Ann Microbiol (2017) 67:321–331
DOI 10.1007/s13213-017-1263-5

ORIGINAL ARTICLE

Isolation and characterization of anticancer flavone chrysin (5,7-dihydroxy flavone)-producing endophytic fungi from *Passiflora incarnata* L. leaves

Prabukumar Seetharaman[1] · Sathishkumar Gnanasekar[1] · Rajkuberan Chandrasekaran[1] · Gobinath Chandrakasan[1] · Murugan Kadarkarai[2] · Sivaramakrishnan Sivaperumal[1]

Received: 27 October 2016 / Accepted: 1 March 2017 / Published online: 22 March 2017

Abstract Chrysin (5,7-dihydroxy flavone, ChR) is a flavone of plant origin, possessing numerous biomedical properties, such as antimicrobial, anti-inflammatory, antidiabetic, anxiolytic, hepatoprotective, anti-aging and anticonvulsant activities. In this study, chrysin-producing fungal endophytes (*A. alternata* KT380662, *C. capsici* KT373967, and *C. taiwanense* PI-3 KX580307) were isolated from the leaves of *Passiflora incarnata* L. and characterised via morphology and internal transcribed spacer (ITS) sequences. Thin layer chromatography and high-performance liquid chromatography profiles of fungal extracts showed Rf values and retention times that closely match those of standard chrysin (ChR). Further, the production of fungal chrysin (FChR) was confirmed through UV-vis spectroscopy, FT-IR, LC-ESI-MS, and $^{1}H_{1}$ NMR analysis. Among the isolated strains, *A. alternata* KT380662 was identified as having a high-level of ChR production, with rates measuring approximately 846 mg L^{-1}. On the other hand, in vitro anticancer and radical scavenging studies proved that FChR has significant cytotoxic activity against human liver carcinoma cells (HepG2). These results clearly imply that the isolated *A. alternata* KT380662 could serve as an alternative source for the commercial production of ChR, which holds anticancer and radical scavenging activities, and the fungal-derived ChR can be used in chemotherapy or in prodrug development.

✉ Sivaramakrishnan Sivaperumal
sivaramakrishnan123@yahoo.com

[1] Department of Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, Tamilnadu, India

[2] Vice-Chancellor, Thiruvalluvar University, Vellore 632 115, India

Keywords Chrysin (5,7-dihydroxy flavone ChR) · Endophytic fungi · *Passiflora incarnata* L. · *Alternaria alternata* KT380662 · LC-ESI-MS · Lung cancer

Introduction

Endophytes are potent bio-resources that produce a wide variety of chemical entities, especially improved natural bioactive compounds. These active compounds, which are secreted by the endophytes and reside inside the plant domains, have been exploited successfully for different medical, agricultural, and industrial applications (Aly et al. 2010). Generally, endophytes have a long-term association with the host, and have the capability to synthesise analogues of active chemical constituents produced by its host because of the intergeneric genetic exchange between the host and the endophyte (Zhao et al. 2011). Therefore, endophytes are receiving global attention related to the isolation of novel and potential anticancer compounds such as paclitaxel (taxol), which can be isolated from the endophytic fungus *Taxomyces andreanea* (Stierle et al. 1993).

Isolation of certain plant bioactive compounds from endophytic fungi is gaining great importance in the medical and agricultural industries due to its cost-effectiveness and low requirement for biomass to obtain a large number of active compounds. Moreover, different physiological and chemical conditions can be optimised to obtain high yields of compounds with low-cost downstream processing. In recent years, many plant-derived compounds such as paclitaxel, podophyllotoxin, camptothecin, vinblastine, aspirin, and lovastatin have been extracted successfully from different endophytic fungal strains (Xiong et al. 2013; Nadeem et al. 2012; Pu et al. 2013; Prabukumar et al. 2015; Parthasarathy and Sathiyabama 2015).

