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Original Article

ANTIFUNGAL ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM *LANNEA COROMANDELICA*– AN INSILICO APPROACH

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ABSTRACT

Objective: The objective of this research was to isolate endophytic fungi from *Lannea coromandelica* having antifungal activity potential and to isolate the secondary metabolite from the dominant fungi and predict the probable mechanism behind its activity.

Methods: The endophytic fungi were isolated from leaves of *Lannea coromandelica* by surface sterilization method. Then fungal biomass was extracted for intracellular metabolites by using ethyl acetate as solvent. The crude extract was filtered, and the filtrate was dried under vacuum at 40 °C. The filtrate was analysed for antifungal activity. The fungi which showed the maximum activity was identified and the metabolite present in the ethyl acetate extract was characterized and identified by GC-MS (Gas-Chromatography Mass-spectrophotometry) analysis. Further, these compounds were docked against the target protein Lanosterol 14-alpha demethylase to unravel and predict the probable mechanism behind the antifungal activity of secondary metabolite.

Results: *Aspergillus flavus, Aspergillus niger, Alternaria alternata* and *Colletotrichum gloeosporioides* were isolated and identified based on their morphological features as endophytic fungi. Among the four dominant fungi, the antifungal activity of *Aspergillus flavus* showed the maximum activity with an inhibitory zone of 26.22 mm against *Candida albicans* and 16.72 mm against *Malassezia pachydermis*. Further, the secondary metabolite was identified by GC-MS (Gas-Chromatography Mass-spectrophotometry) () analysis and found to be Kojic Acid, Octadecanoic acid, n-Hexadecanoic Acid, diethyl Phthlate, 3-Phenyl Propionic Acid. These compounds were docked with the target protein and were able to bind at an active site similar to that of Flucanozole a known inhibitor.

Conclusion: The finding of this work clearly indicates that the metabolite produced by the endophytic fungus could be used as an alternative source of antifungal agents against clinical pathogens.

Keywords: Endophytic fungi, Secondary metabolite, Antifungal activity

INTRODUCTION

In the last 15 y, there has been an alarming increase in life-threatening systemic fungal infections of varying types which are observed in immunosuppressed/immunocompromised person such as AIDS, cancer, and transplant patients. *Candida* sp. have emerged as significant pathogens in recent decades as a consequence of increasing numbers and population of immunocompromised patients [1]. As a result, they have become a major direct cause of death in patients being treated for malignant diseases and emerging immune deficiency diseases [2]. Management of fungal infections have become more complex due to lack of a number of effective antifungal drugs, problems of drug safety, side effects, resistance and effectiveness of drug forms [3]. Therefore, there is an urgent need to improve currently used drugs, to design new drugs and to explore an alternate source of novel drugs through various mechanisms, for better treatment

Endophytic fungi are a group of microorganism colonized in inter and intracellular host plants without causing any disease, plays important physiological roles and ecological roles [4]. After colonization many fungal endophytes may occupy a large area relatively and create an obstacle for other endophytic species by competing [5] or by producing antagonistic metabolites [6, 7]. After the discoveries of endophytes which reside in unique biological niche that is intracellular cells of the plants, created a worldwide curiosity among scientist which led to isolation of endophytes and to study their natural products. It has been observed that plants harbour a myriad of endophytes, possessing enormous metabolic diversity and diverse bioactive compounds that can be used as a therapeutic agent against emerging human diseases such as cancer and infectious diseases [8, 9]. It has been reported that mainly the filamentous fungi, are capable of producing a large number of chemically different secondary metabolites [10], which possess diversified functions and can be used as antimicrobials, antifungal and antivirals. These metabolites have high chemical diversity which comprises of alkaloids, terpenoids, quinones, peptides, xanthones and phenols [4]. Due to the presence of the above-mentioned properties, it may provide numerous lead compounds for the development of new drugs, directly, or as an inspiration for synthetic drugs [8]. A considerable body of research has investigated the diversity, ecological role, secondary metabolites and bioactivity of the endophytic fungi isolated from various medicinal plants [11].

