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Anti-bacterial, Anti-Inflammatory Activity and Bio-Active Compounds Analysis of *Curvularia lunata* an Endophytic Fungi Isolated from *Justicia Tranquebariensis*-Using GCMS

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Abstract: ***Medicinal plants are the local heritage with universal importance. World was gifted with a rich wealth of medicinal plants. Medicinal plants, as source of remedies, are widely used as alternative therapeutic tools for the prevention or treatment of many diseases. Justiciatranquebariensis is a small shrub, which is widely distributed in southern parts of India. From this plant fungal endophytes were isolated on PDA medium and identified as Curvularialunata. The fungus was cultured on potato dextrose broth for production of fungal metabolites. Ethyl acetate extract of the C. lunata was obtained by liquid-liquid partition of broth of endophyte and evaporation. Dried crude extract was tested for anti-bacterial activity by agar well diffusion method. The extract showed antibacterial nature against all the test pathogens (ATCC Cultures). Dried crude extract was also tested for anti-inflammatory activity by egg albumin denaturation method, ability of the extract to inhibit protein denaturation was studied. Ethanolic extract of C. lunata was elucidated using Gas Chromatography-Mass Spectrometry method. Twenty compounds were identified. Among these 20, nearly 12 Compounds are reported as anti-inflammatory principles. From this study, it is obvious that Curvularialunata an Endophytic fungi isolated from Justiciatranquebariensis extracts contain many biologically active compounds, Such as Antioxidant, Anticancer, Antitumor, Antimicrobial, and Anti-inflammatory. Hence further studies are required to isolate and purify the compound to assess the nature of the compound and its mode of action to take this study in to the next level of research in the future.***

Keywords: Endophytic fungi, agar well diffusion, bioactivecompounds, GCMS Analysis, Tetracosamethyl-cyclododecasiloxane, Anti-inflammatory activity.

1. Introduction

The term endophytes (in greek: endo = within, phyte = plant) includes all organisms (bacteria, actinomycetes and fungus) that during the variable period of their lifetime, colonize the living internal tissues of their hosts without causing apparent harm to the host. Endophytes are found in a wide variety of plant tissues such as seeds, ovules, fruits, stems, roots, leaves, tubers, buds, xylem and bark [1]. Endophytes produce bioactive substances to enhance the host's growth and competitiveness in its natural habitat [2]. Plants are capable of synthesizing an overwhelming variety of low-molecular weight organic compound called secondary metabolites, usually with unique and complex structures [3]. Many metabolites have been found to possess interesting biological activities and find applications, such as pharmaceuticals, insecticides, dyes, flavors and fragrances. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases [4]. *Justicia tranquebariensis* is a small shrub, which is widely distributed in southern parts of India. In this genus about 20 species have been chemically investigated and major secondary metabolites isolated were ligans, flavonoids, steroids and tri terpenses. The juice of small and somewhat fleshy leaves of genus *Justicia* is considered by natives of India as cooling and aperients, and is prescribed for the children in the smallpox. Some species of the genus *Justicia* have been used in the traditional system of medicines for the treatment of fever, pain, inflammation, diabetes, diarrhoea and liver diseases. They possess anti-allergic, anti-tumoral, anti-viral and analgesic activities. The leaf juice *Justicia tranquebariensis* has been used to treat jaundice and leaf paste is applied over affected area to skin diseases.

Plant Information

Plant name: *Justiciatranquebariensis*
Description: Subshrubs, leaves 2.5-3 x 2cm, obovate-orbicular, apex obtuse, base cuneate, membraneous, pubescent, petiole 1.5cm, Spikes terminal and axillary to 10cm; bracts 1 x 0.7cm, broadly ovate; calyx teeth 5mm, lanceolate, 3-nerved; corolla bilabiate, tube 5mm, villous inside, upper lobe 7 x 5 mm, lower narrow, white with pink blotches; filaments dilated, 2mm; ovary 1.5 mm. ciliate along the margins, style ciliate. Capsule 8mm, widened above the middle, puberlulus [5].
Habit: herb

Taxonomic Classification
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Scrophulariales
Family: Acanthaceae
Subfamily: Acanthoideae
Genus: *Justicia*
Species: *tranquebariensis*

MEDICINAL IMPORTANCE

Tribal Claims
Local people use this plant drug for inflammations.