In general, passiflora consists of 500 species that are found mostly in warm and tropical regions. Passiflora species such as *P. incarnata*, *P. caerulean* (passion flower), *P. edulis* (purple passion fruit), and *P. mollissima* (banana passion fruit) have been found in India. Among them, *P. incarnata* is known as an ornamental plant that is cultivated for enjoyment of its glamorous purple-coloured flowers. The chemical and biological properties of *P. incarnata* were revealed by Dhawan et al. (2001). *P. incarnata* was found to contain several active compounds, including alkaloids, phenols, glycosyl flavonoids, and cyanogenic compounds. Due to the presence of these active compounds, the passiflora species possess excellent biomedical values for the treatment of diseases like anxiety, opiates withdrawal, insomnia, attention deficit hyperactivity disorder, and cancer. The major compounds present in *P. incarnata* are C-glycosyl flavonoids (vitexin, isovitexin, orientin, chrysin, etc.) and b-carbolinic alkaloids (harman, harmin, harmalin, harmol, and harmalol) (Blumenthal et al. 2000). Wolfman et al. (1994) first demonstrated the anxiolytic effects of chrysin (ChR), which is a naturally occurring flavonoid compound. Chrysin has stupendous biological activities, including antibacterial (Wang et al. 2011), anti-inflammatory (Gresa-Arribas et al. 2010), anti-diabetic (Torres-Piedra et al. 2010), anxiolytic (Brown et al. 2007), hepatoprotective (Pushpavalli et al. 2010), anti-aging (Anand et al. 2012), anticonvulsant (Medina et al. 1990), and anticancer (Khoo et al. 2010) effects. Accordingly, it actively inhibits inflammatory enzymes such as iNOS and COX-2 by inducing the PPAR- (Liang et al. 2001). Hence, in this study, we have aimed to isolate chrysin (5,7-dihydroxy flavone, ChR)-producing potential endophytic fungal strains from *P. incarnata* L. leaves. To our knowledge, this is the first report on isolation of ChR-producing fungal endophytes in this plant for its valuable biomedical applications.

Materials and methods

Chemicals

All chemicals including potato dextrose agar (PDA) [containing potato (200 g/L), dextrose (20 g/L), agar (15 g/L), pH 6.0)], Czapek Dox Broth [sucrose (30.0 g/L), sodium nitrate (3 g/L), dipotassium phosphate (1 g/L), magnesium sulphate (0.5 g/L), potassium chloride (0.5), ferrous sulphate (0.01 g/L)], DMEM medium supplemented with 10% fetal bovine serum, 100 g/mL streptomycin, and 100 U/mL penicillin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT), ethidium bromide, and acridine orange, were purchased from Himedia Laboratories. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich, India. The human liver carcinoma cell line (HepG2) was obtained from the National Centre for Cell Sciences (NCCS), Pune, India.

Plant materials

Healthy symptomless fresh leaves were collected from healthy looking perennial vines of *P. incarnata* L. passiflora located in front of the insect molecular biology laboratory on the Bharathidasan University campus in Tiruchirappalli, Tamilnadu, India. The collected leaf samples were then stored at 4 °C for further studies.

Isolation of endophytic fungi

Initially, the fresh leaves were thoroughly washed three times with sterile distilled water, and surface sterilisation was carried out using a modified version of the protocol described in Cui et al. (2011). Briefly, the leaves were immersed in 75% ethanol for 1 min and 5% NaOCl for 5 min. The plant materials were then sluiced six times with sterilised distilled water to remove any surplus surface chemicals, and excess wetness was removed using sterile filter paper to fully dry the leaves. Finally, the surface-sterilised leaf samples were cut into small pieces (approximately 5 × 5 cm) with a sterile blade, and placed into autoclaved petri dishes containing PDA medium supplemented with chloramphenicol (100 mg/L). These petri dishes were then incubated at 26 ± 2°C for 7–15 days. Pure fungal cultures were obtained as described by Lacap et al. (2003) and Promputtha et al. (2005). The strains were preserved on PDA slants and stored at 4°C for further studies.

