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Woodfordia fruticosa (Linn.) Kurz's fungal endophyte *Mucor souzae*'s secondary metabolites, kaempferol and quercetin, bestow biological activities

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ABSTRACT

Woodfordia fruticosa is reported to have ethnomedicinal values. As exploitation of the host on a large scale is detrimental, the authors attempted to look into its endophytes for secondary metabolites. Thus, the current investigation is directed at the fungal endophytes of the leaves identified based on morphotyping by microscopy and molecular typing by internal transcribed spacer–DNA sequences. *Mucor* sp. was identified as *M. souzae* by molecular typing and its ethyl acetate extract was screened for biological activities. The antioxidant capacity of the extract exhibited 1,1-diphenyl-2-picrylhydrazyl scavenging capacity with an IC_{50} of 58.64 ± 4.38 µg/ml. Bactericidal property assayed by disk diffusion in antagonism to bacterial strains revealed a notable halt to their growth. Bacterial biofilm inhibition capacity stained by acridine orange and ethidium bromide imaged by confocal laser scanning microscopy revealed the loss of microcolonies. HeLa cells subjected to *M. souzae* extract resulted in high degree of DNA fragmentation, revealing apparent apoptosis, and condensation of chromatin was recorded by confocal microscopy. Bioactive kaempferol and quercetin detected via thin-layer chromatography could support the biological activities. Thus, scrutiny on endophytes from untraversed plant with medicinal properties may guide researchers to the discovery of secondary metabolites as therapeutic agents with potential drug applications.

1. INTRODUCTION

Woodfordia fruticosa (Linn.) Kurz (family: Lythraceae) is commonly called "Dhainyaro" and is housed from 200 to about 1,800 m above the ocean surface. Therapeutic values for its various parts are reported [1] with commercial formulations incorporating them. It has, thus, evoked significant curiosity in the scientific community to appraise the therapeutic properties with scientific rationality for its use in medical therapy. There are a few reports on the use of flowers and leaves exhibiting experimentally proved anti-inflammatory, antitumor, anti-leucorrhoeic, and immunomodulatory activities. The biological compounds reported in parts like flowers, leaves, and stem of *W. fruticosa* have been credited to provide therapeutic benefits [2–4].

Endophytic microbes of medicinal flora have gained awareness as they are accepted to be magnificent assets of secondary metabolites endowed with biological capacity [5–10]. As the secondary metabolites produced by fungal endophytes have significance in both academic and industrial scenarios, the study of *W. fruticosa*-associated fungi yields wider appreciation toward its diversity. As a first report about the biological activities of the extract from endophytic fungi residing in *W. fruticosa* leaves, this study reports the fungus *Mucor souzae* as a new source with the ability to yield molecules with application in pharmacy.

2. MATERIALS AND METHODS

2.1. Plant Samples

Leaf samples of *W. fruticosa* (Linn.) Kurz were gathered from Gokarna, which is along the west coast of India, in the taluk of Kumta, and is in the district of Uttara Kannada of Karnataka state. The herbarium specimen has been deposited (*W. fruticosa* # IOE

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LP0006). The specimens were placed in plastic containers, sorted during a stipulated time period (24–48 hours), and were processed for growth and analysis of fungal endophytes.

2.2. Culturing of Leaf Endophytes

The leaves collected were cleaned under tap water (five times), resulting in the clearing of dust and grime particles. The sterilization of cleaned leaf surface was carried out under sterile conditions. The steps involved several rinses with ethanol (70%, v/v) for a minute. The next step was cleaning with sodium hypochlorite (3 minutes, containing 3% accessible chlorine; 4.5% v/v diluted; HiMedia, India). Lastly, a rinse was given using distilled water (sterile), resulting in the removal of any remnants of sterilants on the surface [11]. All leaf bits were then tapped, resulting in the removal of adhering water droplets. Leaf bits (10–15 bits; 0.5–1 cm) were placed in media (sterile water agar; 15 $g.l^{-1}$) containing chloramphenicol (240 ppm; Sigma Aldrich, St. Louis, MO) in a sterile petri dish. Incubation was for 15 days for 16 hours (28°C; dark alternate with light cycles). The fungal endophytes sprouting from the bits of leaves were photographed. Furthermore, they were then placed on potato dextrose agar (PDA) media (HiMedia, India) to induce maturation, including sporulation. Then the grown fungal endophytes were stored (−80°C) on PDA and 15% glycerol (v/v) in cryovials.