Siddha Uses
Leaf is used as expectorant, in Cold, Cough and nasal disorders.

Medicinal Uses
Juices of leaves act as a cooling agent and aperients and also given to children in Small pox. Crushed leaves applied to contusions. Paste made of the leaves applied externally on the swelling to reduce the pain. Root paste applied for tooth ache. Leaf juice, about 15-20ml, is administered orally for every one hour up to half of the day and keeping of leaf paste externally on the sight of snake bite work as an antidote for Cobra bite. Leaf juice is given orally to treat jaundice and leaf paste is applied over affected area to treat skin diseases [6].

2. Materials and Methods

2.1 Collection of plant samples

Healthy and mature plants were collected from the Kalrayan hills, Eastern Ghats, Kallakuruchi district. The plant was randomly collected from different sites and brought to the laboratory in sterile bags and processed immediately to reduce the chances of contamination. Processing of the stem, leaf, flower tissues were started by thoroughly rinsing with tap water. Samples were cut into 0.5-1cm pieces prior to rigorous surface sterilization (70% ethanol for 5s followed by 4% sodium hypochlorite for 90s). Surface sterilization tissues were rinsed with sterile distilled water; blot dried and plated on Sabouraud Dextrose agar (SDA) medium plates amended with gentamicin. The inoculated Petriplates were wrapped with parafilm and incubated at 28^0C-30^0C [7]. After 3days of inoculation, the plates were observed daily for growth of fungi from cultured segments upto one month by light microscopy. The emerging fungi were transferred to fresh Potato Dextrose agar (PDA) plates. The fungus that emerged from tip of the segment was identified on the basis of mycelial morphology and spore characters using fungal manual *Hyphomycetes, Taxonomy and Biology* [8].

2.2 Primary Screening of Endophytic fungi

The Isolated Endophytic fungi in this study were subjected to preliminary screening of Antibacterial activity against human pathogens namely *Staphylococcusaureus* (ATCC-25923), *Klebisella pneumonia* (ATCC-700603), *Escherichiacoli* (ATCC-25922) based on the methods by Agar plug method [7]. Discs (9mm) from 7 days old culture of the fungal isolates maintained in potato dextrose agar medium were picked up by using a sterile cork borer and then agar plug with mycelia was placed on surface of Nutrient agar medium seeded with test organisms separately. The plates were incubated for 2-5days at 37°C. After incubation the diameter of the zone of inhibition was measured. For this screening 4 endophytic fungi namely *Curuvularialunata*, *Exserohilumrostratum*, *Bipolaris*, *Curuvularia* were studied.

2.3 Extraction Fungal Metabolites

The Endophytic fungi selected by primary screening producing larger diameter zone of clearing against the human pathogens were subjected to extraction of fungal

metabolites by fermentation method. The mycelia of *C. lunata* purified on PDA medium were used for this study. The Potato Dextrose broth was used for production of fungal metabolite. Two to three pieces (0.5 X 0.5cm$^{2)}$ of the mycelia of *C. lunata* (5 to 6 days old culture) were taken and inoculated into three 500 ml Erlenmeyer flask containing 300ml of Potato Dextrose broth medium on a rotary shaker for 10-15days. The culture filtrate were filtered to remove the mycelium through a Whatman filter paper. The filtrate was extracted with the same volume of ethyl acetate and the crude extract was dissolved in Dimethyl Sulphoxide (DMSO) and subjected to antimicrobial activity, Anti-inflammatory activity and GC-MS studies [7].

2.4 Evaluation of anti-bacterial activity

The Antibacterial activity of the Crude Culture filtrate of the endophytic fungi *Curuvularia lunata (*JTF3) was assessed agar well diffusion method [9]. Muller Hinton agar (MHA) plates were swabbed with overnight culture of each bacterial suspension to be tested, by evenly spreading out with a sterile cotton swabs. The agar wells were prepared using a sterile cork borer (7mm in diameter). The wells are then filled with 20µl, 40µl and 60µl of the crude culture filtrate of the endophytic fungi dissolved in DMSO. Plates were left in a cooled incubator at 4 °C for one hour for diffusion and then incubated at 37°C in a bacterial incubator. Gentamycin was used as the positive control for tested bacteria with the solvent Dimethylsulphoxide (DMSO) as negative control. The zone of inhibition was recorded and compared with the control.