Identification of endophytic fungi

Isolated fungal genomic DNA was extracted by the CTAB method. Further, the internal transcribed spacer (ITS) region was amplified using universal primers ITS-1 and ITS-4. The total reaction mixture for PCR amplification (40 μL) contained 4 μL template, 2 μL of each primer (10 μM), 20 μL *Taq* PCR mix (Amplicon), and 12 μL double distilled water. The PCR product was amplified with the following cycles: (1) 94 °C for 5 min; (2) 30 cycles of 94 °C for 30 s, (3) 55 °C for 30 s, and 72 °C for 1 min; and (4) 72 °C for 10 min. After run completion, the successfully amplified regions were purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany), and subsequent sequencing was performed by Eurofins Genomics India Pvt Ltd. The sequences obtained were generated using the CAP3 program, and the closest related sequence was ascertained using a BLAST search. Finally, multiple sequence alignment was performed using the CLUSTAL W program. A phylogenetic tree was then constructed using the neighbor-joining method and tree topologies were evaluated by performing a bootstrap

analysis of 1000 data sets using MEGA 6.0 and finally submitted to the GenBank database.

Extraction

For the extraction of active metabolites, isolated endophytic fungal strains were inoculated in 1000 mL Erlenmeyer conical flasks containing 500 mL Czapek-Dox broth, and incubated using an orbital shaker at 26 ± 2 °C at 180 rpm for 15 days. Subsequently, the mycelial biomass was harvested by filtering through a Whatman No. 1 filter paper. Then, the mycelial biomass was dried and extracted twice with 100% ethyl acetate. The filtrate was evaporated under reduced pressure using a rotary vacuum evaporator to condense the yield. Finally, the extracted metabolites were redissolved in 5 mL 100% methanol for further chromatographic and spectroscopic analysis.

Ferric chloride test

To perform a ferric chloride test, a 10% ferric chloride solution was added dropwise into the raw mycelium and extracellular extract. A blackish-red precipitate then developed, which confirmed the presence of flavonoids. ChR-producing potential fungi (both intra- and extra-cellularly) were selected based on the results of thin layer chromatography (TLC) analysis and the ferric chloride test. The mycelia and extra-cellular products were processed distinctly by the extraction procedure.

Thin layer chromatography

For TLC analysis, the fungal methanolic extracts and standard ChR (Sigma, St. Louis, MO) were applied as spots onto a silica gel 60 F254 (1-mm thickness) pre-coated TLC plate. The plate was then retained in a solvent system of toluene: ethyl acetate: acetic acid (36:12:5) at room temperature in a vertical separating chamber (14 cm), after previous saturation with the solvent system for 1 h. After drying, the plate was visualised under UV light (254 nm). The Rf values and the colour of FChR were compared with those of standard ChR.

High performance liquid chromatography

The fungal extract spot with the same Rf value as authentic ChR was scraped off, dissolved in methanol, and then centrifuged. The supernatant was concentrated for HPLC analysis to confirm and quantify ChR. Specifically, an Agilent 1100 apparatus and a Novapak C-18 (3.9 × 150mm particle size) column with a 4 μm particle diameter were employed at a flow rate of 1 mL/min. Ten microliters (10 μL) of the sample was injected each time and detected at 254 nm via a UV detector.

FTIR and LC-ESI-MS

To authenticate further, mass spectral studies of the extract were obtained by LC-ESI-MS (Thermo Finnigan LCO Advantage max ion trap mass spectrometer). Briefly, 25 μL of the sample was introduced into the ESI source through a Finnigan surveyor autosampler. The mass spectra (MS) were scanned in the range of 150–750 m/z, and the solvent was eluted as a given gradient program at 1 mL/min. The maximum ion injection time was set at 200 ns, the ion spray voltage was set at 5.3 kV, and the capillary voltage was set at 34 V. After the MS scan had run for 20 min, the data reductions were performed by Xcalibur 1.4 SRI. The column was used as ODS-2, 250 × 4.6, 5 μm, and the mobile phase was CH_3CN:5 mM $AcNH_4$. The MS positive ionisation mode was ESI (±) or APCI (±). For FTIR analysis, the samples were pelleted using KBr.

