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Antibacterial, antioxidant, and anticancer activities of *Penicillium citrinum* Thom. endophytic in *Jatropha heynei*

Gowdru Basanna Ashoka, Manchanahally Byrappa Shivanna*
Department of PG Studies and Research in Applied Botany, School of Biosciences, Kuvempu University, Shivamogga, India.

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ABSTRACT

In the present study, the leaf, fruit, and root samples of *Jatropha heynei* were incubated using potato dextrose agar, malt extract agar, czapek dox agar, and water agar methods to determine colonization frequency and diversity indices of endophytic fungi. Thirty three endophytic fungal species of 20 genera were recovered from 5,400 segments of *J. heynei* and were identified based on their morphological characteristics. Species richness was high in leaf followed by root and fruit. Diversity indices varied depending on the season and different plant parts. The metabolites of endophytic fungus *Penicillium citrinum* were tested for antibacterial, antioxidant, and cytotoxic studies *in vitro*. The culture filtrate (CF) extract of *P. citrinum* showed high antibacterial activity to *Pseudomonas syringae* (zone of inhibition 17.65 ± 1 mm) and *Staphylococcus aureus* (zone of inhibition 16.32 ± 0.5 mm). The antioxidant potential was determined by cyclic voltammetry method based on the detection of redox potential of metabolites. The CF extract exhibited significant cytotoxic effect in both A549 and MCF-7 cell lines at 500 µg ml^{-1} with IC_{50} values of 280.7 and 283.0 µg ml^{-1}, respectively. Orbitrap high resolution liquid chromatography mass spectroscopy (OHR-LCMS) analysis of CF extract documented 21 bio-active compounds; major compounds include 8-hydroxyquinoline, trigonelline, spectinomycin, psoralidin, nicotinic acid, kanosamine, sulfamethazine, artemisinin, and other compounds with bioactive properties. Fourier Transform Infrared Spectroscopic analysis confirmed the presence of functional groups that are attributed to antibacterial, antioxidant, and anticancer compounds profiled in the OHR-LCMS.

INTRODUCTION

The endophytic fungi, among various microbial communities reported around the world, reside asymptomatically within plant tissues (Petrini, 1991). Endophytic fungi are the reservoir of novel bioactive metabolites capable of improving the plant growth and supporting survival of their host plants in the habitat (Chandra *et al*., 2021). These findings invoked considerable interest in scientists to search for possible agents for innovative medicinal and agricultural product development (Paramanantham *et al*., 2019). Consequently, the identification of bioactive substances with enormous potential has resulted from the separation and screening of naturally occurring substances from endophytes and their host plants. The co-existence of endophytic fungi in their host is a unique relationship which impacts the number and quality of metabolites derived from fungal endophytes (Khare *et al*., 2018).

Almost all studies to date on endophytic fungi of host plant origin focused on the importance of metabolites with antibacterial and antioxidant properties in human welfare (Dwibedi and Saxena, 2020). Following the development of drug resistance in clinical microbial pathogens, the exploration of natural resources of new antibacterial agents hostile to the resistant pathogens and treatment of diseases caused by them has been gaining much importance. Antioxidants are chemical substances that shield cells from harm caused by reactive oxygen species and free radicals that incites carcinogenesis,

*Corresponding Author
Manchanahally Byrappa Shivanna, Department of PG Studies and Research in Applied Botany, School of Biosciences, Kuvempu University, Shivamogga, India.
E-mail: mbshivanna @ yahoo.co.uk

DNA damage, and degenerative disorders including Alzheimer's disease (Adeleke and Babalola, 2021; Fadiji and Babalola, 2020). In this regard, the exploration of endophytic fungi with the ability to produce antioxidant compounds for the treatment of human disorders caused by reactive oxygen species has been taken up (Adeleke and Babalola, 2021; Ambele *et al*., 2019). Literature also indicated that metabolites secreted internally and externally by fungal endophytes may have therapeutic value in the management of a variety of microbial diseases in humans (Aharwal *et al*., 2021).

Cancer, a collective name for a variety of related diseases is caused by the uncontrolled division of cells following mutation and invades and destroys adjacent tissues and organs (Li *et al*., 2018). Cancer is treated by chemotherapy, radiation, immunotherapy, and suicide gene therapy (Ghobrial *et al*., 2005). Nonetheless, the multidrug-resistant feature of cancer cells and the adverse effect of medications still remain a significant impediment for a successful chemotherapy (Gottesman *et al*., 2002). Eventually, studies on the source of new compounds with pharmaceutical importance, drug discovery, and development have received much importance and attention (Newman and Cragg, 2007). The exploration of *Taxomyces andreanae* endophytic in *Taxus brevifolia* (Stierle *et al*., 1995) that produce the anticancer compound paclitaxel (taxol) resulted in further studies demonstrating a diverse range of chemical compounds with various biological activities produced by endophytic fungal communities resident in ethnomedicinal plants (Vieira *et al*., 2012). The fungal endophytes in comparison to their host plants are considered as a potential source for the mass production of biologically active chemicals (Sopalun *et al*., 2021).

The selection of the medicinal plants to isolate endophytic fungus is one of the routine methods to identify novel compounds. The ethnomedicinal plants are the fascinating source of fungal taxa that produce a variety of biologically active substances (de Carvalho *et al*., 2021). In this study, *Jatropha heynei* of Euphorbiaceae a rare and endemic medicinal herb growing in dry tropical regions of Karnataka, was chosen to investigate the diversity of fungal endophytes and to assess the capability of certain endophytic fungal taxa to produce metabolites with bactericidal, free radical scavenging, and cytotoxic properties. Such of the endophytic fungal extracts that contain chemical components with above biological activities were subjected to chemo-profiling. Further, the spectral compounds were analyzed for their functional groups.

METHODS AND MATERIALS

Selection of study region and plant species

Three locations in Surammanahalli village (13.2181° N, 75.6556° E) of Chitradurga district in Karnataka were selected for the collection of plant samples of *J. heynei*. The soil of the study site is red sandy loam soil and receives 593.5 mm of an average annual rainfall and the temperature of 32°C–36°C. The specimen plant sample was characterized based on the morphological characteristics (Gamble, 1934) as *J. heynei* N. P. Balakr, and the identity of the species was confirmed by Dr. K. G. Bhat, an eminent plant taxonomist of Karnataka. The herbarium specimen of the plant was prepared and deposited (Voucher No-KU/AB/05) in the Herbarium Center at Department of Applied Botany, Kuvempu University, Shankaraghatta.