2.5 Evaluation of anti-inflammatory activity

The reaction mixture 5ml consisted of 0.2ml of egg albumin (from fresh hen's egg), 2.8ml of phosphate buffered saline (PBS, pH 6.8) and 0.2 to 2ml of varying concentrations of active so that final concentrations become 0 to 10mg\ml. Similar volume of double-distilled water and DMSO served as control. These mixtures were incubated at 37°C in a BOD incubator for 30min and then heated at 75°C to 10min. After cooling, their absorbance was measured at 660 by using vehicle as blank. Diclofenac sodium at the final concentration of 10mg\ml was used as reference drug and treated similarly for determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula:

$$\% \text{ inhibition} = 100 \text{ X } (Vt \setminus Vc\text{-}1)$$

Where, Vt = absorbance of test sample, Vc = absorbance of control

The extract \ drug concentration for 50% inhibition (IC50) was determined by plotting percentage inhibition with respect to control.

2.6 GC-MS Analysis of the Crude Endophytic Fungal extract

To investigate the presence of compounds in the ethyl acetate extract of the *Curuvularia lunata (*JTF3) was subjected to GC-MS analysis using a Shimadzu GCMS QP 2020 cum auto injector AOC 20i. In this GCMS instrument was used a fused silica column, packed with SH-Rxi-5Sil MS (30m x 0.25mm ID x 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1ml\min. The injector temperature was set at 280°C during the chromatographic run. The 1µl of extract sample injected into the instrument the oven temperature was as follows: 40°C (2min); followed by 280°C at the rate of 10°C min-1 and 280°C, where it was held for 3min. The mass detector conditions were transfer line temperature 240°C, ion source temperature 240°C; and ionization mode electron impact at 70eV, a scan time 0.2 sec and scan interval of 0.1 sec. The fragments from 40 to 550 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2017) library.

3. Results and Discussion

3.1 Taxonomic Identification of the Medicinal Plant *Justicia tranquebariensis*:

The Medicinal plant *Justicia tranquebariensis* collected from Kalrayan Hills used in this study for isolation of Endophytic fungi were identified and authenticated based on the floral features by the Director, Botanical Survey of India (BSI), Coimbatore, Tamilnadu and given the authentication certificate with No: BSI/SRC/5/23/2021/Tech/270 dated 01.03.2021

3.2 Isolation and Identification of Endophytic Fungi

About 18 segments (6 segments of each part respectively) of the medicinal plant were screened for the presence of the endophytic fungi. A total of 15 endophytic fungi were isolated from 18 segments of the medicinal plant *Justicia tranquebariensis* part such as Leaves, Stem, & Flower. The endophytic fungi were identified based on their macroscopic and microscopic features. There were equal distribution of the Endophytes in this study among the three parts that were taken for this study from the medicinal plant

Among the 15 endophytic fungi, the predominant endophytic fungi isolated belonged to the genera of *Chaetomium spp*., (20%) *Curvularia lunata., (*13.3%), *Fusarium sp., (*13.3%) followed by *Bipolaris spp., (*6.6%), *Alternaria longipes (*6.6%) & *Exserohilum rostratum., (*6.6%) The fungi which did not produce any reproductive structure in spite subculturing onto various media such as PDA, SDA and TWA were grouped as *Mycelia sterilia (33.3*%). In this study majority of the fungi belonged to the class Dematiaceous Hyphomycetes followed by ascomycetes. The colonisation frequency was found to be 83.33% for Leaves, Stem, & Flower of the medicinal plant *Justicia tranquebariensis*

