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# Identification and bioactivities of endophytic fungi from *Lagenandra toxicaria* Dalz. and *Kaempferia rotunda* L.

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**ABSTRACT**

Endophytic fungi from *Lagenandra toxicaria* and *Kaempferia rotunda* were isolated and evaluated for their antimicrobial and anthelmintic activities. Based on the morphological and ribosomal large subunit rDNA gene (D1–D2) sequence, LTRH2 and LTRO1 isolated from *L. toxicaria* were identified as *Aspergillus tamarii* and *Aspergillus niger*, respectively. The KMPRO2 isolated from *K. rotunda* was identified as *Aspergillus flavus*. Among the endophytic fungi isolated from *L. toxicaria* rhizome, the LTRH2, LTRO1, and LTRH1 showed conspicuous growth inhibition against all the Gram-positive bacteria tested. *In vitro* nematicidal activity of these endophytic extracts showed significant mortality to *Haemonchus contortus* first instar larva. Among the isolates of *L. toxicaria*, LTRH2 induced mortality to *H. contortus* larva with an LC50 of 2.03 mg/ml and LTRO1 with an LC50 of 3.67 mg/ml. The extracts of KMPRO2 and KMPRH1 also showed similar results with an LC50 of 2.63 and 2.44 mg/ml, respectively. high-performance liquid chromatography mediated polyphenol profiling of these extracts revealed the presence of many phenolic molecules common in both the crude rhizome extract and the endophyte extracts of the respective plants. These fungal extracts are, therefore, recommended for further study as a novel source of phytochemicals with good biological activity.

## 1. INTRODUCTION

Plants do not live alone as single entities rather closely associated with the microorganisms that reside both externally and, especially with those living internally [1]. Multiple microbial communities such as bacteria, archaea, and fungi reside inside healthy plant tissues without producing any prominent disease or clinical symptoms in their host can be considered as endophytes [2]. They devote their entire life or a part of the life cycle in the symplast or apoplast region of healthy plant tissues [2,3]. Endophytes contribute to the overall growth, development, fitness, and diversification of plants [1]. They provide enhanced competitive abilities, increased resistance to herbivores, pathogens, and various abiotic stresses that negatively affect the health and survival of their host [4]. Endophytes are now considered as a repository of novel bioactive natural compounds. There are several recent studies which illustrate the importance of endophytes as a new reserve of antibacterial and other bioactive molecules [5,6]. Over 8,600 bioactive metabolites of fungal origin have been described and it was also reported that they are able to make the same secondary metabolites as the host plant itself [4,7].

The *Lagenandra toxicaria* Dalz. is a semi-aquatic herb that belongs to the Family Araceae, and is an important ingredient in the folklore treatment modalities. It is used in the preparations of ointments for skin itch, renal and bilious complaints [8]. The *L. toxicaria* rhizomes and roots were also reported to be diuretic, carminative, tonic, and also used for wound healing. Its rhizomes have also shown insecticidal and antimicrobial properties [9,10]. Another plant under investigation, *Kaempferia rotunda* L., belongs to the Family Zingiberaceae is commonly known as peacock ginger (Bhuichampaka in Sanskrit), is a fragrant aromatic herb with tuberous rhizome. The plants of the Zingiberaceae Family have been widely used in dietary cuisines and in traditional oriental medications [11]. The *K. rotunda* extracts are known to contain diverse secondary metabolites such as flavonoids, flavonols, stigmasterol, chalcones, quercetin, $\beta$-sitosterol, syringic acid, and protocatechuic acid [12–14]. Several recent studies have also endorsed the fact that *K. rotunda* possesses a wide range of

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pharmaceutical activities such as antibacterial, anti-proliferative, anti-mutagenic, and antioxidant activity [15–17]. The current study was designed to isolate the endophytic fungi from these two plants and to evaluate their potential as bio-control agents.