Endophytic fungal isolation and identification

Jatropha heynei plants at the flowering stage during June–August 2020 were randomly selected from the study sites. The healthy plant samples like leaf, fruit, and root were collected in sterile polypropylene sheets and brought within 24 hours to the laboratory. The specimens were cleaned in running water, then subjected to surface disinfection successively with hydrogen peroxide (0.2%), ethyl alcohol (70%), and Sodium hypochlorite (0.3%) (Achar and Shivanna, 2013) and segmented (1 cm) in an aseptic environment and placed on potato dextrose agar (PDA), malt extract agar (MEA), czapek dox agar (CZA), or water agar media (WA) supplemented with or without chloramphenicol (100 mg l^{-1}) and incubated in a regime of 12/12 hours of light and nUV light (350–400 nm) for 5–7 days at 23°C ± 2°C (Shivanna and Vasanthakumari, 2011). Simultaneously, the surface disinfected leaf samples were pressed on to the surface of PDA medium and incubated as above to ensure that the fungal isolates are not originating as the concomitant source of contamination but arising only from the inner regions of the plant tissues (Unterseher and Schnittler, 2009). The morphological characteristics of fruiting bodies and spores were used to identify the endophytes that were expressed on the incubated plant segments (Rama Rao and Manoharachary, 1990; Subramanian, 1983). *Penicillium citrinum*, an endophytic fungus with high incidence and *in vitro* antibacterial activity, was chosen for the molecular characterization. The internal transcribed spacer (ITS) DNA regions of fungal species were chosen for molecular identification by cetyltrimethylammonium bromide method (Aamir *et al*., 2015). Primers such as ITS1: 5′-TCC GTA GGT GAA CCT CGG-3′ and ITS4: 5′-TCC TCC GCT TAT TGA TAT GC-3′ were employed in order to amplify the ITS region. The reaction mixture for the polymerase chain reaction (PCR) amplification includes 10 µl of genomic DNA and 25 µl of PCR reaction mixture (*Taq* polymerase, 10× buffer, dNTPs, primers, and sterile water). The PCR protocol included 30 reactions of amplification, denaturation (95°C for 1 minute), annealing (50°C for 30 seconds), and extension (73°C for 1 minute) after a preliminary denaturation of 95°C for 5 minutes. The PCR products were purified and the amplification of PCR products was confirmed by the gel electrophoresis technique. The amplified DNA segments were sequenced by the Sanger's technique. The phylogenetic tree was constructed using RAxML GUI software and by a maximum likelihood method of 1,000 bootstrap replications by the GTRGAMMA+I model as suggested by jModelTest (Darriba *et al*., 2012).

Preparation of fungal extract

The endophytic fungus was cultured with occasional shaking at 26°C for 8–12 days in two 500 ml Erlenmeyer flasks consisting of potato dextrose broth (Gagana *et al*., 2020). The culture filtrate (CF) and mycelial parts were separated by using Whatman filter paper No. 1. In a separating funnel, ethyl acetate (Himedia, Mumbai, India) was used to extract the metabolites from the CF. The CF was condensed with rotary vacuum evaporators at room temperature and crude extract was re-extracted using the same solvent. The partially purified extract was pooled and kept at 4°C (Nischitha *et al*., 2020).

In vitro antibacterial assay

The antibacterial potential of the extract was tested by well diffusion technique (Nischitha *et al*., 2020) against

five clinical bacterial pathogens procured from the Institute of Microbial Technology, Chandigarh. Bacterial strains are Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa* (MTCC-4734), *Salmonella typhi* (MTCC-734), *Klebsiella pneumoniae* (MTCC-7028), and *Pseudomonas syringae* (MTCC-1604). Antibiotics such as amoxicillin (Amozlin-250 Dr. Reddy's Laboratories, Hyderabad, India), chloramphenicol (125 mg, Sanofi Aventis Pharma India), and ciprofloxacin (BIOCIP-TZ—Biochem, India) were served as standard controls, while dimethyl sulfoxide (DMSO; Himedia, Mumbai, India) was served as the negative control. The endophytic fungal extract (10 mg ml^{-1}) was dissolved in DMSO and made into concentrations of 100%, 50%, and 25%. A sterilized cork-borer was used to create wells (0.5-mm diameter) in the solidified nutrient agar (Himedia, Mumbai, India) and 20 µl of the extract was poured in to every well and kept for incubation at 37°C for 24 hours. The antibacterial activity of extract and standards was evaluated by measuring the inhibition zone (ZI, mm).

Redox potential of extract by electrochemical assay

The oxidation potential was determined by the cyclic voltammetry technique (Arulpriya *et al*., 2010). The crude endophytic fungal extract (2 ml) was diluted in phosphate buffer (pH 7, 50 Mm of disodium hydrogen phosphate (65%, w/v) and 50 Mm sodium dihydrogen phosphate (35%, w/v). The cyclic voltammograms of the extract were produced using the Electrochemical Workstation CHI 660c (model potentiostat). The experiment used a carbon paste working electrode (3-mm diameter), a carbon paste electrode modified by methanol extract (CE), a counter electrode made of platinum wire, and a saturated calomel reference electrode. To determine the impact of various scan speeds on the anodic oxidation of the extract, the electrode was pre-treated with potassium chloride (1 Molar, 2 mg). The oxidation potential of extract was evaluated at a scan rate of 50 mVs^{-1} (Nischitha *et al*., 2020).

In vitro cytotoxic activity

The cytotoxic potential of *P. citrinum* metabolites was analyzed by using the 3-(4, 5-dimethylthiazole-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell proliferation method (Mosmann, 1983) against MCF-7 human breast cancer cell line and A549 human adenocarcinoma cell line (NCCS, Pune, India). The working solution (1% v/v) was prepared by dissolving the extract in DMEM high glucose medium (Himedia, Mumbai, India). The cell suspension of 200 µl with the appropriate cell number (20,000 cells/well) and without test agent was taken in a 96-well microtiter plate and extract concentrations of 31.25, 62.5, 125, 250, or 500 µg ml^{-1} were then added in to well and kept for incubation at 37°C for 24 hours in 5% CO_2 environment. Curcumin served as a standard, and DMSO served as a negative control. The incubated cell suspension was added with MTT reagent and 100 µl of solubilization solution (DMSO) was added. Finally, absorbance of cell suspension in the microtiter plate was read at 595 nm in ELISA plate reader reader (Agilent biotech, USA). The IC_{50} value was calculated by linear regression formula $Y = mx + c$

where, $Y = 50$, the viability graph was used to calculate M and C values.