One such plant studied is *Lannea coromandelica,* which is commonly known as "The Indian Ash Tree" belonging to the Anacardiaceae family. The bark and leaves of this tree have been used to treat various aliment by villagers and tribal people. *Lannea coromandelica* is considered to have antimicrobial, antifungal and antiviral properties [12]. Apart from this, it is claimed to have anti-inflammatory, zoosporicidal, tissue healing, and tissue regeneration capacity. It is also used in the treatment of skin diseases, injuries, diarrhoea, particularly in young children, ulcers, impotence, vaginal troubles, heart diseases, gout, rheumatism, sore eyes, dysentery, and as an antidote in coma caused by the narcotics [13-15].

In this study an attempt was made to investigate and identify the antifungal metabolite produced by the fungal endophytes by GC-MS (Gas-Chromatography Mass-Spectrophotometry) analysis. Further studies were carried out to evaluate the interactions of metabolites present in the ethyl acetate extract of *Aspergillus flavus* by using *In silico* docking method for antifungal analysis with Lanosterol 14-alpha demethylase as target protein. Our study dealt with the assessment of the interactions between ligands and target protein by molecular docking method in order to calculate the minimum binding energy (kcal/mol) between them.

MATERIALS AND METHODS

Isolation and extraction of metabolite

The endophytic fungi were isolated from healthy leaves of *Lannea coromandelica* by using potato dextrose agar medium and stored at 4 °C. They were identified as *Aspergillus flavus Aspergillus niger*, *Alternaria alternata,* and Collectotrichum *gloeosporioides* based on morphological features [16]. The strains of were inoculated in a production media and incubated at 28 °C for 12 d, and biomass was then harvested. The fungal biomass was extracted for intracellular metabolites by using ethyl acetate as solvent, and this extraction was repeated three times. The crude extract was filtered through whatman No 1 filter paper, and the filtrate was dried under vacuum at 40 °C. The filtrate was analysed for antifungal activity. Further secondary metabolites of *Aspergillus flavus* were identified by using GC-MS analysis, by matching with known compound library.

Antifungal activity (Agar-well diffusion method)

For antifungal assay (SA) Sabouraud's agar medium (Himedia, Mumbai) was prepared freshly and sterilized, then a pinch amount of streptomycin was added and mixed well. Then 20 ml of the media was poured into each petri plate and allowed to solidify. The test fungal cultures (3×10^5cfu/ml) namely *Candida albicans* and *Malassezia pachydermis* were evenly spread over the (SA) Sabouraud's agar medium (Himedia, Mumbai) surface by using sterile cotton swab. Then a well 0.5 cm was made in the medium by using sterile cork borer, 150µg/ml of the ethyl acetate extracts of endophytic fungi was transferred into separate wells. Then these plates were incubated at 27° C for 48-72 h. Fluconazole was used as a control. After incubation period the results were observed and measured, the diameter of inhibition zone around the each well.

In silico–methodology

The isolated endophyte produced several compounds as secondary metabolite which had bioactivity. The compounds identified by GC-MS analysis was screened against the target protein. The target protein Lanosterol 14-alpha demethylase (PDB ID: 3LD6) were retrieved from the (PDB) protein data bank (http://www. rcsb. org/pdb/). The compound details were retrieved from the Pub chem database, and the chemical structure was generated from SMILES notation by using the Chemsketch software (http://www. acdlabs. com). Docking studies carried out in Ligand Fit (Discovery Studio 2.0) [17]. It is based on a cavity detection algorithm and Monte Carlo conformational search algorithm for generating ligand poses consistent with the active site shape; hydrogen bonds were added and CHARMm force field was applied to all molecules. After applying CHARMm force field macro molecule 3LD6 was assigned as receptor. The receptor cavity was searched using flood filling algorithm and partition site was adjusted for the better fitments of molecule in the partition site of receptor. The comparative docking studies of the metabolites obtained from the result of GC MS for all molecules were performed. The determination of the ligand binding affinity was calculated using Dock score. They were used to estimate the ligand-binding energies.