## 2. MATERIALS AND METHODOLOGY

### 2.1. Collection and Identification of Plant Material and Their Endophytes

Healthy plants were collected from their natural habitats in different areas of Thrissur District, Kerala, India. Plant materials were subjected to morphological identification at the Department of Botany, University of Calicut, Kerala, India, and voucher specimens are kept (*L. toxicaria* 7001 and *K. rotunda* 7002) for reference. Isolation of endophytic fungi were done according to the method described previously by Ezra *et al*. [18]. Rhizomes of *L. toxicaria* and *K. rotunda* were used for the isolation of endophytic fungi. Tissues were washed separately in running water for 10 minutes to remove debris and finally washed with double distilled water to minimize the microbial load from the sample surface. Thin sections of plant parts (approximately 2–3 cm in length) were then subjected to surface sterilization using 0.5% sodium hypochlorite for 3 minutes followed by 70% ethanol for 2 minutes and finally rinsing in sterile distilled water for removing alcohol traces. Tissues were then dried under the sterile laminar air flow. The surface treatment was done adopting the methodology of Petrini *et al*. [19], and the effectiveness of surface sterilization was checked according to the method of Schulz *et al*. [20]. The outer tissue layers were removed using sterile scalpel and the internal tissues were cut into smaller pieces of 0.5–1 cm and plated individually in Petri dishes containing potato dextrose agar, to which 0.1% antibiotic solution (stock) was previously added. The potato dextrose agar (PDA) plates were incubated at 25°C for 4 weeks under dark and checked every 24 hours for any fungal growth. Fungal hyphae emerging out of the plant tissues were sub-cultured several times to obtain the pure culture. Isolation procedure was repeated for 10 times to confirm the presence of the same endophyte. Only those endophytic fungi that have been obtained during repeated isolation were further taken for morphological and molecular identification**.** The phenotypic study was based on the culture characteristics and morphology of spores. Morphological changes such as growth rate, color, color variation over different time periods, upside and down side color of the colonies, and surface texture were observed. Also, microscopic features such as the mycelium, conidiophores, conidia were studied using lactophenol cotton blue staining methods [21].

### 2.2. Identification of the Endophytes using Molecular Taxonomic Approach

The clone purified fungal cultures were then used for further taxonomic studies. Ribotyping targets, especially the large-subunit rDNA gene (D1–D2) have shown particular promise for the molecular identification of fungi. The D1/D2 region was amplified by polymerase chain reaction (PCR) from fungal genomic DNA using PCR universal primers: DR–5′-GGTCCGTGTTTCAAGACGG-3′ and DF-5′-ACCCGCTGAACTTAAGC-3′. The amplicon was subjected to automated DNA sequencing on ABI3730xl Genetic Analyzer (Applied Biosystems, Bedford, MA). Each nucleic acid sequence was verified manually to correct any falsely identified bases and trimmed to remove unreadable sequences at both 3′and 5′ ends (considering peak and quality values for each base) using the sequence analysis tools. The edited sequences were then used for similarity searches using BLAST program in the NCBI GenBank DNA database for identification of the fungal strain.

### 2.3. Fermentation and Solvent Extraction of Endophytes

The isolated colonies were cultured in Potato Dextrose Broth for 2 weeks. These fermented broths were then repeatedly extracted with the same volume of ethyl acetate. The solvent extracts were then combined and evaporated to dryness by a rotary evaporator (KNF Rotary evaporator RC 600) giving a final yield of about 0.8%–1.1% [3].

### 2.4. HPLC Mediated Polyphenol Profiling of the Extracts

Polyphenol profiling and quantification was executed using the high-performance liquid chromatography (HPLC) method proposed by Rodriguez-Delgado *et al*. [22], with minor modifications. The analysis was performed on a Prominence UFLC system (Shimadzu, Japan) containing LC-20AD system controller, Phenomenex Gemini C18 column (250 × 4.6 mm, 5 µm), a column oven (CTO-20A), a Rheodyne injector (USA) with a loop of 20 µl volume and a diode array detector (SPD-M20A). The solutions of *K. rotunda* rhizome extracts and the reference compounds (1 mg/ml) were filtered through 0.45 µl polytetrafluoroethylene filter; 20 µl was injected into the HPLC system. Sample peaks were identified by comparing with retention times of standard peaks.

### 2.5. Antibacterial Studies

#### *2.5.1. Disk diffusion*

The isolated endophytic fungi were evaluated for their antibacterial activity against seven strains of bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Shigella flexneri*, *Serratia marcescens*, *Bacillus cereus*, and *Enterococcus faecalis*). The antibacterial effect of the extract obtained from endophytic fungi was tested by a modified agar disc diffusion assay [23] with paper disks. The isolated endophytic fungi were evaluated for their antibacterial activity against seven strains of bacteria (*E. coli*, *K. pneumoniae,* and *S. aureus*). The fermented broths were extracted with the same volume of ethyl acetate. The extracts were then combined and evaporated to dryness by a rotary evaporator (KNF Rotary evaporator RC 600) giving a final yield of about 0.8%–11% [3]. The collected crude extract was weighed and finally dissolved in 1% dimethylsulfoxide (DMSO) and diluted to 50 µg/µl for assay. A sterile paper disc (6 mm diameter, Whatman no. 1) was impregnated with 10 µl of 1% DMSO dissolved culture filtrate using a micropipette and kept under a laminar hood for 20 minutes to dryness. The air-dried paper discs containing 0.5 mg crude extract were used to test the activity against three strains of bacteria. The bacterial suspension (100 µl) from overnight broth culture, adjusted to contain $1 \times 10^8$ colony-forming unit (CFU)/ml of bacteria was spread by a sterile glass rod onto the surface

of solidified Mueller-Hinton Agar Petri plates. The paper discs containing 0.5 mg crude extract were placed on the surface of the Mueller-Hinton Agar medium seeded with test bacterium in separate Petri plates. The paper disc dried after impregnating with only 1 % DMSO of the same volume was considered as vehicle control. The reference antibiotic discs were amoxicillin (10 µg/disc) and tetracycline (5 µg/disc). The plates were incubated at 35°C ± 2°C for 24 hours and the degree of sensitivity was determined by measuring the zone of bacterial growth inhibition [23]. Each test was done in three replicates.