2.3. Isolation of Genomic DNA from Fungal Endophytes, its PCR Amplification, and Molecular Typing

Initially, the fungal endophytes were identified depending on their colony characteristics. Their spores and fruiting bodies were photographed under the microscope (Research Stereo Zoom Microscope; Stereo Discovery V20; Carl Zeiss, Germany). Furthermore, typing was carried out as reported in standard manuals specifically for fungal identification [11]. Endophytic fungi were cultivated on PDA media and the fresh culture was harvested for further work. The DNA from this culture was extracted by cetyltrimethylammonium bromide protocol [12,13]. DNA was kept in a buffer (Tris EDTA; 100 µl). Quantification of the genomic DNA and its purity were checked in a nano-spectrophotometer (Thermo 2000C; Thermo Fisher Scientific, USA). Corresponding to pure desirability (1.6/1.8) indicated no contamination with ribose nucleic acid, protein or contaminant, phenol. The DNA in the resultant sample was confirmed in 1% agarose gel electrophoresis and the results were documented (Geldoc XRT; BioRad, USA). Universal internal transcribed spacer (ITS) primers for fungi were used to amplify the extracted DNA (ITS1: 5′-TCC GTAGGTGAACCTGCG G-3′; ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) [14]. The reaction was carried out in polymerase chain reaction (PCR) tubes (0.2 ml) containing the reaction mixture (25 µl) and genomic DNA (1 µl; 50 ng/µl) in a thermal cycler (Mastercycler gradient; Eppendorf, Germany). The program was as follows: initial denaturation (5 minutes, 95°C followed by 3 minutes, 94°C) and primer annealing (1 minute, 55°C), followed by extension (2 minutes, 72°C and 10 minutes extension), and it was repeated for 35 cycles. The product of this PCR amplification (5µl) was assessed on 1% agarose gel electrophoresis and the results were reported in comparison to a commercially available standard molecular ladder. The amplified PCR samples were sequenced at Bangalore (Chromous Biotech). The resulting DNA sequences were analyzed by BLAST with NCBI.

2.4. Preparation of Endophyte Extracts

Agar pieces (0.5 cm^2) with actively growing fungal endophytic colonies were inoculated onto sterile potato dextrose broth (1 l). The flasks were incubated with alternate light (8 hours) and dark cycles (16 hours) in a stationary phase (25°C ± 2°C) for 15 days. The mycelial mat in the broth was sonicated and the whole mass was filtered. For extraction of bioactive ingredients, the filtrate was transferred to a separating funnel containing an equal volume of ethyl acetate. The mixture was strongly agitated. The solvent layer on the top was separated and condensed to 10 ml in a Heidolph system, an evaporator with rotations at 42°C [15]. The fraction that was dry was solubilized in methanol (5 ml). It was stored (4°C) in a colored glass container for further experimentation.

2.5. Estimation of Total Phenol Concentration in the Endophyte Extract

The phenol content in endophyte extracts was determined according to the calorimetric method [16]. The extract (100 µl) was mixed with a Folin–Ciocalteu reagent (0.75 ml; 1:10 diluted, HiMedia, India) and incubated (22°C, 5 minutes). Saturated sodium carbonate (60 g/l) was added to neutralize it. Further incubation was carried out in the dark (1.5 hours, 22°C). The blue color was measured at 725 nm (Hitachi U-3900 UV/visible spectrophotometer). Phenol content was estimated using the standard curve of gallic acid (25–250 µg/ml; Sigma Aldrich, St. Louis, MO). The concentration of phenol in the extract was reported as 153.39 gallic acid equivalents (GAE mg/mg extract). All the estimations were carried out in triplicate.

2.6. Estimation of Total Flavonoid Content

The sample extract (100 µl) was combined with the methanolic solution of aluminum chloride (2%; 100 µl). After incubation (30 minutes), the absorbance of the reaction was read at 430 nm. The flavonoid content was reported using the Rutin standard analytical curve (10–100 µg/ml; Sigma Aldrich, St. Louis, MO). The flavonoid concentration in the extract was expressed in mg Rutin (mg/g of extract). All estimations were carried out in triplicate.

2.7. 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Assay

DPPH was obtained from Sigma Aldrich (St. Louis, MO). The procedure for estimation of the antioxidant potential was followed as described by a previously reported protocol [17]. The results were measured at 517 nm using a Spectra max 340 Colorimeter from Molecular devises, USA. A methanolic DPPH solution with radicals (300 µM) was prepared. The final mixture included an extract (5 µl) mixed with a DPPH solution (95 µl) at 37°C for 30 minutes. The scavenging activity of DPPH radical (percent) was estimated in comparison with methanol (negative control), ascorbic acid, and quercetin (25–250 µg/ml; positive controls; Sigma Aldrich, St. Louis, MO). The values (IC_{50}) representing the concentration of the endophyte extract required to scavenge DPPH (50%) radicals was reported.

2.8. Biological Activity Studies

2.8.1. Agar disk diffusion method

Escherichia coli (MTCC 724) and *Pseudomonas aeruginosa* (ATCC 27853) were the Gram-negative bacteria used in the study. *Staphylococcus aureus* (MTCC 96) and *Bacillus subtilis* (MTCC 441) were the Gram-positive bacteria used in the study. They were obtained from the Institute of Microbial Technology (Chandigarh, India). The endophyte extract (1, 2, 5.0, and 10.0 µg) and positive control (streptomycin 2 µl of 1 mg/ml; Sigma Aldrich, St. Louis, MO) were used. The samples were introduced on Whatman's paper disks (10 mm) placed on Mueller Hinton agar as per a previously reported protocol [18]. They were incubated for 15–18 hours at 37°C. The inhibition zone diameter was reported in comparison with streptomycin.