^{1}H NMR analysis

A $^1H_1$ (500 MHz) spectrum was recorded on a Bruker AV III (500 MHz ($^1H_1$) NMR spectrometer using tetramethylsilane (TMS) as an internal reference. The chemical shifts are expressed in parts per million (ppm).

DPPH scavenging activity

The free radical scavenging effects of ChR and FChR were determined using a modified version of the DPPH method described by Brand-Williams et al. (1995). Appropriate dilutions of the ChR and FChR (20 μg/mL to 100 μg/mL) were mixed with 1 mL of a 0.135 mM methanolic solution of DPPH radicals. Absorbance was then measured at 517 nm after a 30 min incubation period in the dark. BHT was used as the standard, and the inhibition percentage was calculated using the following formula:

$$\% \text{ of inhibition} = (\text{Abs control} - \text{Abs sample}/\text{Abs control}) \times 100$$

In vitro anticancer studies

Cell viability: MTT assay

The cytotoxic effects of ChR and fungal ChR (FChR) were measured using the MTT assay [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Mossman 1983). Briefly, human hepato cellular carcinoma cells (HepG2) were seeded in a 96-well plate at a density of 2.0 × 10^4

cells. After 24 h, different concentrations of ChR and FChR (10–100 μg/ml) were added to each well, and plates were incubated for 24 h in ambient conditions of 5% CO_2 and 95% humidity at 37 °C. At the end of incubation period, 20 μl MTT solution [5 mg/mL in phosphate-buffered saline (PBS)] was added to each well, and the plates were covered with aluminum foil and incubated again for 4 h at 37 °C. Next, 100 μL of 100% DMSO was added to each well to dissolve the purple MTT-formazan product. Absorbance was measured using a 96-well plate reader (Bio-Rad, Hercules, CA) at 570 nm with the 630 nm absorbance as the reference. The percentage of inhibition was calculated using the following formula:

$$\% \text{ of cytotoxicity} = (\text{Abs of control} - \text{Abs of Sample} / \text{Abs of control}) \times 100$$

Where, Abs of control = Mean OD of untreated cells; Abs of Sample = Mean OD of treated cells. The IC_{50} value was determined as the concentration of extract required for reduction of the absorbance to half that of the control. All assays were performed in triplicate, and the results are presented as mean ± SD.

Acridine orange/ethidium bromide staining

A cell suspension of each sample containing 5×10^5 cells was treated with 25 μl of acridine orange (AO) and ethidium bromide (EB) solution (3.8 μM of AO and 2.5 μM of EB in PBS) and examined with a fluorescent microscope (Zeiss, Jena, Germany) using a 450–490 nm UV filter. A total of 300 cells per sample was counted in triplicate for each dose point. Based on the staining intensity, the cells were scored as viable, apoptotic, or necrotic. Morphological changes such as membrane integrity and nuclear morphology were also observed and photographed. The percentage of apoptotic and necrotic cells was then calculated.

Hoechst 33528 Staining

Cells (HepG2) were treated with IC_{50} concentrations of ChR and FChR. After a 24 h incubation, both treated and untreated cells were harvested and stained with Hoechst 33258 (1 mg/mL, aqueous) for 5 min at room temperature. For fluorescent microscopy, a drop of the cell suspension was placed on a glass slide, and a cover slip was placed atop the suspension to reduce light diffraction; 300 random cells, in duplicate, were observed at 400× in a fluorescent microscope (Zeiss) fitted with a 377–355 nm filter. The percentage of cells reflecting pathological changes was subsequently calculated.