3.3. Description of Endophytic fungi

S. No	Endophytic Fungi	Description
1.	***Alternaria spp***	*Alternaria spp.* Produced black to olivaceous-black colored colonies with reverse dark brown, and were suede-like to floccose. Microscopically, branched acropetal chains (blastocatenate) of multicellular conidia (dictyoconidia) were produced sympodially from simple, sometimes branched, short conidiophores. Conidia were obclavate, obpyriform, often with a short conical or cylindrical beak, pale brown, smooth-walled
2.	***Bipolaris spp***	Colonies were moderately fast growing, effuse, and grey to blackish brown, suede-like to floccose with a black reverse. Microscopic morphology showed sympodial development of pale brown pigmented, pseudoseptate conidia on a geniculate or zig-zag rachis. Conidia were produced through pores in the conidiophore wall (poroconidia) and were straight, fusiform to ellipsoidal, rounded at both ends, smooth to finely roughened, germinating only from the ends (bipolar)
3.	***Curvularia lunata***	Colonies are fast growing, suede-like to downy, brown to blackish brown with a black reverse. Conidiophores erect, straight to flexuous, septate, often geniculate (producing conidia in sympodial succession) sometimes nodulose. Conidia are ellipsoidal, often curved or lunate, rounded at the ends or sometimes tapering slightly towards the base, pale brown, medium reddish brown to dark brown, 3-10 (usually 3-5) septa, conidial wall smooth to verrucose. Hilum protuberant in some species.
4.	***Exserohilum rostratum***	Colonies are grey to blackish-brown, suede-like to floccose in texture and have an olivaceous-black reverse. Conidia are straight, curved or slightly bent, ellipsoidal to fusiform and are formed apically through a pore (poroconidia) on a sympodially elongating geniculate conidiophore. Conidia have a strongly protruding, truncate hilum and the septum above the hilum is usually thickened and dark, with the end cells often paler than other cells, walls often finely roughed. Conidial germination is bipolar.
5.	***Fusarium spp.***	Colonies are usually fast growing, pale or bright-coloured (depending on the species) with or without a cottony aerial mycelium. The colour of the thallus varies from whitish to yellow, pink, red or purple shades. Species of Fusarium typically produce both macro-and microconidia from slender phialides. Macroconidia are hyaline, two to several-celled, fusiform to sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell. Microconidia are one or two-celled, hyaline, smaller than macroconidia, pyriform, fusiform to ovoid, straight or curved. Chlamydospores may be present or absent.
6.	***Chaetomium spp***	*Chaetomium* is a common ascomycete characterised by the formation of darkly-pigmented, globose, ovoid, barrel to flask-shaped, ostiolate ascocarps (perithecia) beset with dark-coloured terminal hairs (setae) which are straight, branched or curved. Asci are clavate to cylindrical, typically eight-spored and evanescent. Ascospores are one-celled, darkly-pigmented, and smooth-walled, of varying shape, mostly ovoid, ellipsoidal or lemon-shaped. Chlamydospores and solitary conidia may also be produced.

3.4 Primary screening of antibacterial activity of Endophytic fungi:

Among the four Endophytic fungi that were subjected to preliminary screening *Curuvularia lunata (*JTF 3) showed significant activity against the human pathogens tested by producing larger zones namely 20mm, 17mm, & 15mm against *S. aureus, K. pneumoniae,* and *E. coli.* The other strain of *Bipolaris spp., (*JTS 2) also showed significant activity against S. aureus, *K. pneumoniae, and E. coli*by producing zones of 12mm, 14mm, and 14mm in diameter**.** . But the endophytic fungi *Curuvularia lunata (*JTF 3) were selected for the further study as it showed very good activity against the human pathogens tested in this study**.**

3.5 Antibacterial Activity of Crude Culture Filtrate of *Curvularia lunata (*JTF3) Isolated from the Medicinal Plant *Justicia tranquebariensis***.**

The Ethyl acetate extract of the Endophytic fungi *Curuvularia lunata (*JTF3) isolated from the Medicinal Plant *Justicia tranquebariensis* showed good growth during the production of bioactive secondary metabolites in the potato dextrose broth. The fungal extract showed good activity against *E. coli (*18mm) and *K. pneumoniae* (18mm) at different concentrations namely 20µl, 40µl & 60 µl respectively

3.6 Evaluation of invitro anti-inflammatory activity of endophytic fungi *Justicia tranquebariensis* extract using egg albumin denaturation method

Endophytic fungi are considered to be a dependable source of novel natural compounds with a high level of biodiversity and may also yield several compounds of therapeutic importance which is currently attracting scientific investigations worldwide. The naturally isolated anti-arthritic agents function by suppressing the different types of inflammatory mediators involved in inflammation process to evaluate the anti-arthritic property of thavasumurungai botanical name *J. tranquebariensis* extract with this belief that the treatment of extracts releases various bioactive substances that could play a role in generating a particular pharmacological activity. Similarly, protein denaturation method, membrane stabilization and albumin denaturation test were done for this purpose. Anti-inflammatory studies Inhibition of albumin denaturation Denaturation of proteins is the main cause of inflammation. As part of the investigation on the mechanism of the anti-inflammatory activity, ability of the extract to inhibit protein denaturation was studied. Selected extracts were effective in inhibiting heat induced albumin denaturation IC50. The production of auto antigens in certain arthritic disease may be due to denaturation of protein and membrane lysis action. The maximum % inhibition of protein denaturation, albumin denaturation and membrane stabilization action were observed as 58.25% mg and IC 50 Value 38.92% respectively. Diclofenac 84.81% was used as a standard anti-inflammation drug.