2.8.2. Assay of the loss of biofilm by confocal laser scanning microscopy

The biofilm structure was observed in confocal laser scanning microscopy (CLSM) (LSM 710; Carl Zeiss, Germany) and reported. The experiment was carried out as per a previously reported procedure [19]. The overnight bacterial cultures, Gram-negative bacteria, and *P. aeruginosa* (ATCC 27853), along with *E. coli* (MTCC 724), were prepared. A 6-well plate was used for the assay. Cover slips were introduced in all the wells. The media (2 ml) was poured over them. The controls consisted of a cover slip with media. To the experimental wells, overnight-grown bacterial cells (750 µl) were added. The endophyte extracts (200 µl, 750 µg) were then introduced into the wells. The 6-well plates were incubated at 37°C for 24 hours. The cover slips in wells with biofilm formed on them were carefully removed. The biofilm was washed with phosphate buffer saline. The film was stained with ethidium bromide and acridine orange (24 µl; concentration 1 µg in sterile distilled water, 400 µl; HiMedia, India). Furthermore, they were carefully washed (thrice). The cover slips containing stained biofilms were placed on a glass slide in an inverted position. The samples were imaged and CLSM images were presented.

2.8.3. Evaluation of M. souzae extract-induced cell cytotoxicity

2.8.3.1. Animal cell lines and their culturing with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) assay. Human cell lines, HeLa (cervical carcinoma), obtained from the National Center for Cell Science (NCCS, Pune, India) were used. They were cultivated in Dulbecco's Modified Eagle Medium media (Sigma Aldrich; St. Louis, MO). Fetal bovine serum was added to it (10%; Gibco BRL, USA). MTT assay of the fungal extract was reported by MTT (Sigma Aldrich, St. Louis, MO) [20]. Three wells were used for each setup in triplicate.

2.8.3.2. DNA fragmentation assay. HeLa cells (5×10^4 cells/ml) were treated with *M. souzae* extract (60 mg/ml) and camptothecin (2 mg/ml; Sigma Aldrich, St. Louis, MO). The cells (treated and controls) were harvested and a DNA fragmentation procedure was carried out as reported earlier [20].

2.8.3.3. Apoptosis assessed by nuclear morphology. HeLa cells were treated with *M. souzae* (60 µg/ml) and camptothecin (2 µg/ml). They were stained with acridine orange and propidium iodide [Acridine orange/propidium iodide (AO/PI); 1 mg/ml] as reported earlier [20]. Changes after treatments in the apoptotic nuclear morphological were observed by CLSM LSM 710 (Carl Zeiss, Germany).

2.9. Chromatographic Analysis by Thin-Layer Chromatography (TLC)

TLC was carried out on precoated (0.25 mM silica gel) glass plates (10 × 5 cm; 60 F254, Merck, Germany). Prior to use, they was activated in an oven (110°C, 1 hour). The ethyl acetate extract (10 µ, 5 mg/ml) was applied by means of a capillary tube. The standards quercetin and kaempferol (10 µl and 1 mg/ml, respectively; Merck, Germany) solubilized in methanol were also applied along with the extracts by using the capillary tube. The plates were developed in glass chambers by solvent system hexane–ethyl acetate–methanol (10:75:15; v/v). The secondary metabolites of *M. souzae* ethyl acetate extract were visualized by spraying with anisaldehyde sulfuric acid (HiMedia, India) with subsequent heating at 115°C. The chromatogram images were visualized under UV365 and the images were captured by a digital camera.

2.10. Statistical Analysis

All the experimental results and reported values were in triplicate. The results are reported as mean ± standard deviation (SD) after subjecting to variance scrutiny, followed by Tukey's test. This lead us to analyze the differences among the *M. souzae* ethyl acetate extract and control conditions at p-value < 0.001.

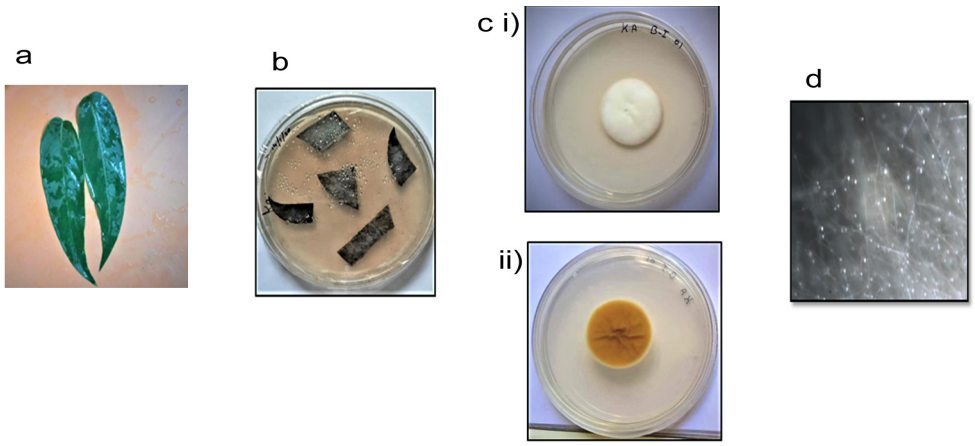
3. RESULTS AND DISCUSSION

Fungal endophytes as asymptomatic symbiotic colonizers are present in the internal tissues of host plants, an association sound to the host, wherein they reside as in several scenarios that are neither mutualistic nor pathogenic, and have been reported with the capacity to produce the same biologically active compound as reported in their host plant. Thus, it resulted in several leads and discoveries of unique secondary metabolites that have been used as drugs [21]. In this direction, our work is on the study of endophytic fungi of *W. fruticosa* (Linn.) Kurz (family: Lythraceae) tree leaves, an endemic tree from the western coastal region, Karnataka, India, that provides a view into the fungal endophytic consortia.