Percentage of cell viability was calculated from the ELISA absorbance data by the following formula:

$$\text{Percentage cell inhibition} = \frac{\text{Absorbance of control–Absorbance of sample}}{\text{Absorbance of sample}} \times 100$$

Chemical profiling of *P. citrinum* metabolites by orbitrap high resolution liquid chromatography mass spectroscopy (OHR-LCMS)

Based on the antibacterial, antioxidant and cytotoxic activities of *P. citrinum* crude extract, sample was subjected to OHR-LC-MS (Thermo Scientific Xcalibur, ver. 4.2.28.14). The analytic instrument consists of an outer electrode and a central electrode which function as analyzer and detector. Both positive and negative ionization modes were employed for direct infusion with the mass (m/z) which ranged from 50 to 8,000 amu. In this study, the data acquisition software Thermo Scientific Xcalibur (version 4.2.28.14) and the data processing software Compound Discoverer 2.1 SP1 was employed. The column material, Hypersil Gold 3 µm 100 × 2.1 MM (Thermo Scientific), was used in conjunction with a mobile phase of 0.1% formic acid in distilled water and methyl alcohol. The OHR-LCMS analysis was done at Sophisticated Analytical Instrument Facility (SAIF) in IIT Bombay, Mumbai, India. Search engines for open chemistry databases like Pub-Chem and Chem-spider were used to check the biological properties of detected compounds. Chem-Sketch software was used to draw the chemical structures.

Fourier transform infrared spectroscopic (FTIR) analysis

The FTIR observations in the range of 4,000–400 cm^{-1} were determined with FTIR accouterment (Bruker optic, Germany). The extract of 150 µl (0.5 mg ml^{-1}) was mixed with methanol and then with newly prepared FTIR reagent (4.5 ml). The spectral data were obtained with resolution of 4 cm^{-1}.

Statistical analysis

The data were analyzed to determine the colonization frequency (%) of endophytic fungal species, as well as the diversity indices like Simpson and Shannon evenness and species richness indices and antibacterial activity (Duncan's multiple range test $p < 0.05$). All experiments were done with three trials. Results of data were shown as the mean and standard error. Phylogenetic tree was constructed using the RAxML GUI version 2.0.0.0 with maximum likelihood method.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungal community

The isolation of fungal endophytes in *J. heynei* was carried out during July 2019–May 2020 (rainy, winter, and summer seasons), at three different locations and by four isolation methods such as PDA, MEA, CZA, and Water agar methods. The assemblages of endophytic fungal species varied depending on the season which played vital role in the colonization of fungi, as suggested by season-wise isolation. The endophytic fungal colonization frequency was more in the winter season when compared to the rainy and summer seasons. The occurrence of the endophytic fungal assemblages in plant parts varied based on the nutritional supplement; fungal

incidence was more in PDA followed by Water agar, MEA, and CZA media (Table 1). Thirty three endophytic fungal species were recovered from 5,400 segments of *J. heynei* and fungal species were characterized based on their morphological features. *Cladosporium cladosporioides*, *Aspergillus*, and *Penicillium* species were dominant during rainy season while *C. cladosporioides*, *Colletotrichum truncatum*, and *Fusarium* sp. were dominant in the winter season. *Aspergillus flavus*, *C. cladosporioides*, *P. citrinum*, and *Fusarium* sp. were predominantly found in the summer season and in all parts of *J. heynei*. The fungal emergence was more in the leaf than in the root and fruit regions. Based on the preliminary study of antibacterial activity in certain fungal species, an isolate of *P. citrinum* (Fig. 1) with good antibacterial activity was used for molecular identification. Based on the ITS regions of the rDNA, the fungal isolate identified as *P. citrinum* (sequence deposited to NCBI GeneBank, Accession. No. OP049986) and based on the maximum likelihood method *P. citrinum* grouped with the most closely related reference species and formed separate clades in the phylogenetic tree (Fig. 2). The diversity indices of Shannon and Simpson and evenness indices of the endophytic fungi were high in the leaf than in the root and fruit (Table 2). Amongst the regions of the plant, the leaf followed by root harbored both anamorphic as well as teleomorphic stages of ascomycetes. Similar findings were recorded in previous research work (Gagana *et al*., 2020).

The qualitative mycochemical analysis of metabolites produced by *P. citrinum* detected the presence of alkaloids, glycosides, sterols, phenols, tannins, and triterpenoids. The secondary metabolites in fungal endophytes produced by distinct metabolic pathways (Tan and Zou, 2001) have been reported for the antimicrobial, antioxidant, and other biological activities (Chandrappa *et al*., 2013).

Antibacterial potential of fungal metabolites *in vitro*

The antibacterial activity of *P. citrinum* extract against selected bacterial strains is detailed in Table 3. *Penicillium citrinum* extract caused more antibacterial activity against Gram-negative strain *P. syringae* (17.65 ± 1 mm) and Gram-positive strain –*S. aureus* (16.32 ± 0.5 mm) than the rest of the strains. The activity of extract against the above strains was lesser than that of ciprofloxacin and choramphenicol standards. However, the activity was almost equivalent to that of amoxicillin control. Further, the extract was less in effectiveness against other test bacterial strains. The colony suppression of bacterial pathogens could be attributed to the inhibition of DNA or protein synthesis or damage caused to the integrity of cell membranes, ultimately leading to cell death (George *et al*., 2019). The inhibitory effect of endophytic fungal extract against clinical bacterial pathogens could be caused by the presence of active bactericidal compounds or synergistic effect of other bio-active chemicals present in the extract.

Antioxidant activity of fungal metabolites by cyclic voltammetry

Since the redox potential and antioxidant properties have a strong link, the reducing ability of extract was assessed by cyclic voltammetry. The CF generated positive oxidative peak at Ipa, 0.14 V (Fig. 3) when anodic current potential was present. The compounds with strong scavenging ability are known to oxidize at low potential (Gagana *et al*., 2020).The high oxidation potential suggested that CF extract exhibited considerable antioxidant activity. At pH 7, the anodic peak was generated between 0.1 and 0.2 V. The shift in anodic peak potential toward a positive

Table 1. Colonization frequency (%) of endophytic fungi in *J. heynei* during rainy and winter seasons of 2019–2020.