#### *2.5.2. Broth microdilution assay*

Broth microdilution assay was executed for the determination of minimum inhibitory concentration (MIC). The assay was executed according to the method proposed by Wiegand *et al*. [24] and Bussmann *et al*. [25] with slight modifications. Endophytic fungal extracts were serially diluted in sterile Muller-Hinton broth (MHB) to obtain an initial concentration range from 12 to 0.023 mg/ml. The assay was executed in sterile 96-well microtiter plates. The 1–10 wells (test) of each row were filled with 500 µl of endophytic fungal extracts dissolved in sterile MHB. The 11th column served as growth control well and 12th column served as sterility control well. The sterility control wells received 1,000 µl sterile MHB alone, whereas growth control wells received 500 µl sterile MHB containing standard antibiotics such as tetracycline in an initial concentration 5 µg/ml against gram positive strains and amoxicillin in an initial concentration 16 µg/ml against gram negative strains. Inoculated each well containing endophytic fungal extracts (1–10) and growth control well with 500 µl of the bacterial suspension. This results in the desired bacterial inoculum of $5 \times 10^5$ CFU/ml. Now the final concentration of endophytic fungal extracts ranges from 6 to 0.011 mg/ml (wells 1–10). Final concentration of the standard antibiotic in the growth control well changes to 0.25 and 8 µg/ml for tetracycline and amoxicillin, respectively [24,25].

### 2.6. Anthelmintic Studies

The anthelmintic potentials of fungal extracts were studied using 1st instar larvae of *Haemonchus contortus* by *in vitro* larvicidal assay. First stage *H. contortus* larvae were obtained by incubating eggs (obtained from infected animals) at 27°C for 24 hours. Approximately 100 motile larvae of first instar (L1) were collected in 100 µl water. To this equal volume of serially diluted fungal extracts were added to get a final concentration of 1.25, 2.5, 5, and 10 mg/ml. Albendazole (ABZ) (1 µg/ml) and DMSO (1%) served as positive and vehicle controls, respectively [26]. All samples were then incubated for 24 hours at room temperature. The loss of motility of the larvae after 24 hours of treatment was recorded. A number of larvae found non-motile/dead were counted under a dissecting microscope at 40× magnification [27] to calculate the mortality percentage.

### 2.7. Statistical Analysis

The data obtained are expressed as mean ± standard error. Data from larval mortality assay were transformed by probit analysis against the logarithm of extract concentration in Statistical Package for the Social Sciences version 5.0.