3.1. Fungal Endophytes by Morphotyping and Molecular Typing

In the present study, the fungal endophytes of the leaves were sporulated and reported by morphotyping (Fig. 1) and molecular typing by analysis of ITS in their DNA sequence. A set of 21 endophytes were obtained from 82 bits of leaves.

DNA from the endophytes was isolated. The ITS region of DNA was amplified. A clear band at 600 bp (Fig. 2) obtained on agarose gel was also reported. The band was purified and



a. *Woodfordia fruticosa* leaves
b. Leaves were plated on malt agar (2%)
c. The pure colony of fungus (*Mucor* spp.) isolated and grown on potato dextrose agar

i) Image of the top view of fungus growth
ii) Image of the bottom view of the fungus growth with the dish inverted

d. *Mucor* spp. spores and mycelia visualized in Stereozoom microscope

Figure 1: An approach of the reporting fungal endophyte, *M. souzae*, from leaf segments of *W. fruticosa*.

Table 1: Details of the fungal endophytes from leaves of *W. fruticosa.*

Endophyte key	Endophyte identified	DNA (ng/100ml)	Total DNA	Similarity index (%)	Accession No. w.r.t. GenBank
WF-6	*Mucor* spp	196	1.83	*M. souzae* (98%)	NR_165210.1
WF-14	*Colletotrichum* spp	182	1.81	*Colletotrichum* spp. (92%)	MG980301.1
WF-23	*Alternaria* spp	193	1.76	*Alternaria* spp (91%)	KC978055.1
WF-33	*Penicillium* spp.	195	1.71	*Penicillium* spp. (94%)	MG600580.1
WF-37	*Fusarium* spp	191	1.82	*Fusarium* spp. (90%)	MN844733.1
WF-42	*Aspergillus* spp	189	1.78	*Aspergillus* spp. (94%)	MT106901.1

sent for DNA sequence analysis. The obtained results of DNA sequences were analyzed by the BLAST tool at NCBI. *Woodfordia fruticosa* endophytes identified by molecular typing include *Mucor* spp., *Colletotrichum* spp., *Alternaria* spp., *Penicillium* spp., *Fusarium* spp., and *Aspergillus* spp.

Mucor sp. was identified as *M. souzae* by the colony, spore morphology (Fig. 1ci, ii and d) and ITS analysis (Table 1). This isolate reported was taken up for further studies. Its ethyl acetate extract was screened for biological activities and secondary metabolites were reported.

3.2. Total Secondary Metabolites (Phenol and Flavonoid) Contents

In the current study, crude extracts from fungal endophytes were screened and our work was the first study reporting antioxidant and antibacterial capacities of the extracts of *M. souzae*, endophytic fungi of *W. fruticosa* tree leaves. Endophyte extracts exhibited several biological properties, like antioxidant and antibacterial with biofilm inhibition capacities.

Phenol concentration was expressed as GAE (mg/g of extract). Flavonoid concentration was reported as Rutin equivalents (mg/g of extract). *Mucor souzae* extract contained a total phenol

Table 2: Total secondary metabolites content (phenol and flavonoid) in the extract of fungal endophytes reported from *W. fruticosa*.

Endophyte code	Endophyte fungi identified	Total phenol content*	Total flavonoid content**
WF-6	*M. souzae*	151.39 ± 0.124[a]	72.05 ± 0.32[a]
WF-14	*Colletotrichum* spp	143.98 ± 0.44[b]	71.76 ± 1.16[ab]
WF-23	*Alternaria* spp	143.87 ± 1.23[b]	66.77 ± 1.02[c]
WF-33	*Penicillium* spp.	135.46 ± 0.94[c]	71.62 ± 0.98[ab]
WF-37	*Fusarium* spp	142.19 ± 1.09[bc]	69.19 ± 1.78[b]
WF-42	*Aspergillus* spp	150.98 ± 1.23[ab]	68.98 ± 2.04[bc]

Data expressed are mean (n = 3) ± SD.
Same letters mean no statistical differences among values as per the test (Tukey's) at $p < 0.001$.
Values followed by different letters within columns are significantly different according to the test (Tukey's) at $p < 0.001$.
*Total phenol content; GAE (mg/g endophyte extract).
** Total flavonoid content; Rutin equivalent (mg/g endophyte extract).

Table 3: Scavenging DPPH radical by *M. souzae* extract of *W. fruticosa* leaves.

Endophyte extracts/ positive controls	DPPH (IC_{50}) (µg/ml)[a]
M. souzae extract	58.64 ± 4.38
Ascorbic acid	29.31 ± 4.21
Quercetin	23.58 ± 3.23

[a] Data expressed are mean (n = 3) ± SD.

content of 151.39 ± 0.124 (GAE; mg/g of extract). The total flavonoid content of 72.05 ± 0.32 (Rutin equivalents; mg/g of extract) was reported in the extract (Table 2).

3.3. DPPH Radical Scavenging Property of the Extract

The test compounds with hydrogen-donating capacity can decolorize DPPH. The activities were recorded and compared to the reference standards, ascorbic acid and quercetin. Endophyte, *M. souzae* ethyl acetate extract exhibited DPPH scavenging activity with the IC_{50} value of 58.64 ± 4.38 µg/ml. The reference compounds, ascorbic acid and quercetin, exhibited IC_{50} values of 29.31 ± 4.21 and 23.58 ± 3.23 µg/ml, respectively, as reported in Table 3.

