0.5% sodium hypochlorite (NaOCl) and finally rinsed with 3 sets of sterile distilled water. Samples were then air dried on sterile blotting paper.

Isolation and Characterization of endophytic fungi

After proper drying, outer tissues of the surface sterilized plant part were excised and inner tissues were taken for isolating endophytes. Three or four excised segments were placed on Petri plates containing PDA (Potato Dextrose Agar) media. To prevent bacterial contamination the inoculated medium was amended with streptomycin (25µg/ml). The plates were then sealed with parafilm and incubated in the BOD incubator (Macro Scientific works Pvt. Ltd.) at 28 ±2ºC. The inoculated plates were monitored every day for growth of endophytic fungi. The pure culture slants were preserved and maintained at 4ºC for routine use. The colonies of isolated fungus from root were studied for their macroscopic features i.e. color, shape and growth of cultured colonies *etc*. and ultimately the isolate was identified as *Geotrichum pseudocandidum* (Gokhale *et al*., 2017)

Extraction of secondary metabolites from the root of Oroxylum indicum

The dried roots were ground into a fine powder form. The root powder (5g) was dissolved in 100ml ethanol and was incubated in an orbital shaker120 rpm for 24 hours at 25 ±2^{o}C. After incubation period ethanolic crude root extract was filtered and evaporated to dryness. Residue was dissolved in 1 ml distilled water which was then acidified, neutralized again filtered and used as aqueous extract.

GC-MS analysis

Gas chromatography-mass spectrometry (GCMS) analysis of crude fungal extracts was carried out at the advanced instrumentation research facility (AIRF), GC-MS analysis of the crude was performed on a Shimadzu GC-MS-QP-2010 plus system. RTx-5 SilMS column (30 m × 0.25 mm id × 0.25 µm film thickness) was used with the following operating conditions: oven temperature program from 80°C-250°C at 5°C/ min with holding time of 4 min and from 250°C-310°C at 20°C/ min with holding time of 5 min, and the final temperature was kept for24 min. The injector temperature was maintained at 270°C, pressure 81.7 kPa, total flow 16.3 ml/min, column flow 1.21 ml/ min, linear velocity 40.5cm/ sec, purge flow 3.0 ml/ min, split ratio 10.0,ion source temperature 230°C, scan mass range m/z 40-600, and interface line temperature 280°C.

The identification of compounds was performed by comparing the mass spectra with data from NIST (National Institute of Standards and Technology, US), Wiley, Pesticide Library 3rd edition, Drug Library, GC/MS Metabolite Mass Spectral Database and FFNSC (Flavor and Fragrance Natural and Synthetic Compounds) libraries.

RESULTS

GC–MS Analysis of endophytic fungus

The GC-MS analysis was used as a tool to identify several compounds extracted from different fractions of ethyl acetate, chloroform and n–butanol isolated from endophytic fungi *G. pseudocandidum* (Fig 4-6). The highest area percent (as obtained in GC-MS chromatograms) was obtained in all fractions for 3–Cyclohexene–1–ol, 4–mehtyl–1–(1–methylethyl)–(R) – also known as Terpinen–4–ol (Table 2-4). The second major common compound in all three fractions is (–)–5–Oxatricyclo (8.2.0.0 (4, 6)) Dodecane, 12–Tri, also called Caryphyllene oxide.

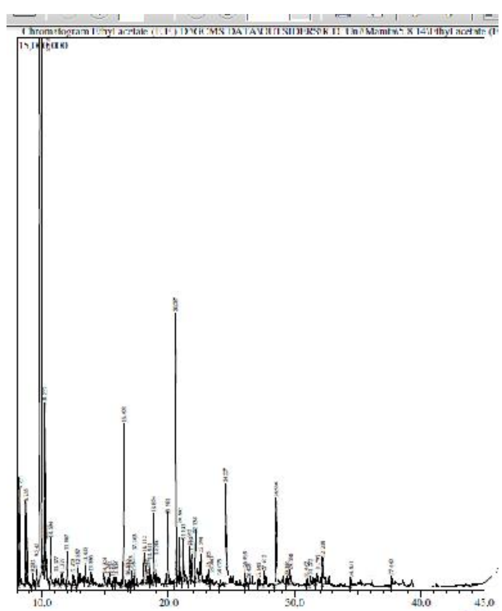


FIGURE 1: GC/MS Chromatogram of volatile compounds from *Geotrichum pseudocandidum* ethyl acetate fraction.