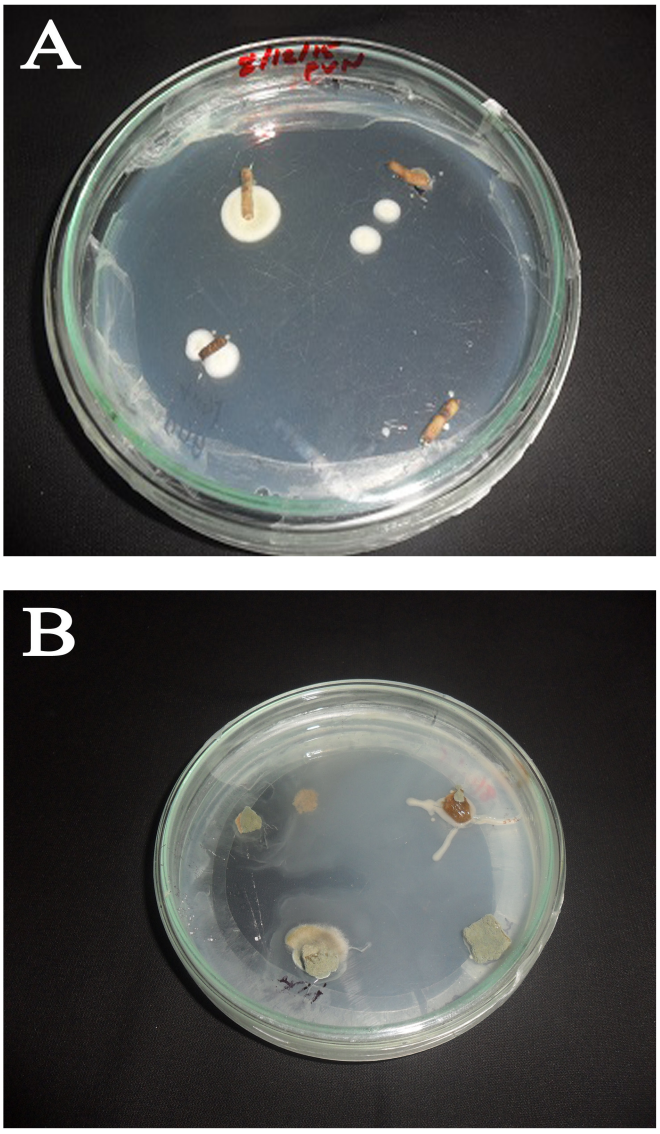


**Figure 1:** Endophytic fungi emerging from (a) *L. toxicaria* and (b) *K. rotunda* rhizomes in PDA plates.

## 3. RESULTS

### 3.1. Isolation and Identification of Endophytic Fungi

Rhizomes from *L. toxicaria* Dalz and *K. rotunda* L. were separately processed to investigate the presence of endophytic fungi (Fig. 1). A total of four endophytic fungi were isolated to pure culture from *L. toxicaria* rhizomes and three from the rhizomes of *K. rotunda* (Table 1). Among the isolates, two strains from *L. toxicaria* (LTRO1 and LTRH2) and one strain from *K. rotunda* (KMPRO2) were identified morphologically and also using molecular techniques. The LTRO1 strain was able to develop an aspergillum-like-spore bearing structure. They possess septate hyphae with conidiophores, hyaline and are smooth-walled. The LTRH2 strain appears cinnamon in color with rough conidia and white mycelium on PDA culture. KMPRO2 Conidiophores were heavy-walled, pale green colored, coarsely roughened and approximately 1 mm in length. Also, the phialides appeared biseriate. Alongside

**Table 1:** Endophytic fungi isolated from rhizomes of *L. toxicaria* and *K. rotunda.*

| SI. No | Host plant | Plant part used for isolation | Isolated endophytic fungi | Identification according to D1/D2 gene sequences | Gen bank accession number |
|---|---|---|---|---|---|
| 1 | *L. toxicaria* | Rhizome | LTRO1 | *A. niger* | MW684710 |
| 2 | *L. toxicaria* | Rhizome | LTRO2 | NI | – |
| 3 | *L. toxicaria* | Rhizome | LTRH1 | NI | – |
| 4 | *L. toxicaria* | Rhizome | LTRH2 | *A. tamarii* | MW684711 |
| 5 | *K. rotunda* | Rhizome | KMPRO1 | NI | – |
| 6 | *K. rotunda* | Rhizome | KMPRO2 | *A. flavus* | MW684712 |
| 7 | *K. rotunda* | Rhizome | KMPRH1 | NI | – |

NI = Not Identified.

**Table 2:** Antibacterial activity of crude plant rhizome extract and isolated endophyte extracts (0.5 mg/disk) against different bacteria.

| Endophytic fungi | The diameter (in mm) of zone of inhibition | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S. aureus* (MTCC 3160) | *E. coli* (MTCC 443) | *K. pneumonia* (MTCC 661) | *S. flexneri* (MTCC 9543) | *S. marcescens* (MTCC 2645) | *B. cereus* (MTCC 430) | *E. faecalis* (MTCC 3159) |
| LTRO1 | 21.5 ± 0.56 | 12.16 ± 0.70 | 6 ± 0.34 | 0 | 0 | 18 ± 0.24 | 16 ± 0.08 |
| LTRO2 | 16.33 ± 0.84 | 0 | 0 | 0 | 0 | 0 | 0 |
| LTRH1 | 15.83 ± 0.30 | 14.71 ± 0.88 | 7 ± 0.67 | 0 | 0 | 8.34 ± 0.41 | 10.18 ± 0.34 |
| LTRH2 | 22.66 ± 0.71 | 15.5 ± 0.22 | 8.66 ± 0.33 | 0 | 0 | 17 ± 0.12 | 20 ± 0.48 |
| *L. toxicaria* rhizome | 20 ± 0.23 | 17 ± 0.19 | 14 ± 0.34 | NT | NT | NT | NT |
| KMPRO1 | 9.16 ± 0.65 | 0 | 0 | 0 | 0 | 0 | 8.11 ± 0.08 |
| KMPRO2 | 13.17 ± 0.47 | 13.83 ± 0.79 | 6 ± 0.44 | 0 | 0 | 9.33 ± 0.1 | 10 ± 0.22 |
| KMPRH1 | 15.16 ± 0.30 | 9.5 ± 0.42 | 6 ± 0.90 | 0 | 0 | 7 ± 0.84 | 8.54 ± 0.88 |
| *K. rotunda* rhizome | 23 ± 0.63 | 19 ± 0.18 | 17 ± 0.22 | NT | NT | NT | NT |
| DMSO (1%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Amoxicillin (10µg/disc) | 28 ± 0.24 | 31.15 ± 0.63 | 8 ± 0.88 | 26 ± 0.13 | 24 ± 0.11 | 21 ± 0.34 | 21 ± 0.26 |
| Tetracycline (5µg/disc) | 33 ± 0.13 | 28 ± 0.13 | 18 ± 0.19 | 15 ± 0.61 | 18 ± 0.77 | 31 ± 0.08 | 27 ± 0.1 |