3.4. Antibacterial Activity

3.4.1. Agar disk diffusion method

The extracts inhibited bacterial growth resulting in clear ZOI indicating an antibacterial effect. Inhibitions of both Gram-negative and Gram-positive bacteria were recorded (Fig. 3). This antibacterial property of the *M. souzae* ethyl acetate extract was screened using four microbes (Fig. 3), *S. aureus* (MTCC 96) and *B. subtilis* (MTCC 441) (the Gram-positive bacteria) and *E. coli* (MTCC 724) and *P. aeruginosa* (ATCC 27853) (the Gram-negative bacteria). The resulting inhibition zones (ZOI) were also recorded. Endophyte extract (10 µg) significantly arrested the growth of *P. aeruginosa* (5 mm, ZOI) and *S. aureus* (2 mm, ZOI), followed by *E. coli* and *B. subtilis* (4 and 3 mm, ZOI, respectively).

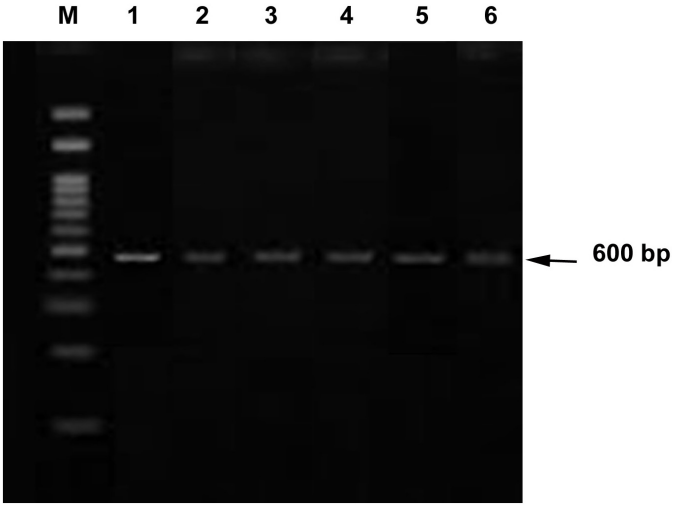


Figure 2: Amplifications of ITS in the DNA of endophytes and its analysis on agarose gel (1% w/v).Lane M: DNA ladder (100–1 kbp); lane 1: *Mucor* spp; lane 2: *Colletotrichum* spp; lane 3: *Alternaria* spp; lane 4: *Penicillium* spp; lane 5: *Fusarium* spp; and lane 6: *Aspergillus* spp.

3.4.2. CLSM investigation to record biofilm loss

Gram-negative strains of *P. aeruginosa* (ATCC 27853) and *E. coli* (MTCC 724) and the biofilm forming microbes were incorporated for this study and the loss of biofilm was visualized by CLSM. From sample observations of controls, *P. aeruginosa* (Fig. 4a) and *E. coli* (Fig. 4c) formed a biofilm on the support, and live cells were visualized and stained green by acridine orange. The addition of the extract (200 µl, 750 µg) resulted in a significant loss of biofilm that was recorded by CLSM. Patches and broken-down biofilm architecture along with diminished microcolonies containing dead cells of *P. aeruginosa (*Fig. 4b*)* and *E. coli* (Fig. 4d) were observed to be red as they were stained with ethidium bromide.