Endophytic fungal species	Colonization frequency (%)/plant parts		
	Root	Leaf	Fruit
Anamorphic ascomycetes			
Alternaria alternata	0.11	1.45	0
Aspergillus spp.[5]	4.61	2.02	5.41
Cladosporium spp.[2]	7.24	5.46	4.27
Colletotrichum spp.[2]	0	3.56	0
Fusarium sp.	2.70	0	0
Gliocladium sp.	0	0.08	0
P. citrinum	4.78	4.43	11.45
Penicillium spp.[3]	1.37	0.62	0
Pestalotiopsis spp.[2]	0	0.21	0
Vertilicillium sp.	0.03	0	0
Telomorphic ascomycetes			
Chaetomium spp.[3]	1.09	0.74	0.62
Curvularia spp.[4]	0.1	4.34	0
Helminthosporium halodes	0	0.87	0
Nigrospora oryzae	0.15	0.33	0
Phoma sp.	0	0.20	0
Total frequency	21.90	24.18	15.73
Zygomycetes			
Zygorhinchus sp.	0	0.04	0
Morphotypes	8.33	5.17	2.73

Data is an average of 3 trials with 225 segments in each replicate. The frequency of fungal endophytes was estimated based on the number of segments colonized by each fungus over the total number of segments investigated.
Aspergillus spp. = *A. flavus* (1.89, 1.13, 4.16), *A. niger* (3.07, 0.90, 1.25), *A. ochraceus* (1.20, 0.11, 0), *A. candidus* (0.91, 0.39, 0), *A. fumigatus* (0.45, 0.08, 0).
Cladosporium spp. = *C. cladosporioides* (5.79, 4.32, 2.08), *C. herbarum* (0.34, 0.14, 2.91).
Colletotrichum spp. = *C. trunchatum* (0, 2.55, 0), *Colletotrichm* sp. (0, 0.11, 0).
Penicillium sp. = *P. citrinum* (2.49, 2.67, 11.45), *P. chrysogenum* (0.11, 0, 0), *P. commune* (0.17, 0.22, 0), *Penicillium sp*. (0.02, 0.19, 0), *Penicillium sp*. (0.8, 0.19, 0).
Pestalotiopsis spp. = *P. psidi* (0.17, 0.22, 0), *P. theae* (0, 0.11, 0).
Chaetomium spp. = *C.globosum* (0, 0, 0.62), *C. indicum* (0.14, 0.73, 0), *Cheatomium sp.* (1.2, 2.01, 0).

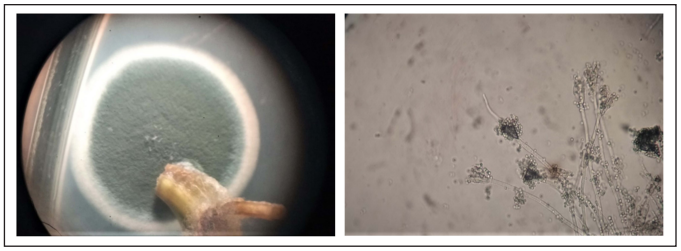

Figure 1. Photograph showing colony and spore structure of *P. citrinum.*

value indicated the presence of oxygen-free radicals that can be scavenged in the fungal extract (Keffous *et al*., 2016). The presence of flavonoids and phenolic substances is linked to the good antioxidant property of ethyl acetate extract (Sochor *et al*., 2013). The antioxidant compounds have always been demonstrated for their antibacterial, antiinflammatory, antiviral, and anticancer activities (Cai *et al*., 2004; Prasher and Sharma, 2021).

The primary drawback of spectrophotometric assays was identified as interference from biomolecules which absorbs the same wavelength while evaluating the antioxidant activity (Amamra *et al*., 2018). In this study, the electrochemical measurement demonstrated its uniqueness in determining antioxidant activity by enabling the detection of the substantial potential for oxidation of certain chemicals at shifting pH and in various reaction settings. This implied that the cyclic-voltammetric technique could be used in the place of spectrophotometric assay of antioxidant compounds (Nischitha *et al*., 2020).

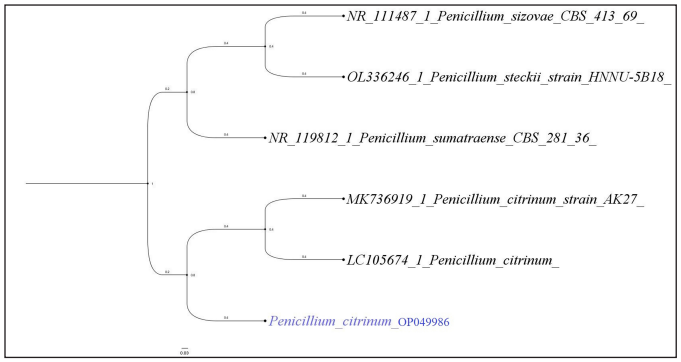

Figure 2. Phylogenetic analysis of rDNA ITS sequences of *P. citrinum* (GenBank Accession No. OP049986) isolated from *J. heynei*.

In vitro cytotoxic activity of fungal metabolites

The MTT colorimetric assay is frequently used to assess the cytotoxicity *in vitro* and gauge the rate at which the enzyme mitochondrial dehydrogenase converts MTT into formazan. A visible spectrophotometer measures the quantity of blue formazan, and it is positively correlated to the number of cells living because

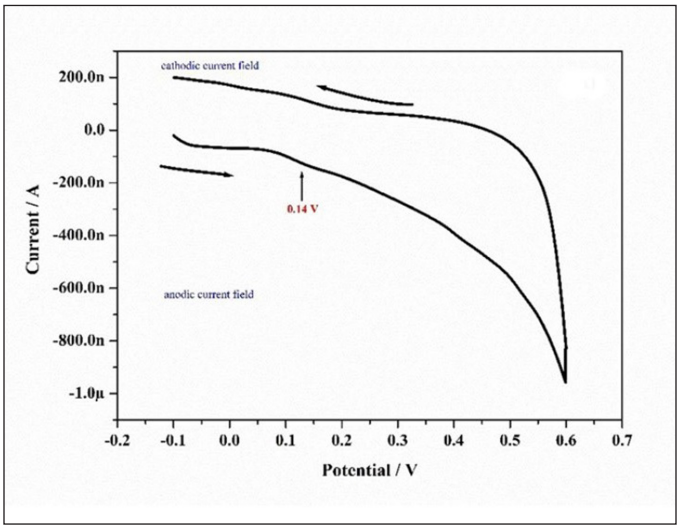

Figure 3. Voltammogram of the ethyl acetate extract of *P. citrinum* culture filtrate. The measurement was performed at a scan rate of 50 mVs^{-1} with a 3-mm diameter carbon paste electrode as the working electrode, saturated calomel as the reference electrode, and a platinum wire as the auxiliary electrode at pH 7.0.

Table 2. Species abundance, diversity, and evenness of endophytic fungal populations in root, leaf, and fruit regions of *J. heynei*.

Plant parts	Species richness	Diversity index		Evenness index	
		Simpson ($D^{'}$)	Shannon ($H^{'}$)	Simpson ($E^{'}$)	Shannon ($J^{'}$)
Root	22	0.8476	2.205	0.4123	0.7134
Leaf	32	0.8937	2.62	0.4293	0.756
Fruit	6	0.6769	1.401	0.6763	0.7817

The data are based on the average values of three distinct locations and three replicates; 100 segments/sample (root, leaf, and fruit).

Table 3. *In vitro* antibacterial activity of metabolites of *P. citrinum* in *J. heynei*.