3.7 GC-MS analysis of Crude Culture Filtrate of Curuvularia *lunata (*JTF3) isolated from the Medicinal Plant *Justicia tranquebariensis*

The Crude Culture Filtrate of Endophytic fungi *Curuvularia lunata (*JTF3) Extract isolated from the Medicinal Plant *Justicia tranquebariensis* was subjected to GC-MS which was carried out by an instrument Thermo GC-Trace Ultra Ver: 5.0 at CIMF Instruments, Periyar University, Salemand was quantitatively analysed for the identification and analysis of potential bioactive phytochemical Compounds. In this graph, nearly 20 Retention peaks are separated based on the retention times of GC reference compounds were given to three decimal places. There are two different peaks were observed in the interpreted graph as long and short sharp peaks. Among these, 20 peaks were observed such as PROPANOIC ACID, ETHYL ESTER, ACETIC ACID, PROPYL ESTER, ACETIC ACID, 2-METHYLPROPYL ESTER, 1-BUTANOL, 3-METHYL-, ACETATE, ACETOPHENONE, 1-TETRADECANOL, BENZOIC ACID, 3-HYDROXY-, METHYL ESTER, 1-HEXADECANOL, CYCLONONASILOXANE, OCTADECAMETHYL-, 1-OCTADECENE, N-HEXADECANOIC ACID, 1-OCTADECENE, 10 (E), 12 (Z)-CONJUGATED LINOLEIC ACID, OCTADECANOIC ACID, N-NONADECANOL-1, CYCLODECASILOXANE, EICOSAMETHYL-CYCLODECASILOXANE, EICOSAMETHYL-, BIS (2-ETHYLHEXYL) PHTHALATE, TETRACOSAMETHYL-CYCLODODECASILOXANE and the molecular ions, fragmentation patterns were studied by mass spectrometer at various retention time from 3.0-28 mins.

Most of the identified compounds have been reported to possess interesting biological activities. Among these 20 compounds, nearly 12 compounds such as Acetophenone, 1-Tetradecanol, 1-Hexadecanol, Cyclononasiloxane, octadecamethyl-, n-Hexadecanoic acid, 1-octadecene, 10 (E), 12 (Z)-Conjugated linoleic acid, Octadecanoic acid, n-Nonadecanol-1, Cyclodecasiloxane, Eicosamethyl-, Bis (2-Ethylhexyl) Phthalate, Tetracosamethyl-cyclododecasiloxane are reported as anti-inflammatory principles. At the same time, 1-Hexadecanol and n-Hexadecanoic acid may have different biological activities such as Antioxidant, Anticancer, antitumor, Antimicrobial, Anti-inflammatory. Hexadecanoic acid methyl ester inhibits the cyclooxygenase II enzymes and produce a selective anti-inflammatory action. The biological activity of antimicrobial effects of propanoic acid-ethyl ester, 1-Hexadecanol, n-Hexadecanoic acid and Tetracosamethyl-cyclododecasiloxane are due to the fact that fatty acids, fatty acid ester and aliphatic chains (long chain alkanes and alkenes) normally accumulated in the lipid layer of the cell membrane and mitochondria. Consequently, they disturb the integrity of cell structure which becomes permeable. Unsaturated fatty acids such as Conjugated linoleic acid have also known to lower blood cholesterol levels

4. Conclusion

The efficiency of the bioactive compound was found to be the same when tested before and after the extraction. However further studies are required to test the compound at a higher concentration by following the MIC method. This study has revealed the multi-potential of the Secondary metabolites produced by this endophytic fungi which were found to be possesses medicinal value against the clinical isolates tested and were found to possess anti-inflammatory activity in this study. Hence further studies are required to isolate and purify the compound to assess the nature of the compound and its mode of action to take this study in to the next level of research in the future.

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