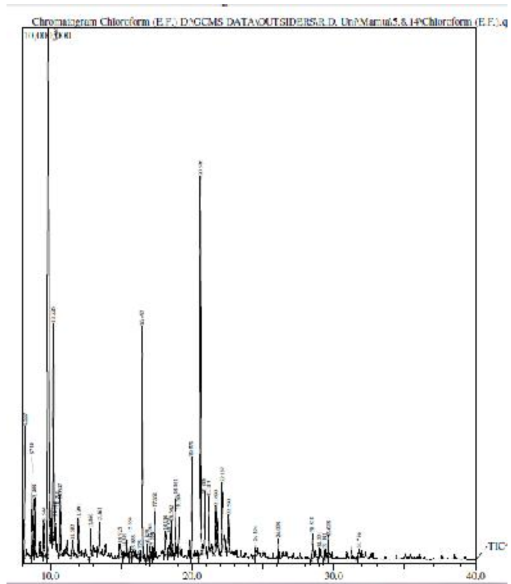


FIGURE 2: GC/MS Chromatogram of volatile compounds from *Geotrichum pseudocandidum* chloroform fraction.

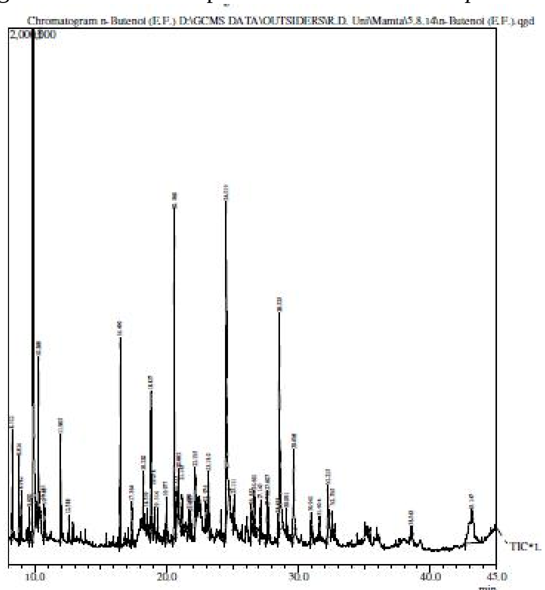


FIGURE 3: GC/MS Chromatogram of volatile compounds from *Geotrichum pseudocandidum* n–butanol fraction extract.

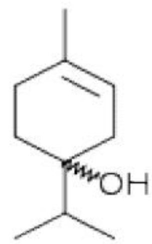
Quantitative estimation of important flavonoids in different fractions of root extract

In the GC-MS analysis of ethanol and methanol extract of root of *O. indicum*, maximum number of compounds (37) were isolated in ethanol extract, whereas in methanol extract , the number of compounds isolated were 35. Baicalein (Rt 40.70) and chrysin (Rt 41.79) compounds were detected in both extract of root.

Four compounds are common in ethanol and methanol fractions from root of *Oroxylum indicum* and in chloroform, ethyl acetate and n-butanol fractions of metabolites from isolated fungus. The compounds with their significance and chemical structures –

1) **3-Cyclohexene-1-ol,4-mehtyl-1-(1-methylethyl)-, (R) –** (C10H18O)

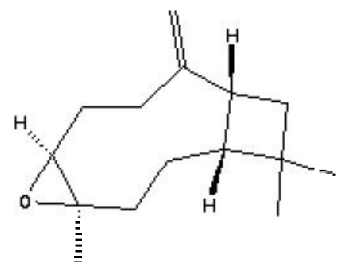
This compound is also known as Terpinen-4-ol with molecular weight 154.249g/mol. It acts as an antibacterial agent, an antioxidant, an anti-inflammatory agent, an antiparasitic agent, an antineoplastic agent, an apoptosis inducer and a volatile oil component. It is a terpineol and a tertiary alcohol. It is considered as the primary active ingredient of tea tree oil. Tea tree oil is toxic when taken by mouth, but is widely used in low concentrations in cosmetics and skin washes. Tea tree oil has been claimed to be useful for treating a wide variety of medical conditions. It shows some promise as an antimicrobial. This oil may be effective in a variety of dermatologic conditions including dandruff, acne, lice, herpes, and other skin infection.