Zone of inhibition (mm)/test bacterial strains	Ethyl acetate (conc. %)			Amoxicillin	Ciprofloxacin	Chloramphenicol
	100	50	25			
St	8.23 ± 0.5	4.12 ± 1	3.01 ± 13	12.33 ± 0.5	27 ± 1	24.17 ± 0.5
Kp	12.01 ± 0	8.32 ± 1	6.02 ± 1	12.33 ± 0.6	25.33 ± 2	23.1 ± 1
Sa	16.32 ± 0.5	12.02 ± 1	9.33 ± 0.5	15.33 ± 0.5	28.33 ± 2	23.33 ± 1
Ps	17.65 ± 1	14.33 ± 2	10.03 ± 2	16.66 ± 0.6	26.66 ± 1	21.66 ± 1
Pa	14.33 ± 1	10.66 ± 1	6.66 ± 1	17.33 ± 0.6	25.66 ± 1	21.33 ± 1

Antibacterial activity was evaluated using Duncan's multiple range te st ($p = 0.05$).
The values represent the mean standard error of three replicates. Three replicates ($n = 3$) of crude extracts were diluted in DMSO at a concentration of 10 mg ml^{-1}. Each well was provided with 20 µl of sample.
Gram-positive bacteria: *Sa Staphylococcus aureus*; Gram-negative bacteria: *St Salmonella typhi, Kp Klebsiella pnuemoniae, Ps Psuedomonas syringae*, and *Pa pseudomonas aeruginosa*.

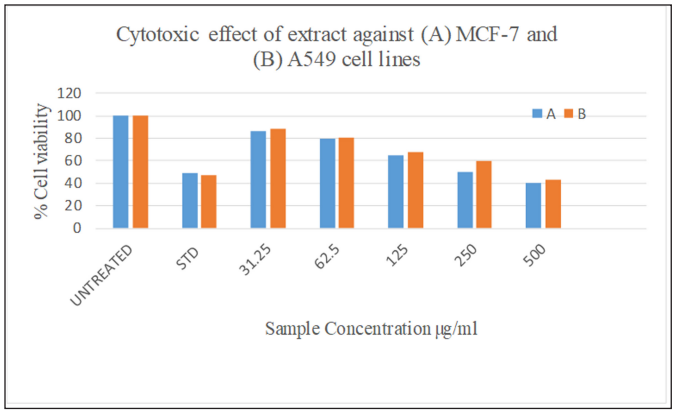

Figure 4. Cytotoxic effect of *P. citrinum* extract *in vitro* against (A) MCF-7 and (B) A549 cancer cell lines.

of the reduction reaction which can occur when the enzyme mitochondrial reductase was produced (Chapdelaine, 2001). The effect of the endophytic fungal extract against human cancer cell lines A-549 and MCF-7 is shown in Figures 4 and 7. The extract exhibited moderate cytotoxicity to both A549 and MCF-7 cell lines at the concentration of 500 µg after the period of 24 hours with IC_{50} values of 280.7 and 283.0 µg ml^{-1}, respectively. The standard curcumin showed 48% cell viability (IC_{50} of 52.41 µg ml^{-1}) at concentration of 10 µM. The cytotoxic effect of fungal extract could be attributed to the involvement of anticancer and antiinflammatory metabolites in the extract (Ghasemzadeh *et al*., 2010).

The observation of the presence of antibacterial, antioxidant, and cytotoxic activities in the fungal extract prompted the authors for further investigation of extract for metabolite profiling using OHR-LCMS method.

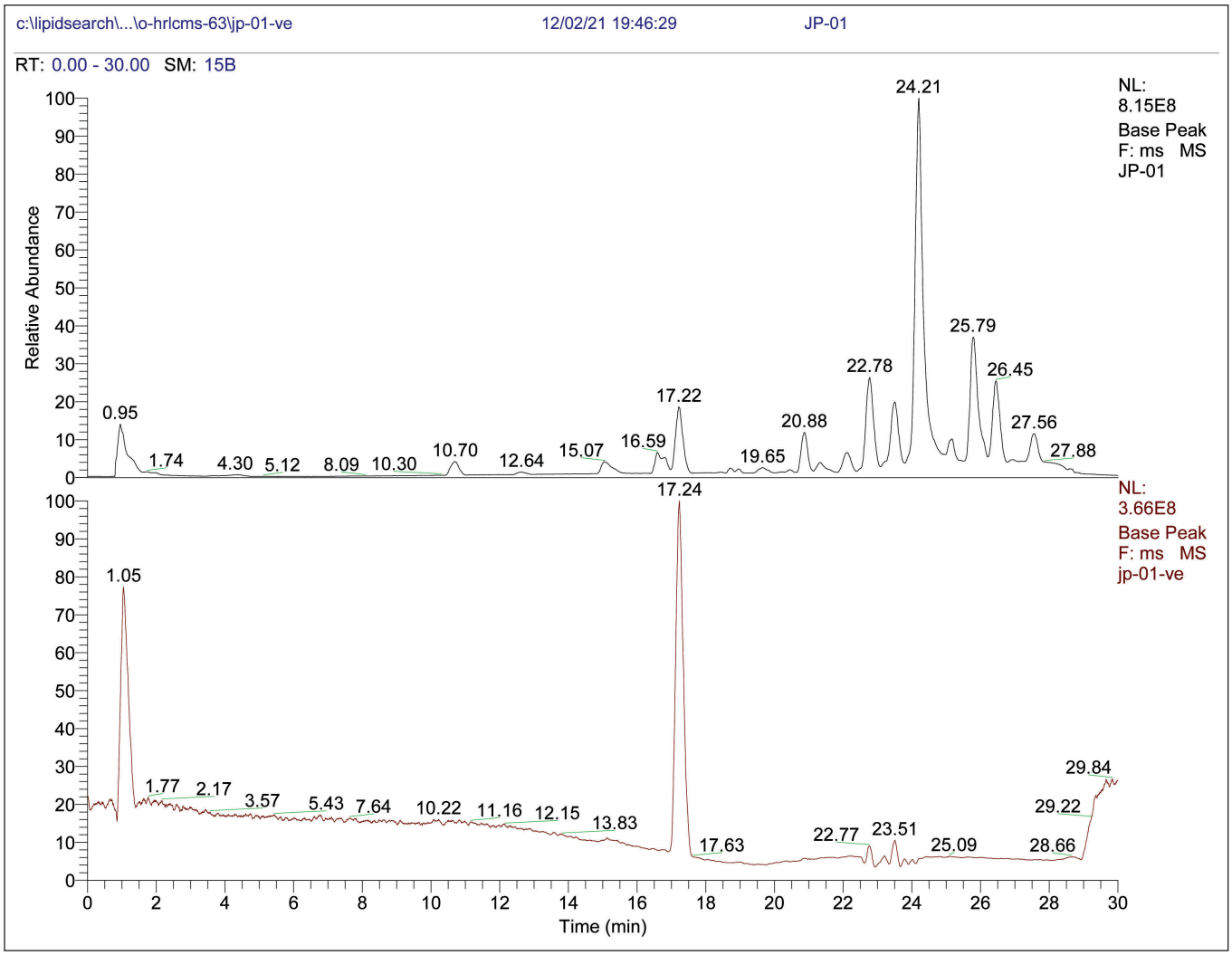

Figure 5. OHR-LCMS chromatogram of metabolites of *P. citrinum*.