RESULTS AND DISCUSSION

The endophytic fungi, namely *Aspergillus flavus, Aspergillus niger, Colletotrichum gloeosporioides,* and *Alternaria alternata* were isolated from *Lannea coromandelica*. Since ethyl acetate is the best solvent for extracting antifungal compounds [18]. The ethyl acetate extract of endophytic fungi were further taken for testing their activity against the test dermatophytic fungi namely *Candida albicans* and *Malassezia pachydermis.*

Antifungal activity of *Aspergillus flavus* shows the maximum inhibitory zone of 26.22 mm against *Candida albicans* and 16.72 mm against *Malassezia pachydermis* (table 1). Whereas *Aspergillus niger* didn't show significant inhibition against the test pathogens and the inhibition zone ranges from 8.95 mm to 12.56 mm respectively. *Alternaria alternata* did not have any significant inhibition against *Candida albicans* with a zone of inhibition of 9.57 mm but shows moderate inhibition against *Malassezia pachydermis* with an inhibitory zone of 15.11 mm. On the contrary, *Colletotrichum gloeosporioides* showed a significant inhibition zone of 21.13 mm activity against *Candida albicans* but did not show any significant inhibition towards *Malassezia pachydermis* with a zone of 12.5 mm.

Table 1: Antifungal activity of endophytic fungi against fungal pathogen

Endophyte	*Candida albicans*	*Malassezia pachydermis*
Aspergillus flavus	26.22±0.20	16.72±0.21
Aspergillus niger	8.95±0.06	12.56±0.40
Alternaria alternata	9.57±0.47	15.11±0.12
Colletotrichum gloeosporioides	21.13±0.12	12.5±0.5
Control	42.8±0.42	38.56±0.2

Values are means of three replicates±Standard Deviation

A list of endophytic fungi isolated from a number of medicinal plants have been claimed to possess antifungal activities by some researchers [19-21]. Most of them were anamorphs of fungi distributed in some common endophytic genera such as *Colletotrichum* sp., *Alternaria* sp., *Ovulariopsis* sp., *Pestalotiopsis* sp., *Phomopsis* sp., and *Phoma* sp., of which most are known to produce various bioactive products [22, 23]. The findings of this study coincide with the report of Kaushal Kanwer Shekhawat [24], which states that the *Aspergillus flavus* an endophytic fungus of *Melia azedarach* displayed greater fungicidal activity against *Penicillium chrysogenum* and *Fusarium oxysporum* with a inhibition zone of 17.05±0.2 than *Trichoderma koningii* with a inhibition zone of 12.08±0.9). Yet in another study the antifungal metabolites,named Asperfumoid, Physcion, Fumigaclavine, Fumitremorgin and Helvolic acid were obtained from an endophytic fungus, *Aspergillus fumigatus,* isolated from the leaf of *Cynodon dactylon* inhibited the growth of *Candida albicans* [25]. *Aspergillus clavatonanicus,* an endophytic fungal strain from *Taxus mairei* yielded Clavatol and Patulin which also possesses inhibitory activity against several plant pathogenic fungi, namely *Botrytis cinerea, Didymella bryoniae, Fusarium oxysporum, Rhizoctonia solani* and *Pythium ultimum* [26]. An endophytic fungus, *Aspergillus niger* isolated from marine brown alga *Colpomenia sinuosa* released the compound Asperamide A and B sphingolipid and their corresponding glycosphingolipid possessing a hitherto unreported 9-methyl-C20-sphingosine moiety displayed moderate activity against *Candida albicans* with a zone of inhibition of 20 mm [27]. Isofusidienol were produced by *Chalara* sp. an endophytic fungus isolated from *Artemisia vulgaris* exhibited antifungal activity against *Candida albicans* [28].