3-Cyclohexene-1-ol, 4-mehtyl-1-(1-methylethyl)-, (R)-

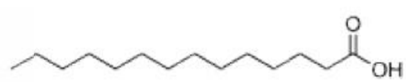
2**) (-)-5-OXATRICYCLO [8.2.0.0(4, 6)] DODECANE, 12-TRI (C15H24O)**

This is the second major common compound in all three fractions with molecular weight 220g/mol. It is also called Caryphyllene oxide. It acts as a Potential insect attractant. Beta-caryophyllene oxide was identified as components in the essential oil obtained from peucedanum dhana seeds. (Aggarwal, 1981). It is used as general flavoring agents used in foods, including condiments and seasonings.



(-)-5-OXATRICYCLO [8.2.0.0(4, 6)] DODECANE,12-TRI

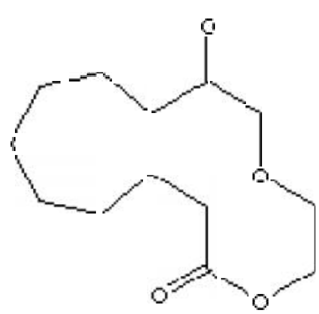
3) **Tetradeconic acid** (C14H28O2)-This compound is also known as Myristic acid. Its molecular weight is 228.37g/mol. It is a common saturated fatty acid and has a sufficiently high hydrophobicity to become incorporated into the fatty acyl core of the phospholipids bilayer of the plasma membrane of the eukaryotic cell and hence acts as a lipid anchor in biomembranes. The ester isopropyl myristate is used in cosmetic and topical medicinal preparations where good absorption through the skin is desired.



Tetradecanoic acid

4) **Ethylene brassylate** ($C_{15}H_{26}O_4$)

Ethylene brassylate with molecular weight of 270.364g/mol is a macrocyclic musk, like natural. It is exceptionally tenacious and a good fixative. It plays well with other musks and is probably best used in combination. The compound is present with maximum area% in the n- butanol fraction, among the 5 fractions studied.