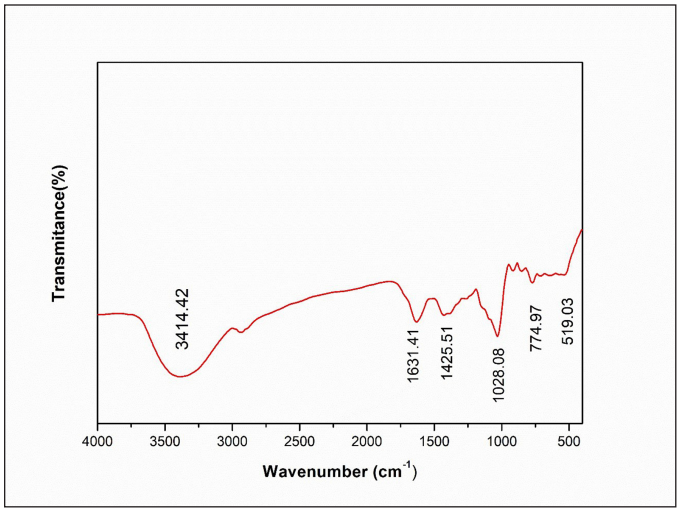

Figure 6. FTIR chromatogram of ethyl acetate extract of *P. citrinum.*

Chemical profiling of fungal metabolites by OHR-LCMS

OHR-LCMS is one of the most recent and effective current techniques for identifying chemicals contained in biological samples (Wu *et al*., 2012). In this study, the chemical composition of crude extract was evaluated by using OHR-LCMS. The method involves the separation as well as identification of Chemical constituents in accordance with their retention period, data base metabolite class, difference (library), experimental m/z, MS/MS fragments, and suggested compounds. The positive and negative ionization modalities of MS data were reported. The ethyl acetate extract of *P. citrinum* detected 21 bio-active compounds by the positive ionization mode (+ESI) and also negative ionization mode (−APCI) (Fig. 5). The major chemicals with bio-active properties detected by positive mode are 8-hydroxyquinoline **(1)**, trigonelline **(2)**, 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione **(3)**, sulfamethazine **(4)**, psoralidin **(5),** spectinomycin **(6)**, kanosamine **(7)**, 4-aminobenzoic acid **(8)**, 2,3-dimethoxy-5-(6,7,8-trimethoxychroman-3-yl)cyclohexa-2,5-diene-1,4-dione **(9)**, 2,4,6-trihydroxy-5-((1S)-1-((1aR,4R,4aR,7S,7bR)-4-hydroxy-1,1,4,7-tetramethyldecahydro-1H- cyclopropa[e]azulen-7-yl)-3-methylbutyl)isophthalaldehyde **(10)**, phenethylamine **(11)**, (2S,3R,4S,5S,6R)-2-[3-hydroxy-5-[2-(4-methoxyphenyl) ethenyl]phenoxy]-6-(hydroxymethyl)oxane-3,4,5-triol **(12)**, 6,6'-oxybis(4-methylbenzene-1,2-diol **(13)**, anthranillic acid **(14)**, (S)-5-hydroxy-1,7-diphenylheptan-3-one **(15)**, cyclosporine **(16)**, 3-(3,4,5-trimethoxyphenyl)propanoic acid **(17)**, artemisinin **(18)**, and (E)-N-(4-acetamidobutyl)-3-(4-hydroxy-3-methoxyphenyl)acrylamide **(19)**. Those compounds detected by both positive and negative ionization modes include nicotinic acid (+/−) **(20)** and 3'-adenosine monophosphate (+/−) **(21)**. Most compounds detected in the negative ionization mode are carbohydrates (glucose, mannose, xylose, and malic acid) and citric acid as well as small peptides (Table 4 and Fig. 8).

Among the above, compounds numbered **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9,** and **10** are previously documented for antimicrobial properties (Holloway, 1982; Janiak and Milewski, 2001; Kannabiran, 2016; Nagata *et al*., 2006; Okoro *et al*., 2019; Sharifi-Rad *et al*., 2020; Torres *et al*., 2021; Turcios and Papenbrock, 2019; Zhou *et al*., 2012). Compounds **11**, **12**, and **13** are reported for antioxidant activities (Huang *et al*., 2017; Strong, 1996; Zeb, 2021), while compounds **5** and **10** are both antimicrobial and antioxidant (Nagata *et al*., 2006; Sharifi-Rad *et al*., 2020). Certain compounds numbered **14**, **15**, **16**, **19**, and **21** are related to antiinflammatory and anticancer activities (Abubakar *et al*., 2018; Cronstein, 1994; Prasher and Sharma, 2021; Tedesco and Haragsim, 2012; Ting *et al*., 2019). Compounds with multiple pharmacological activities include 8-hydroxyquinoline **(1)** (Torres *et al*., 2021), trigonelline **(2)** (Zhou *et al*., 2012), and psoralidin **(5)** (Sharifi-Rad *et al*., 2020). Apart from the above, Compound 3-(3, 4, 5-trimethoxyphenyl) propanoic acid **(17)** is antileishmanial (Ferreira *et al*., 2010), while artemisinin **(18)** produced from this endophytic fungi is a sesquiterpene lactone consist of peroxide bridge responsible for their antimalarial properties (Klayman, 1985). Therapies based on a combination of artemisinin are now considered as standard treatment world-wide against *Plasmodium falciparum* and other species of *Plasmodium* and it is usually extracted from the Chinese traditional medicinal plant *Artemisia annua* (Arsenault *et al*., 2008). Nicotinic acid **(20)** in the extract is