Results

Isolation and identification of endophytic fungi

Initially, the morphological characterisation of *A. alternata* PI-1, *C. capsici* PI-2 and *C. taiwanense* PI-3 were confirmed by their colony and spore growth pattern after 7–10 days in PDA medium (Fig. 1). Strain PI-1 has black or grayish colour colonies, and forms a copious mycelial development; also it produces primarily the hyaline that converted the mycelia into dark brownish colour, septate, multicelled and improperly branched. The conidiophores form small clusters, simple, flexuous, sometimes geniculate and smooth, whereas the conidia were oblique septa and obpyriform in nature. Strain PI-2 exhibits gray to dark gray colour colonies with irregular and cottony mycelial growth; PI-2 forms a falcate type of conidia. Strain PI-3 in PDA medium had white to grayish aerial mycelia with aseptate, hyaline conidia, thickly cylindrical with rounded ends. The isolated stains were then confirmed based on their molecular characterisation through rDNA sequencing. The sequences of all three isolates were aligned with the CLUSTAL W program and subjected to phylogenetic tree construction based on the maximum parsimony method encompassing 1000 bootstrap replications, which showed 99% homology with *A. alternata*, 87% homology with *C. capsici* and 98% homology with *C. taiwanense* from nucleotide BLAST analysis (Fig. 2). The trimmed sequences were deposited with GenBank, and the NCBI database with the corresponding accession numbers: KT380662, KT373967 and KX580307. Morphological and molecular characterisation revealed that the isolated strain PI-1 belongs to the genus *Alternaria* and species *alternata*, strain PI-2 belongs to the genus *Collidiotrichum* and species *capsici*, and PI-3 belongs to the genus *Collidiotrichum* and species *taiwanense*, respectively.

Selection of ChR-producing endophytic fungi

The HPLC chromatograms revealed that strains PI-1, PI-2, and PI-3 have very similar retention times compared with standard ChR (Fig. 3). Interestingly, the standard ChR had a retention time of 11.860 when eluted in a reverse phase C18 column, while the PI-1, PI-2, and PI-3 strains had retention times of 11.679, 11.168, and 11.230 min, respectively at peak extraction. In addition, the ChR yield was quantified in the liquid medium by comparing the peak area and peak height with that of standard ChR. Our findings indicated that the yield of the PI-1 strain was 846 mg/g, PI-2 was 377 mg/g, and PI-3 was 343 mg/g. An appreciable amount of FChR produced by strain PI-1, which was selected and exploited further for characterisation and application studies.

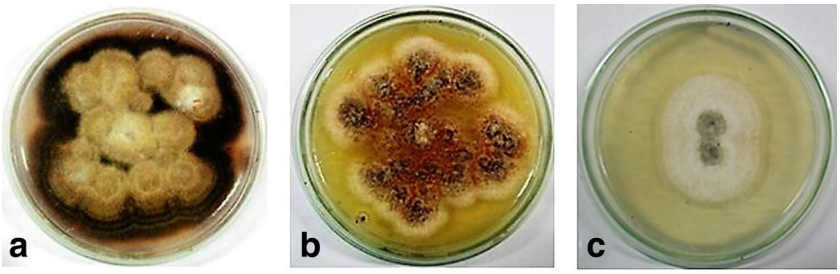

Fig. 1 Morphological characterisation of the mycelial growth on potato dextrose agar (PDA). **a** *Alternaria alternata* PI-1, **b** *Colletotrichum capsici* PI-2 and **c** *Colletotrichum taiwanense* PI-3

TLC and UV-vis spectroscopy

Initially, the standard ChR and fungal extracts form a blackish red colour after the addition of 10% ferric chloride, which confirms the presence of flavonoids. The authentic chrysin (ChR) was overlaid with fungal extracts that exhibited the same Rf values (0.6) as soon as visualisation under a short UV light (254 nm) exhibited black spots (Fig. 4). In addition, the UV-vis spectroscopic analysis of the standard ChR and fungal extracts exhibited very similar absorbance ranges at 268 nm and 316 nm (Fig. 5).