NT = Not tested.
Values are mean ± SE.

the cultural characteristics, lactophenol cotton blue-stained microscopic images were also used for identification purposes. The rDNA gene sequences of the three were done and compared with the sequences in Genbank by blast analysis. Based on the D1/D2 Region-PCR analysis, the nucleotide sequence of the fungal culture LTRO1 showed 100% homology with *Aspergillus niger* and LTRH2 showed 100% similarity with *Aspergillus tamarii*. The isolate from *K. rotunda* (KMPRO2) showed 100% homology with *Aspergillus flavus*. Therefore, by combined analysis of the fungal morphological characters according to Genera of Hyphomycetes (Carmichael 1980), the strains LTRO1 and LTRH2 are identified as *A. niger* (Genbank accession number: MW684710) and *Aspergillus tamari*, respectively (Genbank accession number: MW684711). The strain KMPRO2 is identified as *A. flavus* (Genbank accession number: MW684712).

### 3.2. HPLC Mediated Polyphenol Profiling of the Extracts

Qualitative phytochemical characterization of the extracts showed the presence of a high amount of phenolic compounds compared to other secondary metabolites. Therefore, the polyphenol content in the rhizome as well as in the endophyte extracts of *L. toxicaria* and *K. rotunda* was estimated using high-performance liquid chromatography. Thirteen standards of polyphenolic compounds; gallic acid, catechol, chlorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, ellagic acid, myricetin, cinnamic acid, quercetin, kaempferol, and apigenin were initially analyzed by HPLC at 1 mg/ml concentration. Retention time was noted and later used for the identification of these molecules in the extracts. The phenolic compounds detected were reconfirmed and quantified by spiking with the individual standards. It was observed that several of the compounds were present in both the ethyl acetate extracts of rhizome and its endophyte isolates. Though *L. toxicaria* rhizome extracts showed the presence of eight phenolic compounds, only myricetin and ellagic acid was present in a considerable amount (≥ 0.5 mg/g). One of the endophyte isolates of *L. toxicaria*, LTRH2, had a very similar result whereby, other than myricetin and ellagic acid, only kaempferol was present in a considerable amount. Further, only apigenin was found in higher amounts in the other endophyte, LTRO1 (Table 6). As compared to this, *K. rotunda* was richer in polyphenolic contents. The rhizome extracts had a considerable quantity of quercetin and cinnamic acid other than

**Table 3:** MIC of endophytic fungal extracts against different bacterial strains.

| Endophytic fungi | MIC (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S. aureus* | *E. coli* | *K. pneumoniae* | *Shigella* | *Serratia* | *Bacillus* | *Enterococcus* |
| LTRO1 | 3 | > 6 | > 6 | 0 | 0 | 6 | 6 |
| LTRO2 | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| LTRH1 | 6 | > 6 | > 6 | 0 | 0 | > 6 | > 6 |
| LTRH2 | 1.5 | > 6 | > 6 | 0 | 0 | 3 | 3 |
| KMPRO1 | > 6 | 0 | 0 | 0 | 0 | 0 | 6 |
| KPMRO2 | 6 | > 6 | > 6 | 0 | 0 | > 6 | > 6 |
| KMPRH1 | 3 | > 6 | > 6 | 0 | 0 | > 6 | 6 |

**Table 4:** Mortality percentage to *H. contortus* (L1) larvae by extract treatment.

| Concentration of extract (mg/ml) | Endophytic fungi | | | |
|---|---|---|---|---|
| | LTRO1 | LTRH2 | KMPRO2 | KMPRH1 |
| 10 | 80.50 ± 1.17 | 92.42 ± 0.52 | 90.30 ± 0.51 | 92.37 ± 0.97 |
| 5 | 64.80 ± 2.23 | 78.08 ± 2.08 | 73.42 ± 1.15 | 74.36 ± 1.45 |
| 2.5 | 41.45 ± 2.09 | 61.62 ± 1.39 | 52.35 ± 3.13 | 55.87 ± 3.21 |
| 1.25 | 9.69 ± 1 | 30.45 ± 0.59 | 20.16 ± 1.2 | 22.79 ± 2.85 |
| ABZ (1 µg/ml) | 100 ± 0 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| DMSO (1%) | 0 | 0 | 0 | 0 |

Values are mean ± SE.