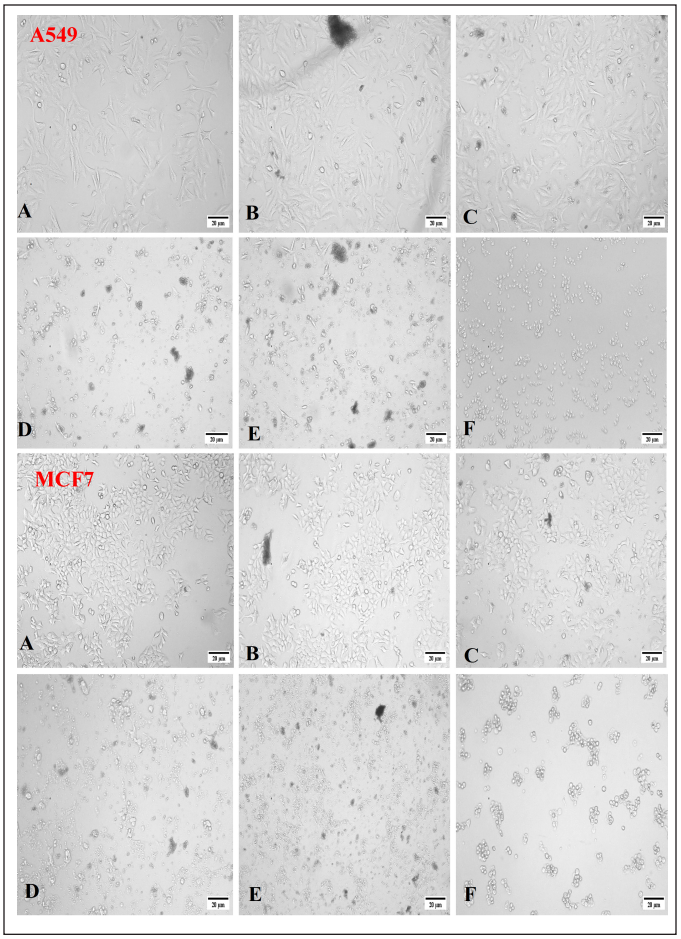

Figure 7. Morphology showing the cytotoxic effect of *P. citrinum* CF extract against A549 and MCF-7 cancer cell lines at different concentrations. A = 31.25 µg, B = 62.5 µg, C = 125 µg, D = 250 µg, E = 500 µg, F = 10 µM of standard.

Table 4. Metabolite profiling of *P. citrinum* by OHR-LCMS analysis.

Sl. No	Compound	Class of compunds	Molecular formula/ weight	mz Cloud Best Match	mz cloud fragments	Biological properties	References
1	8-Hydroxyquinoline	Alkaloid	C_9H_7NO 145.0528	97.4	60 42	Antimicrobial, antiviral, analgesic	Torres *et al*. (2021)
2	Phenethylamine	Alkaloid	$C_8H_{11}N$ 121.0891	94.4	94.4 9.7	Antioxidant	Strong (1966)
3	Trigonelline	Alkaloid	$C_7H_7NO_2$ 137.0477	99.3	99.3 97.9	Antibacterial, antiviral, hypoglycemic and neuro-protective	Zhou *et al*. (2012)
4	(S)-5-hydroxy-1,7-diphenylheptan-3-one	Ketone	$C_{19}H_{22}O_2$ 282.1620	96.7	105 96.7	Antiviral, antiproliferative	Abubakar *et al*. (2018)
5	3-isobutylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione	Organo-oxygen compound	$C_{11}H_{18}N_2O_2$ 210.1368	95.8	154 95.8	Antibacterial activity	Kannabiran (2016)
6	Cyclosporine A	Cyclic polypeptide	$C_{62}H_{111}N_{11}O_{12}$ 1201.8414	95.8	-	Antiinflammatory and immunosuppressive agent	Tedesco and Haragsim (2012)
7	Sulfamethazine	Sulfonamide	$C_{12}H_{14}N_4O_2S$ 278.0837	95.4	108 95.4	Antibiotic	Turcios and Papenbrock (2019)
8	(2S,3R,4S,5S,6R)-2-{3-hydroxy-5-[(E)-2-(4-hydroxy-3-methoxyphenyl) ethenyl]phenoxy}-6-(hydroxymethyl) oxane-3,4,5-triol	-	$C_{21}H_{24}O_9$ 420.1420	96.4	149 96.4	Antioxidant	Zeb (2021)
9	2,3-dimethoxy-5-(6,7,8-trimethoxychroman-3-yl) cyclohexa-2,5-diene-1,4-dione	Quinone	$C_{20}H_{22}O_8$ 390.1315	93.1	-	Antifungal and antileishmanial	Okoro *et al*. (2019)
10	3-(3,4,5-trimethoxyphenyl) propanoic acid	Monocarboxylic acid	$C_{12}H_{16}O_5$ 240.0998	92	97.3 92	Antileishmanial activity	Ferreira *et al*. (2010)
11	Psoralidin	Phenolic compound	$C_{20}H_{16}O_5$ 336.0998	89.8	89.8 72	Antibacterial,antioxidant, anticancer,	Sharifi-Rad *et al*. (2020)
12	2,4,6-trihydroxy-5-((1S)-1-((1aR,4R,4aR,7S,7bR)-4-hydroxy-1,1,4,7-tetramethyldecahydro-1H-cyclopropa[e] azulen-7-yl)-3-methylbutyl) isophthalaldehyde	Sesquiterpenoid	$C_{28}H_{40}O_6$ 472.2825	85.4	-	Antimicrobial and antioxidant	Nagata *et al*. (2006)
13	(E)-N-(4-acetamidobutyl)-3-(4-hydroxy-3-methoxyphenyl) acrylamide	Hydroxy cinnamic acid	$C_{16}H_{22}N_2O_4$ 306.1580	81.6	134 81.6	Cytotoxic and antiinflammatory	Ting *et al*. (2019)
14	Spectinomycin	Glycoside	$C_{14}H_{24}N_2O_7$ 332.1584	80.7	105 80.7	Antimicrobial	Holloway (1982)
15	Artemisinin	Phenolic compound	$C_{15}H_{22}O_5$ 282.1467	79.0	247 79	Antimalarial drug	Klayman (1985)
16	6,6'-oxybis(4-methylbenzene-1,2-diol)	Aromatic ether	$C_{14}H_{14}O_5$ 262.0841	77.3	149 77.3	Antioxidant	Huang *et al*. (2017)
17	Kanosamine	Amino sugar	$C_6H_{13}NO_5$ 179.0794	74	-	Antifungal	Janiak and Milewski (2001)
			Primary metabolites				
1	Nicotinic acid	Amino acid.	$C_6H_5NO_2$ 123.0320	75.1	78 75.1	Vitamin B3, reduces blood cholesterol.	Gille *et al*. (2008)
2	3'-Adenosine Monophosphate	Organic compound	$C_{10}H_{14}N_5O_7P$ 347.0631	89.4	-	Antiinflammatory	Cronstein (1994)

Continued

Sl. No	Compound	Class of compunds	Molecular formula/ weight	mz Cloud Best Match	mz cloud fragments	Biological properties	References
3	4-Aminobenzoic acid	Organic compound	$C_7H_7NO_2$ 137.0477	98.6	108 98.6	Antibacterial and antitubercular	Küçükgüzel *et al*. (1999) and Jani *et al*. (1990)
4	Anthranilic acid	Carboxylic acid	$C_7H_7NO_2$ 137.0477	98.6	119 98.6	Antiinflammatory and anticancer	Prasher and Sharma (2021)