Fourier transform infrared spectroscopy

Infrared spectroscopy (IR) spectroscopy was used to predict the functional group present in FChR, and then compared with standard ChR (Fig. 6). An intense band in the region of 3500–3000 cm^{-1} appeared, which can be attributed to the symmetrical and asymmetrical stretching modes of O–H. A strong vibrational stretch of the carbonyl group ©=O) was noted at 1650.54 cm^{-1}. All of the C–H stretching frequencies were in the range of 2918.34 cm^{-1}. Transmittance at 1262.45 cm^{-1} corresponds to the C–C bending vibration frequency.

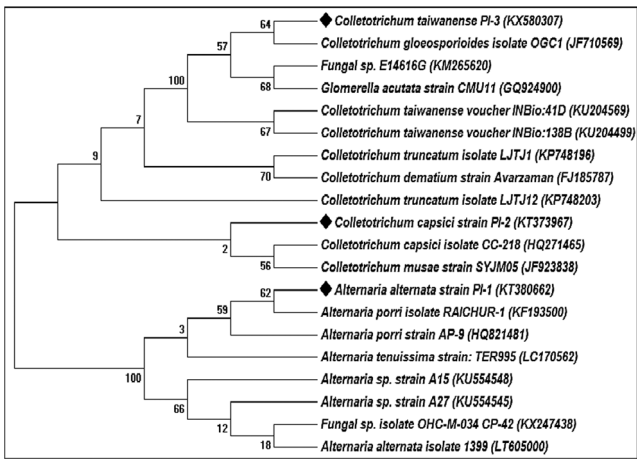

Fig. 2 Phylogenetic relationship of endophytic fungi isolated from the leaves of *P. incarnata*. A phylogenetic tree was constructed based on the ITS-1 and ITS-4 sequences using the maximum parsimony method in MEGA ver. 6.0. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1000 replicates) is shown next to the branches

Interestingly, in these spectra, most of the frequencies are gathered at 1500 cm^{-1}, demonstrating that the chrysin contains more aliphatic units than aromatic units. Based on the IR spectral data, the isolated sample was confirmed to be chrysin (5,7-dihydroxy flavone ChR) and the stretching frequencies were in agreement with the general molecular stoichiometry.

LC-ESI-MS analysis

An additional chromatographic technique was employed to confirm the ChR based on its mass values using LC-ESI-MS. The FChR displays the molecular ion at m/z = 255, which clearly confirms the separated constituent to be chrysin. The ion peak m/z = 255 was detected by the positive mode [M + H] + (Fig. 7).

$^{1}H_{1}$ NMR spectra

The $^{1}H_{1}$-NMR spectra of separated FChR were recorded in DMSO-d6 solution, and depicts the signals at 6.995 (1H, s), 6.470 (1H, s), and 6.295 ppm (1H, s). The chemical shifts of 7.893 (d, H2, H6') and 7.559 (m, H3', H4', H5') clearly suggest no substitution in the B ring of the flavone. The signal at d 12.778 ppm was assigned to the C-5 hydroxyl. Further, methoxy protons appear in the region of d 3.8 ppm. It was very apparent that the aliphatic and aromatic protons in the $^{1}H_{1}$-NMR spectra were in agreement with the general molecular formula of the flavanone chrysin ring systems. Based on the proton NMR spectral data, we confirmed that the extracted fungal entity is the flavonoid chrysin skeleton system (Fig. 8). Further, this identity was proved by mass spectral analysis.

MTT assay

Cellular responses to the fungal chrysin and standard chrysin were evaluated against the human breast carcinoma cell line (MCF-7) by MTT assay. The standard and fungal ChR both significantly inhibited the proliferation of MCF-7 cancer cells in a dose-dependent manner. The IC_{50} values were obtained by plotting cell viability against different concentrations of the FChR and ChR. The inhibitory concentrations were 34.066 μg/mL for ChR and 37.97 μg/mL for FChR (Fig. 9a).