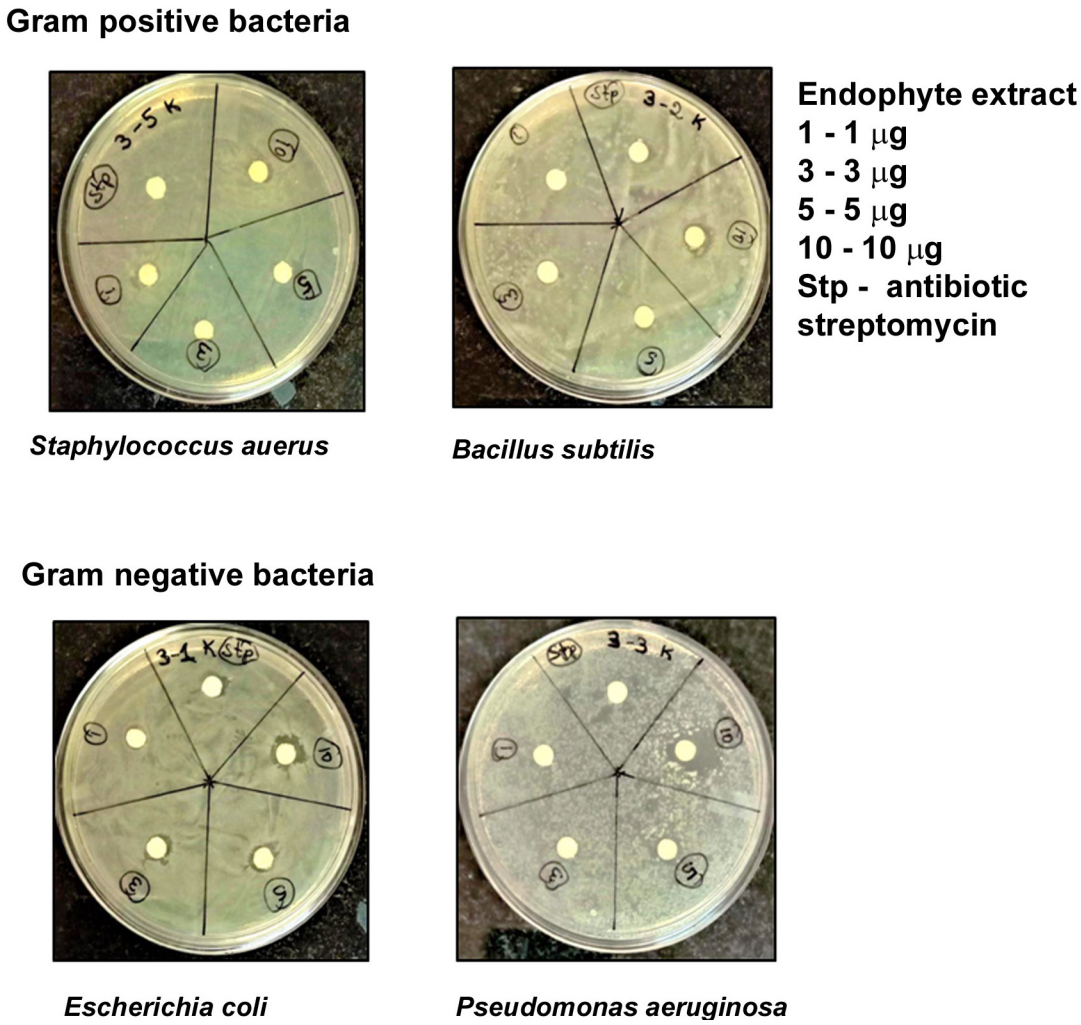


Figure 3: Antibacterial assay of *M. souzae* ethyl acetate extract by the disk diffusion method.

3.5. Kaempferol and Quercetin Presence in Ethyl Acetate Extract by TLC

Woodfordia fruticosa was reported to be rich in secondary metabolites (kaempferol and quercetin). Previously, *Mucor fragilis* isolated from *Sinopodophyllum hexandrum* (Royle) Ying rhizomes harvested at high ranges in the Taibai Mountains (China) had the ability to produce podophyllotoxin and kaempferol [22]. In the present study, secondary metabolite from *M. souzae* extract needed to be characterized. In this direction, we carried out a preliminary study by TLC. Kaempferol (3,4′,5,7-tetrahydroxyflavone) and quercetin (3,3’,4’,5,7-pentahydroxyl-flavone) flavonoids were detected via TLC analysis compared with the standards (Fig. 5). The developed bands were observed under the UV light.

Flavonoids have been reported to show up to a sixfold stronger antibacterial activity in comparison to the standard antibacterial drugs in the market. They are low molecular weight polyphenolics with the core structure being the C6–C3–C6 skeleton. The amphipathic features (hydrophilic and hydrophobic moieties) with substituents (alkyl chains, heterocyclic moieties containing oxygen or nitrogen, and prenyl groups) contribute to antibacterial properties. The mode of action includes cytoplasmic membrane function alterations, inhibition of energy metabolism, and decreased cell attachment resulting in disrupted biofilm formation, changes in permeability of cell membrane, and attenuation of pathogenicity to cite a few [23].

Pure kaempferol exhibited disruption of bacteria. It was carried out by interaction with the head group with a polar charge in the model membrane used in the study under *in-vitro* conditions [24]. Pure quercetin exhibited its antibacterial capacity by blocking the charges on amino acids in the porins [25].

3.6. Evaluation of *M. souzae* Extract-Induced Cell Cytotoxicity via Apoptosis

Maximum cell death was observed and IC_{50} values were reported: *M. souzae* extract (IC_{50} 61 ± 3.5 µg/ml) and camptothecin (IC_{50} 2.3 ± 1.6 µg/ml). DNA fragmentation experiment results exhibited fragmentation of the same to smaller entities (Fig. 6i). A smear was observed indicating nonspecific DNA degradation. These results corroborate apoptotic induction capacity of *M. souzae* extract [26]. To confirm apoptosis, HeLa cells were stained with