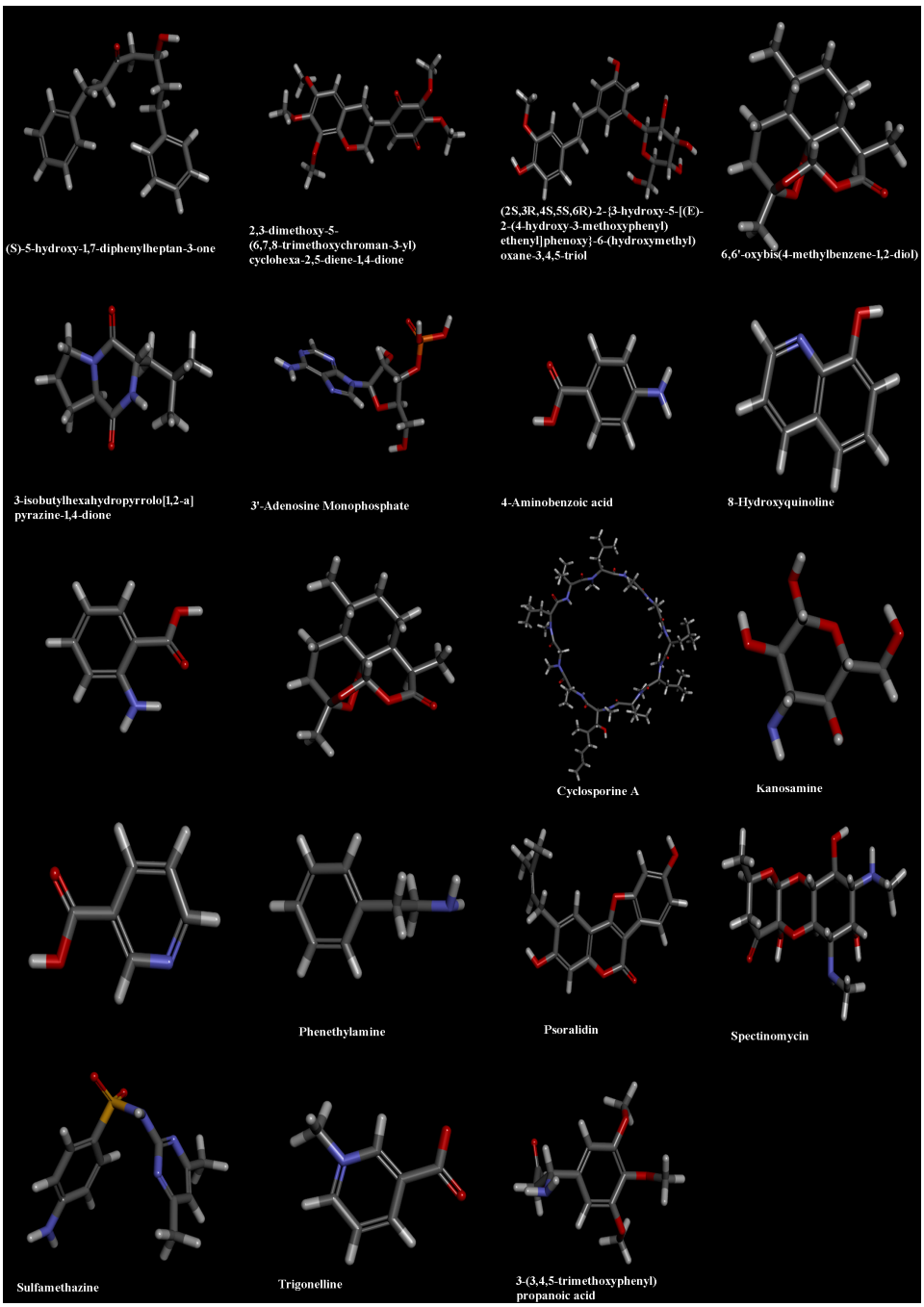

Figure 8. Structures of certain important compounds identified in ethyl acetate extract of *P. citrinum* endophytic in *J. heynei.*

vitamin B3 which reduces the blood cholesterol level (Gille *et al*., 2008) (Table 4).

The FTIR spectrum of *P. citrinum* extract (Fig. 6) confirmed the presence of functional substituents of antibacterial and antioxidant chemicals as evaluated by the OHR-LCMS. A broad stretching peak at 3,414.42 cm^{-1} was assigned to the stretching vibration of amide groups (N–H), indicating the presence of alkaloids, while the band at 2,800.42 cm^{-1} was attributed to carboxyl acid's (C=O) presence and (O–H) groups that indicated the presence of polyphenols and flavonoids; a sharp peak at 1,634.16 cm^{-1} (C=C) alkenes, and 1,425.51 cm^{-1}, aromatic compounds (C=C), and 1,028.08–519.03 cm^{-1} (C–H) groups. Functional groups like carboxylic acids, anhydrides, alcohols, phenols, amines, amides, and esters showed that the fungal extract contained biologically active metabolites (Nischitha and Shivanna, 2021). This observation further supported the antioxidant activity due to phenols and flavonoids as shown by cyclic voltammetry in this study.

CONCLUSION

This study documented that variation in the occurrence of endophytic fungal assemblages in different plant parts depends on the season and isolation methods. Among the large number of endophytic fungi isolated, *P. citrinum* was the dominant endophyte. This study indicated that *P. citrinum* produced compounds with antibacterial, antioxidant, and cytotoxic potentials. This finding is supported by the metabolite profiling of fungal metabolites by OHR-LCMS and further determination of functional groups by FTIR spectroscopy. In this study, several compounds known to literature have been documented that expressed antimicrobial and antioxidant as well as other pharmacological activities. This study also documented the presence of several unknown compounds that might be of future pharmaceutical importance. Further research is needed before these bioactive chemicals can be used to benefit humans.

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LIST OF ABBREVIATIONS

CF: Culture filtrate, DMSO: Dimethyl sulfoxide, ITS: Internal transcribed spacer, PCR: Polymerase chain reaction, OHR-LCMS: Orbitrap high resolution-liquid chromatography mass spectroscopy, FTIR: Fourier transform infrared spectroscopy.

AUTHOR CONTRIBUTIONS

A.G.B. carried out the research, conducted the experimental work and wrote the first draught. M.B.S. reviewed and edited the manuscript before it was finalized. The final manuscript was read and approved by all authors.

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CONFLICTS OF INTEREST

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

ETHICAL APPROVALS

The study does not use animals used as human test subjects in *in vivo* experiments.

DATA AVAILABILITY

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

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