**Table 5:** $LC_{50}$ and $LC_{90}$ values for *H. contortus* larval mortality by endophytes using probit analysis.

| Endophytic fungi | $LC_{50}$ (mg/ml) (LCL–UCL) | $LC_{90}$ (mg/ml) (LCL–UCL) | Chi-square value |
|---|---|---|---|
| LTRO1 | 3.672 (2.025–6.728) | 13.33 (7.107–154.9) | 4.683 |
| LTRH2 | 2.038 (1.680–2.387) | 8.255 (6.575–11.419) | 1.083 |
| KMPRO2 | 2.631 (2.261–3.017) | 9.378 (7.554–12.657) | 1.232 |
| KMPRH1 | 2.443 (2.086–2.810) | 8.817 (7.112–11.891) | 1.578 |
| *L. toxicaria* rhizome extract | 3.213 (2.201–4.857) | 10.383 (6.353–33.492) | 8.990 |
| *K. rotunda* rhizome extract | 2.917 (1.890–4.655) | 8.344 (5.099–27.137) | 13.672 |
| ABZ (µg/ml) | 0.23(0.910–1.211) | 0.78(1.231–3.621) | 0.416 |

myricetin, and ellagic acid (Table 7). Furthermore, KMPRH1, the endophyte isolate of *K. rotunda*, was having a considerable amount of syringe acid other than myricetin and ellagic acid. Of the total extract samples studied, KMPRO2 showed the presence of highest polyphenolic abundance. The ethyl acetate extracts of KMPRO2 were abundant in myricetin, kaempferol, ellagic acid, syringic acid, ferulic acid, coumarin acid, and caffeic acid.

### 3.3. Antibacterial Activity of Endophytic Fungi

#### *3.3.1. Disk diffusion assay*

Culture extracts of all the four endophytic fungi from *L. toxicaria* and all the three endophytes from *K. rotunda* were tested for antibacterial activity by disc diffusion assay against seven strains of bacteria (*E. coli*, *K. pneumoniae* and *S. aureus*, *K. pneumoniae*, *Shigella flexneri*, *S. marcescens*, *B. cereus*, and *E. faecalis*). Of the four isolates from *L. toxicaria,* LTRH2, LTRH1, and LTRO1 extracts inhibited five out of seven pathogenic bacteria including both Gram positive and Gram negative strains (Table 2). The LTRH2 extracts exhibited a zone of inhibition of 22.66 ± 0.71 mm against *S. aureus* and 20 ± 0.48 mm against *E. faecalis* (Both are Gram positive strains). The zone of inhibition was 15.5 ± 0.22 mm against the Gram negative bacteria, *E. coli* (Table 2). The activity of LTRH2 was followed by LTRO1, which also exhibited inhibitory activity against all the three bacterial strains tested. In the case of fungal isolates from *K. rotunda*, KMPR02 and KMPRH1 extracts inhibited five out of seven bacterial strains. Also prominent activity was observed against Gram positive strains. In general, two Gram negative bacterial strains *S. flexneri* and *S. marcescens* showed strong resistance against the fungal extracts. None of the tested extracts were effective against these Gram negative bacteria. On the other hand, *S. aureus* was found to be most susceptible and was found to be inhibited by majority of the endophytic fungal extracts.

#### *3.3.2. Broth microdilution assay*

Table 3 shows the MICs of the endophytic fungal extracts from *L. toxicaria* and *K. rotunda* against Gram-positive and Gram-negative

**Table 6:** Estimation of different polyphenols in ethyl acetate extracts of *L. toxicaria* rhizome and its endophytes.

| Sl No | Polyphenolic molecule tested | *L. toxicaria* rhizome | LTRO1 | LTRO2 | LTRH1 | LTRH2 |
|---|---|---|---|---|---|---|
| 1 | Catechol | 94.4 | ND | ND | ND | ND |
| 2 | Chlorogenic acid | ND | ND | ND | ND | ND |
| 3 | Caffeic acid | 44.99 | 58.019 | ND | ND | 462.559 |
| 4 | Syringic acid | 8.72 | ND | 28.385 | ND | ND |
| 5 | P-Coumaric acid | 9.96 | 50.385 | 25.264 | 966.61 | ND |
| 6 | Ferulic acid | 60.83 | 46.654 | ND | ND | 450.318 |
| 7 | Ellagic acid | 911.75 | ND | 46.845 | 1,926.29 | 992.318 |
| 8 | Myricetin | 6,344.52 | ND | 8.081 | ND | 1,560.68 |
| 9 | Cinnamic acid | 355.12 | 19.808 | 8.236 | 88.244 | ND |
| 10 | Quercetin | ND | 239.769 | ND | ND | ND |
| 11 | Kaempferol | ND | ND | ND | ND | 2,180.77 |
| 12 | Apigenin | ND | 583.269 | 241.23 | ND | ND |
| 13 | Gallic acid | 4,626.58 | ND | ND | ND | ND |

Values are amount of the polyphenolic molecule in µg per gram of the extract.
ND = Not detected.