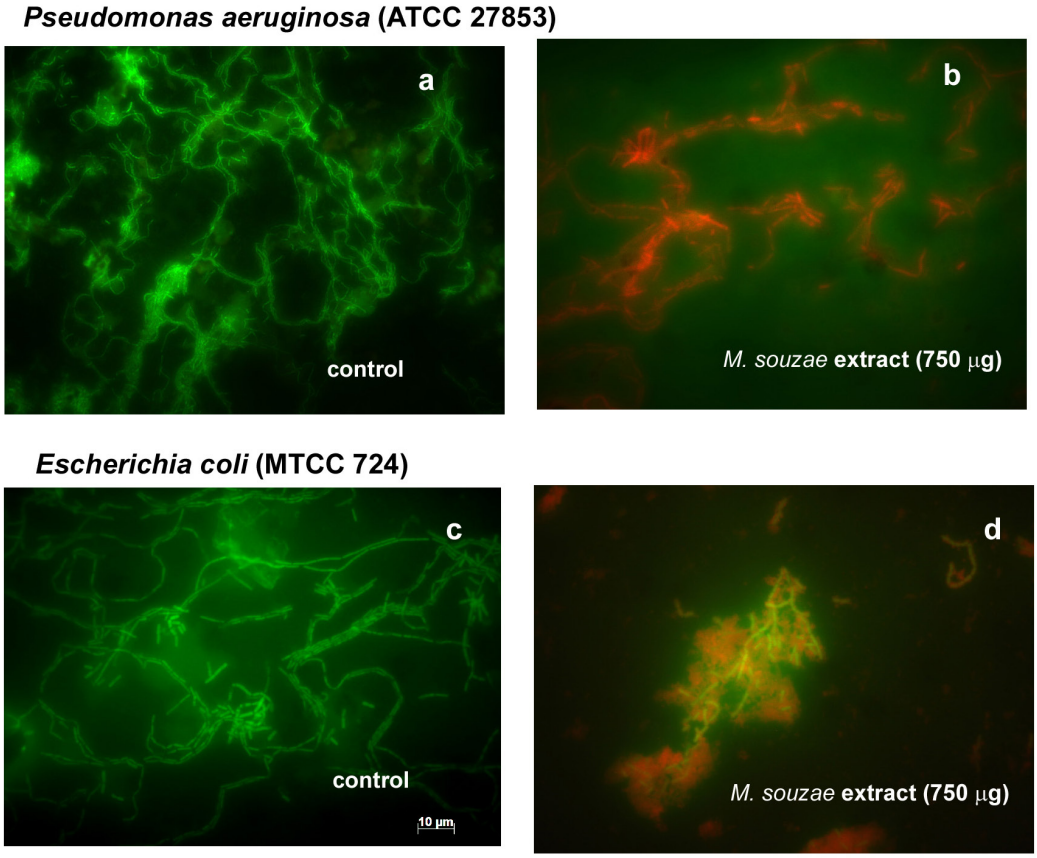


Figure 4: Biofilm inhibition assay of *M. souzae* ethyl acetate extract. Controls, *P. aeruginosa* (a); *E. coli* (c); *M. souzae* extract + *P. aeruginosa (b); and M. souzae* extract + *E. coli* (d).

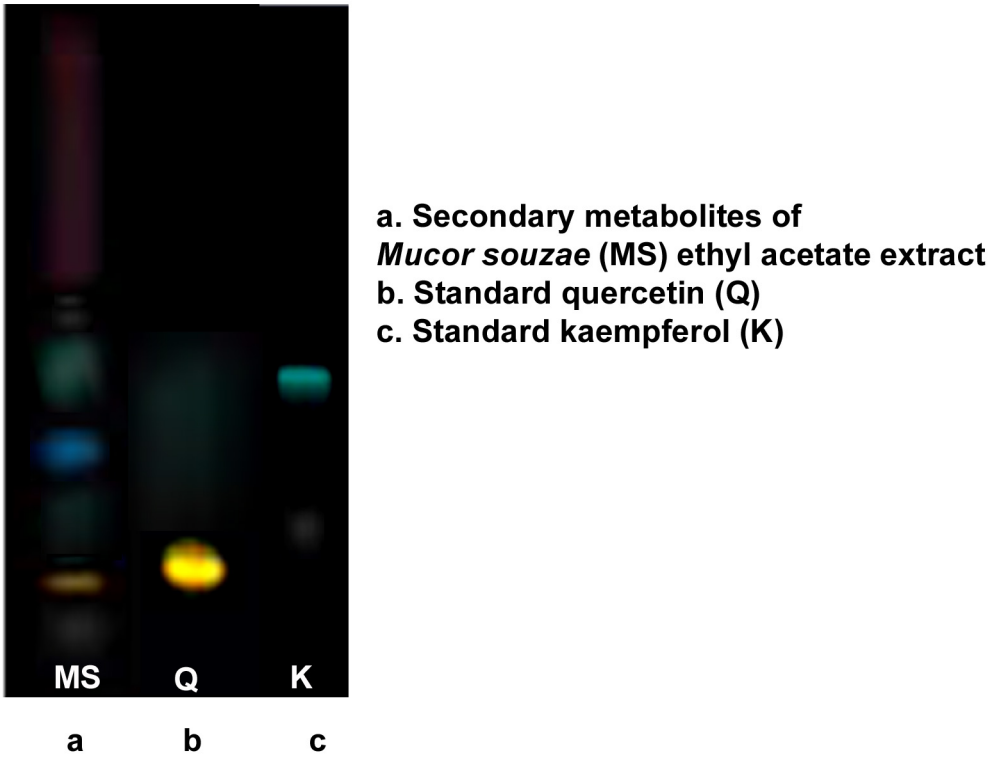


Figure 5: Thin-layer chromatography detecting the presence of quercetin and kaempferol in *M. souzae* ethyl acetate extract as visualized in UV.