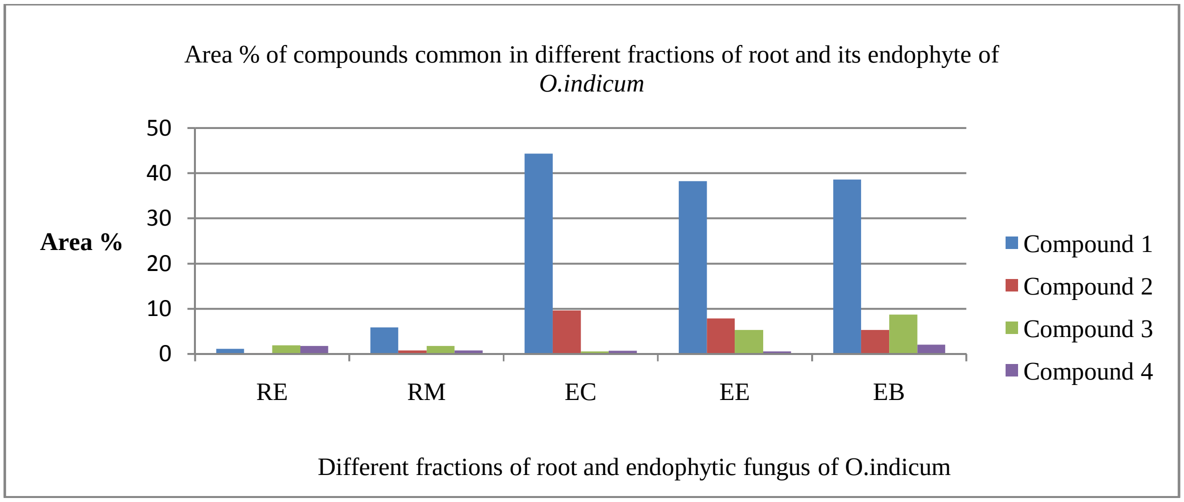


Ethylene brassylate

TABLE 1: Summarizes the area % and the peak of the compounds 3-Cyclohexene-1-ol, 4-methyl-1-(1-methy lethyl)-, (R)- (1); (-)-5- Oxatricyclo [8.2.0.0 (4,6)] Dodecane, 12-Trim (2); Tetradeconic acid (3) and Ethylene brassylate(4) in the 5 fractions.

	RE	RM	EC	EE	EB
1	1.14	5.84	44.36	38.19	38.63
2	0.21	0.79	9.59	7.88	5.30
3	1.95	1.70	0.52	5.31	8.74
4	1.71	0.81	0.73	0.52	2.09

For better comprehension of the differences among the various fractions, graphs are plotted accordingly (Graph 1)



GRAPH 1: Area % of different compounds in different fractions.

The compounds which are commonly present in the all fractions of Endophytic fungal extract and root extract of O. indicum, are showing highest difference in area % for compound 13-Cyclohexene-1-ol, 4-methyl-1-(1-methy lethyl)-, (R)- in endophytic fungi than root extract. Compound 2 (-)-5-Oxatricyclo [8.2.0.0 (4, 6)] Dodecane, 12-Trim is also with higher area % in the fungal extract fractions than the root fractions of *O. indicum*. Whereas the compound 3, Tetradeconic acid has a higher area % in the ethyl acetate and n- butanol fractions of the fungal extract than the root fractions. Compound 4, Ethylene brassylate is showing its highest area in n-butanol fraction of endophytic fungus.

DISCUSSION

The characteristic properties of the plant *Oroxylum indicum* and presence of similar characteristic exhibiting compounds in the fungal extracts suggest some intriguing relation between endophyte and its host plant. Some other economically important compounds found in different fractions of the fungal extract are discussed.

Presence of phytol in the ethyl acetate fraction of the fungal extract can be one such compound establishing the link. *Phytol* is an acyclic diterpene alcohol and a constituent of chlorophyll. *Phytol* is commonly used as a precursor for the manufacture of synthetic forms of vitamin E and vitamin K1 (NCBI, CID=5280435)

The compound trans **(-)**-beta-caryophyllene in the chloroform fraction of the fungal extract with an area % of 5.21 and in the methanolic extract of root with area % 0.39. Occurring in many essential oils, beta-caryophyllene, particularly oil of cloves, has a role as a non-steroidal anti-inflammatory drug, a fragrance, a metabolite and an insect attractant. It is an enantiomer of a (+)-beta-caryophyllene (NCBI, CID=5281515).

A compound named sclareolide obtained in the fungal extract fractions is an economically important product. It is a close analog of sclareol, a plant antifungal compound (Jasinski *et al*., 2001).

Now, it is a well-established fact that both the plant and their endophytes can produce an array of common secondary metabolites from similar precursors. One of the most-studied bioactive compounds with anticancer activity is the synthesis of taxol from yew plant and from its fungal endophyte *Taxomyces andreanae.* (Khare *et al*., 2018)

The fact that the characteristic properties of the plant are highly affected by the endophyte residing in, as a symbiont, needs to be studied with a better approach. Since, Endophyte isolation and preservation is a much effective way towards environmental sustainability, the lacunae in the studies must be found and filled.

The industrial production of the metabolites utilizing the endophytic microflora is a cost effective, highly productive and eco- friendly method. For the society, to fulfill the ever increasing demands, the industrial production also provides a better option over chemical synthesis of the compounds which might possess any kind of threat to the mankind, directly or indirectly.

The endophytes serve as an active metabolite pool and by utilizing them neither the environment nor the plant species will be exploited.

A more comprehensive view of the ecology and evolution of endophytes and host plants is needed (Saikkonen *et al*., 1998) and the present study is an attempt to find out the relationship between endophytes and its host on the basis of their biochemical assets.

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