**Table 7:** Estimation of different polyphenols in ethyl acetate extracts of *K. rotunda* rhizome and its endophytes.

| Sl No | Phytochemical standards | *K. rotunda* rhizome | KMPRO1 | KMPRO2 | KMPRH1 |
|---|---|---|---|---|---|
| 1 | Catechol | 23.33 | ND | ND | ND |
| 2 | Chlorogenic acid | ND | ND | ND | 297.647 |
| 3 | Caffeic acid | 199.8 | 116.567 | 1,000.64 | 123.706 |
| 4 | Syringic acid | ND | 641.067 | 3,470.21 | 2,541.38 |
| 5 | P-Coumaric acid | 133.1 | 404.5 | 1,340.12 | 127.588 |
| 6 | Ferulic acid | 77.09 | 115.267 | 1,960.21 | 207.559 |
| 7 | Ellagic acid | 1,678.2 | 444.533 | 3,215.93 | 2,800.76 |
| 8 | Myricetin | 5,061.75 | ND | 3,897.1 | 2,911.18 |
| 9 | Cinnamic acid | 878.27 | 165.233 | ND | 160 |
| 10 | Quercetin | 3,431.02 | ND | ND | ND |
| 11 | Kaempferol | 436.46 | ND | 3,714.14 | ND |
| 12 | Apigenin | ND | 1,024.23 | ND | ND |
| 13 | Gallic acid | ND | ND | ND | 94.268 |

Values are amount of the polyphenolic molecule in µg per gram of the extract.
ND = Not detected.

pathogenic bacteria. Among the fungal extracts of *L. toxicaria* rhizome, the LTRH2 showed the lowest MIC value of 1.5 mg/ml against *S. aureus*. Against other Gram-positive strains *B. cereus* and *E. faecalis*, the LTRH2 extracts exhibited the MIC values of 3 mg/ml (Table 3). The LTRO1 also showed strong antibacterial efficacy against *S. aureus*. In the case of fungal isolates from *K. rotunda*, the least MIC value was obtained with the KMPRH1 extract followed by KMPRO2 extract against *S. aureus*. All the other extracts showed MIC values ≥ 6 mg/ml concentration (Table 3). The very high MIC values against many strains indicate only a very limited antibacterial potency. In general, Gram positive bacteria showed some susceptibility to the fungal extracts, whereas the Gram negative bacterial strains showed high resistance against the endophytic fungal extracts.

### 3.4. Anthelmintic Activity

*In vitro* larval mortality assay envisaged the potential of extracts from endophytic fungi in causing mortality to the 1st instar *H. contortus* larvae. Two isolates from *L. toxicaria* (LTRO1 and LTRH2) and two isolates from *K. rotunda* (KMPRO2 and KMPRH1) were tested using this assay and all the four extracts showed significant dose dependent mortality to the L1 larvae. At the highest test concentration (10 mg/ml), LTRH2 exhibited 92.42 ± 0.52 percent mortality and LTRO1 showed a mortality of 80.50 ± 1.17 percent mortality (Table 4). The isolates from *K. rotunda* were found effective against the *H. contortus* larvae with a maximum mean percent mortality of 92.37 ± 0.97 and 90.30 ± 0.51, respectively, with the extracts of KMPRH1 and KMPRO2 isolates at this concentration. The mortality induced by these four

fungal extracts was statistically significant ($p > 0.001$) at all the tested concentrations when compared with vehicle control. The lethal concentration required to induce 50% ($LC_{50}$) and 90% ($LC_{50}$) mortality to *H. contortus* larvae was calculated by probit analysis and is shown in Table 5. Crude extracts of LTRO1 induced 50% lethality at 3.672 mg/ml, whereas LTRH2 showed 50% larval mortality at 2.038 mg/ml. In the case of *K. rotunda*, the KMPRH1 and KMPRO2 extracts showed $LC_{50}$ values of 2.443 mg/ml and 2.631 mg/ml, respectively, with 24 hours treatment. Commercially available anti-worm medication, ABZ treatment also caused significant mortality to L1 larva and we obtained 100% larval mortality with 1 µg/ml ABZ treatment. Probit analysis showed an $LC_{50}$ value of 0.23 µg/ml and $LC_{50}$ value of 0.78 µg/ml for this larvicidal assay (Table 5) in our experimental system.