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides,* an endophytic fungus from the plant *Artemisia mongolica,* displayed antimicrobial activity against bacteria as well as against fungus, *Helminthosporium sativum* [29]. In another study *Colletotrichum* sp. isolated from *Artemisia annua*, produces bioactive metabolites that showed varied antibacterial and antifungal activity [30]. Similar to our study, (KA) kojic acid, a natural pyrone, is reported as a potent chemo sensitizing agent of complex III inhibitors disrupting the mitochondrial respiratory chain in fungi thereby acting as an antifungal agent [31].

In the present study Lanosterol 14-alpha demethylase (PDB ID: 3LD6) has been chosen as a target protein, which has been isolated from *Homo sapiens* with a resolution of 2.80A and has 461 amino acids. The compounds (table 2) identified by GC-MS analysis was screened against the target protein.

Table 2: Chemical constituents present in ethyl acetate extract of *Aspergillus flavus*

S. No.	Chemical constituents	ID
1	Kojic Acid	3840
2	Octadecanoic acid	5213
3	n-Hexadecanoic Acid	985
4	Diethyl Phthlate	6781
5	3-Phenyl Propionic Acid	107
6	Fluconazole (std)	3365

Molecular docking was done at site 3, which was chosen as the active site with a volume 38.125Å with point count of 305 in equal grid spacing of 0.5 (X), 0.5(Y), 0.5(Z) direction respectively. The chemical constituents identified from the ethyl acetate extract of endophytic *Aspergillus flavus* were docked with a sphere site being defined as 18.348 (X),-9.95 (Y), 5.039 (Z) using "2 500 120, 4 1200 300, 6 1500 350, 10 2000 500, 25 3000 750" Number of Monte Carlo Trials.

Table 3: Docking analysis of identified compounds with target protein 3LD6

S. No.	Compounds	Dock score in Kcal/mol	Internal energy in Kcal	Interacting amino acid residues
1	Kojic Acid	22.933	-0.544	ASN430,PRO431,ARG425,ASP429,VAL416,GLN428
2	Octadecanoic acid	37.045	-3.862	TRP415,ARG425,PRO431,VAL416,GLN428,ASP420,ASN422,GLU417
3	n hexadecanioc acid	39.491	-6.698	ASN422,VAL416,ASN430,ARG425,GLU417,GLN428
4	Diethyl phylate	31.548	-2.665	ASP420GLU417,VAL416,ASN422,TRP415,SER414,ARG425,PRO 431
5	3-Phenyl propionic acid	29.286	-0.76	ASP420,GLU417,VAL416,ASN422,TRP415,SER414, ARG425,PRO431
6	Flucanozole	17.152	-4.484	ASN429,VAL416,GLN428

Table 3 and fig. 1 represents the results of the docked compounds after docking. The result shows the highest dock score of 39.491 Kcal/mol with an internal energy of-6.698 Kcal for n-hexadecanioc acid at the binding site of the target protein which is better than the standard inhibitor Flucanozole, with a dock score of 17.152 Kcal/mol and with an internal energy of-4.484 Kcal (fig. 2). In this study n-hexadecanioc acid has been found to be more stable than the standard with an internal energy of-6.698 K cal. Moreover, all the compounds were able to bind at active site of the target protein, and the interacting amino acid is depicted in (table 3) thereby indicating that these ligands may be used as an inhibitor similar to that of Flucanozole.