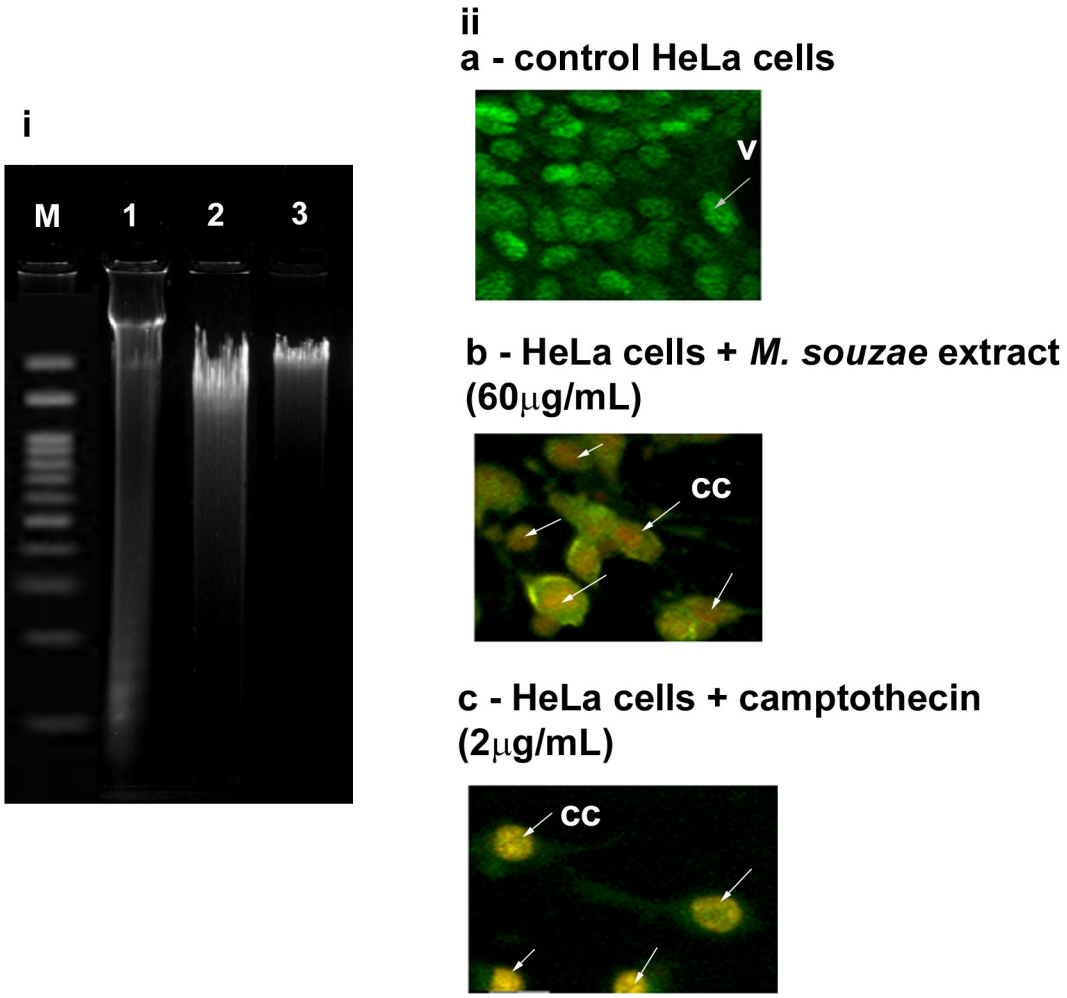


Figure 6: Evaluation of *M. souzae* extract-induced cell cytotoxicity via apoptosis. i) DNA was subjected to fragmentation. The resultant samples were analyzed by agarose gel electrophoresis. Lane M: DNA ladder (1 Kbp); lane 1: DNA of control (only HeLa cells); lane 2: DNA of HeLa cells + *M. souzae* extract (60 mg/ml); lane 3: DNA of HeLa cells + camptothecin (2 mg/ml). ii) Induction of apoptotic nuclear morphology in HeLa cells by *M. souzae* extract (iib) and camptothecin (iic) in comparison to control (iia) cells. They were dual-stained with AO and PI and visualized under a confocal laser scanning microscope. V, viable cells; CC, chromatin condensation in apoptotic cells.

AO and PI. After this dual-stain, an emission of green fluorescent wavelengths by AO and orange fluorescent wavelengths by PI were captured by CLSM. Apoptosis with chromatin condensation was visualized as an orange fluorescence in the nuclear region (Fig. 6ii, b and c) of HeLa cells when they were treated with *M. souzae* and camptothecin. The control cells were visualized as green, indicating live cells (Fig. 6ia). Chromatin condensation affirmed HeLa cell cytotoxicity by *M. souzae* extract as an apoptotic affair.

Cancer cell lines facilitate research on new cytotoxic entities by being useful tools in the anticancer studies and the structure–activity relationship that aids in revealing the potencies of new molecules with therapeutic properties. In this context, studies have revealed the hydroxylation at the C5 position as in the case of quercetin, which can be considered important for cytotoxic activity. Similarly, hydroxylation at 3, 5 7, 4` and its derivative, kaempferol-3-O-p-D-glucopiranoside, have reported cytotoxic capacities [27].

4. CONCLUSION

The current report on the fungal endophyte *M. souzae* screened from a consortium of endophytes from *W. fruticosa* tree leaves by its characterization via morphotyping and molecular typing by ITS–DNA sequence analysis adds to the current trend of knowledge with respect to several studies indicating *Mucor* spp. as a rich source of secondary metabolites [28–30]. However, this study identifies *Mucor* spp. investigated only at the crude extract level. Although kaempferol and quercetin from ethyl acetate extract were reported by TLC analysis in comparison to the use of standards, further investigations are required to determine other secondary metabolites from *M. souzae* that could lead to identification of many more new molecules toward therapeutic applications. Chemical investigation to identify potential active ingredients is still ongoing. Data collection is a treasure toward unraveling the capacity of *Mucor* endophytic species as it is reported to be a true fungal factory required for exploitation of a broad range of applications.

5. ACKNOWLEDGMENTS

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6. CONFLICT IN INTEREST

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

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. ETHICAL APPROVALS

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

9. DATA AVAILABILITY

All data generated and analyzed are included within this research article.

10. PUBLISHER'S NOTE

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