## 4. DISCUSSION

Many previous studies have reported the occurrence of fungal endophytes of medicinal importance inside different regions of medicinal plants [28,29]. The present study was carried out to isolate, identify, and evaluate the antibacterial and nematicidal potentials of endophytic fungi from *L. toxicaria* Dalz and *K. rotunda* L. rhizomes. Remarkably we have isolated a total of four and three endophytic fungi from the rhizomes of *L. toxicaria* and *K. rotunda,* respectively. Based on morphological and molecular studies, LTRH2 and LTRO1 isolated from *L. toxicaria* were identified as *A. tamarii* and *A. niger*, respectively. The KMPRO2 isolated from *K. rotunda* was identified as *A. flavus*. The genus *Aspergillus* belongs to the Family Trichocomaceae of Order Eurotiales in the Class Eurotiomycetes of Phylum Ascomycota [30]. Tawfike *et al*. [31] earlier reported the presence of Ascomycetes inside plant tissues**.** *Aspergillus* genus is a ubiquitous fungus that is pathologically as well as therapeutically important [32]. Disk diffusion and Broth micro dilution assays were used to determine the antibacterial potentials of endophytic fungi isolated from both plants. Strong antibacterial activity was demarcated as MIC < 5 mg/ml [25]. The LTRH2 (*A. tamarii*), isolated from *L. toxicaria*, was the most effective endophyte among others to control bacterial growth of both Gram-positive and Gram-negative strains, followed by LTRO1 (*A. niger*). The inhibitory potentials of *A. tamarii* against Gram-positive bacteria *S. aureus*, *E. faecalis,* and *B. cereus* were found high compared to Gram-negative strains *E. coli* and *K. pneumoniae*. *A. tamarii* isolated from *Lycoperdon umbrium* showed similar growth inhibition against *Salmonella typhi*, *S. aureus*, *Bacillus subtilis,* and *E. coli* in earlier studies [33]. The current study also showed the potential of LTRO1 (*A. niger*) isolated from *L. toxicaria* rhizomes against the tested bacteria. A previous study reported the antimicrobial activity of the ethyl acetate extract of *A. niger* isolated from the stilt rhizomes of *Rhizophora apiculata* [34]. According to their study, *Proteus mirabilis* and *Pseudomonas aeruginosa* were highly sensitive to *A. niger* extract. Another study testified that *A. niger* extracts possess aromatic-nitrogenous compound, tensidol which induced high inhibition against *P. aeruginosa*, *S. aureus*, *Staphylococcus epidermidis*, and *Bacillus* sp. [35]. Further studies are in progress to confirm the presence of tensidol in our endophytes. In the case of *K. rotunda*, KMPR02 (*A. flavus*) and KMPRH1 (unidentified) isolates showed effective inhibition against both Gram-positive and Gram-negative bacterial strains. Derivation of two furan compounds, namely 5-acetoxymethyl furan-3-carboxylic acid and 5-hydroxymethyl furan 3-carboxylic acid by the fermentation of *A. flavus*, isolated from *Cephalotaxus fortunei* and their potent antibacterial activity against *S. aureus* was reported earlier by Ma *et al*. [36]. The present study also highlighted the high resistance of two Gram-negative bacterial strains *S. flexneri* and *S. marcescens* against endophytic fungal extracts from both plants. None of the endophytic fungal extracts were active against these two Gram-negative bacteria.

The greatest problems encountered during the use of anthelmintics are increasing nematode resistance and slow degradation in the soil [37]. Studies on the use of endophytic fungal metabolites against animal parasitic nematodes are very less. Hence, we decided to extend our studies on the nematicidal activity of the extracts that gave good results in the bactericidal assays Our study clearly envisages the potentials of the ethyl acetate extracts from *A. tamarii, A. niger, A. flavus* and KMPRH1 cultures in causing significant larvicidal activities against nematode larvae. A concentration-dependent increase in the death toll of nematode larvae was observable with all the tested extracts.

In the frequent search by the pharmaceutical industry for drug leads, natural resources, particularly plants seem to be a precious repository. Phenols and terpenoids are indeed considered as safe alternatives to commercial anthelmintic drugs and several studies have explained the importance of plant derived flavonoids against worms [38]. Flavonoids such as genistein, quercetin, and kaempferol induced high mortality to nematodes and trematodes in several earlier studies [39,40]. In order to identify and characterize the polyphenolic compounds of fungal extracts, we took the advantage of HPLC analysis. The HPLC studies of our extracts confirmed the presence of at least six different phenolic compounds in a considerable quantity. It was further revealed that the pattern of presence of these molecules was comparable between the rhizome and its endophytes. Myricetin was the most abundant molecule in both rhizomes extract and the endophyte extracts. *Kaempferia rotunda* rhizome, which is known for its aroma, was having higher polyphenolic content so also its endophyte isolates. Out of the nine polyphenolic molecules detected, four of them having more than 0.5 mg per gram of the extract were identified in both rhizome and endophyte extracts. On the other hand, in *L. toxicaria* rhizome, only myricetin and ellagic were present in considerable amounts, which is also true for its endophytes. The molecular mechanism of the observed biological activity is not known from this study. But the presence of these phenolic molecules in the crude plant extracts as well as in the newly isolated endophytes indeed give us an alternative source for these molecules. Further bio-guided fractionation studies are needed to identify the individual components and this can lead to the isolation of the active ingredients from this new source.

## 5. CONCLUSION

In fact, this is the first report on the isolation, identification, and bioactivities of endophytic fungi from *L. toxicaria* and *K. rotunda*. Fascinatingly, the present study results provide a solid platform for the development of novel anthelmintic and antimicrobial agents from endophytic fungi of *L. toxicaria* and *K. rotunda*. It is noteworthy that endophytic fungi, among the novel biotypes, are

in the top priorities now, which would make drastic changes to medicinal, pharmaceutical, and agriculture industries in the future.

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## 7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## 8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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