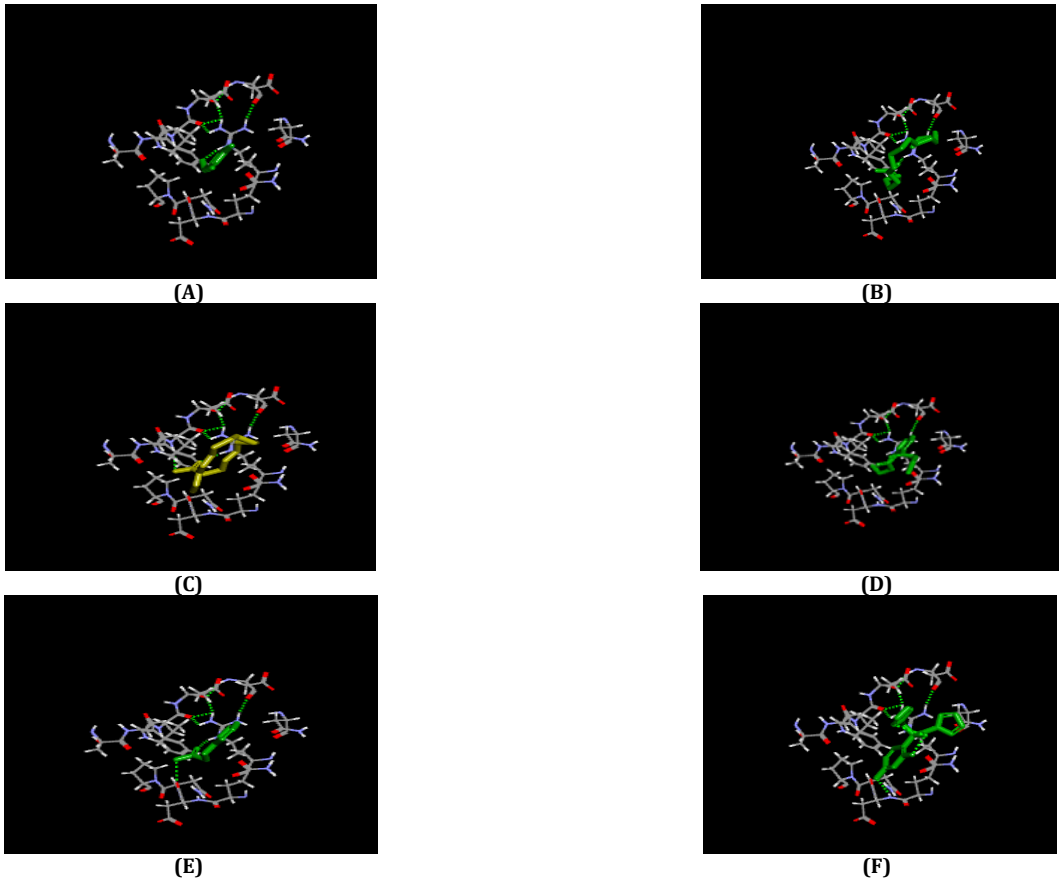

Fig. 1: Illustration of compounds docked with target protein 3LD6 (A) Kojic acid (B) Octadecanoic acid (C) n-Hexadecanoic acid (D) Diethyl phylate (E) 3-Phenyl propionic acid (F) Flucanozole fig. 2: Dock Score and internal energy of compounds with target protein 3LD6

The Flucanozole inhibits fungal CYP51, lanosterol demethylase by binding reversibly to the heme cofactor located in the enzyme active site in a competitive manner [32]. As a result methylated sterol precursors of ergosterol gets accumulated which causes membrane disruption thereby preventing fungal growth [33]. In our studies, on the basis of analysis of interacting amino acid (table 3) it has been found out that these ligands also interacts with the target protein similar to that of Flucanozole, Hence it may be predicted that these ligands may also bind to the heme cofactor, thereby rendering the enzyme inactive.

CONCLUSION

The compounds isolated from an endophyte, *Aspergillus flavus* in this study namely Kojic acid, Octadecanoic acid, n-Hexadecanoic acid, Diethyl phylate and 3-Phenyl propionic acid, are known compounds for their antimicrobial activity. Hence, the synergetic action of these compounds might be the possible reason for its activity against the fungal pathogens like *Candida albicans* and *Malassezia pachydermis.* Moreover, this is the first report that Kojic acid has been isolated from the endophytic fungi grown in a very common Indian tree *Lannea coromandelica.* Observations in this research have important implications for the production of kojic acid at a low cost which may turn to be of wide significance in health, pharmaceutical, food and cosmetic industries. Increase in the yields of these products may also be enhanced by studying the biochemical pathways, new developments in fermentation technology, membrane technologies and genetic manipulation.

CONFLICT OF INTERESTS

Declared none

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