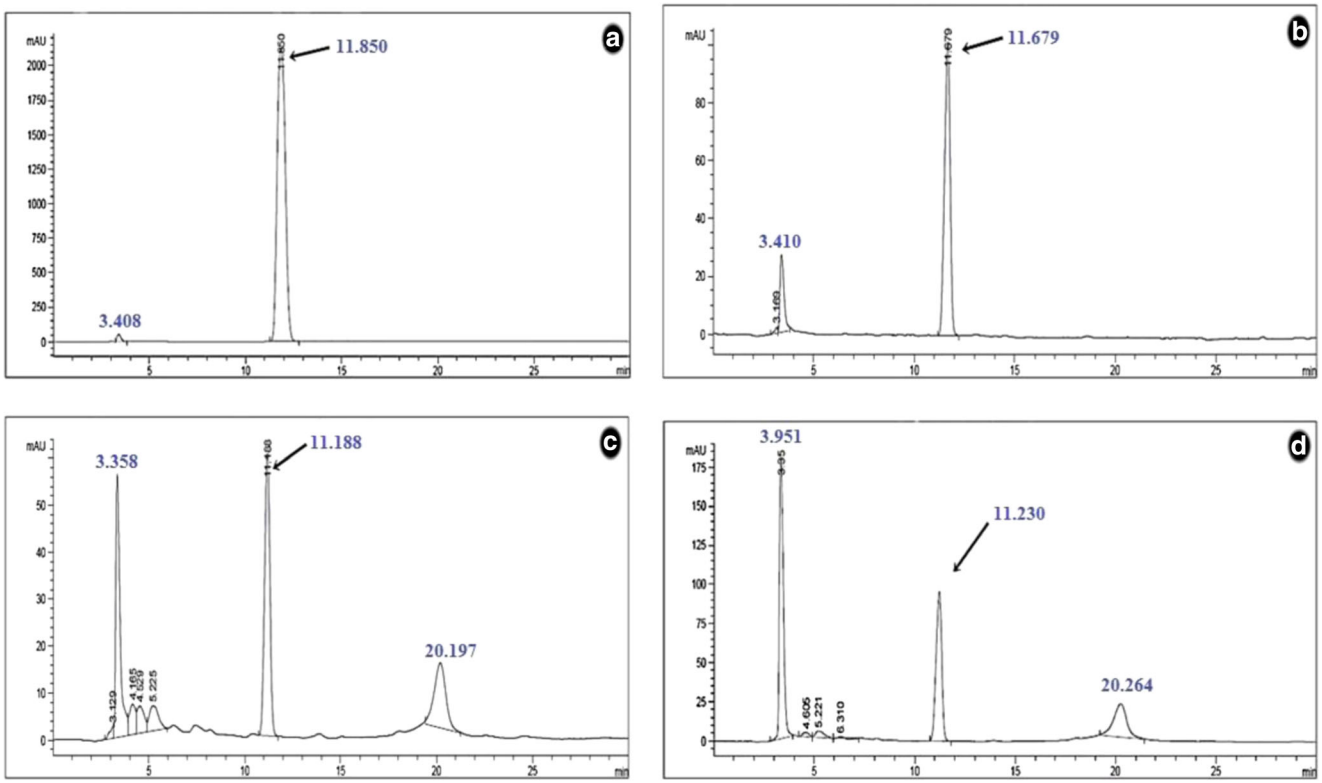

Fig. 3 HPLC results. **a** Standard chrysin (ChR). **b** Fungal chrysin (FChR) of strain PI-1. **c** FChR of strain PI-2. **d** FChR of strain PI-3. Retention peaks were seen at 11.860, 11.679, 11.168, and 11.230 min, respectively, and the results clearly depict the high yield of ChR (846 mg/g) in strain PI-1

AO-EtBr staining

To distinguish live and dead cells based on their morphological features, treated and untreated cells were stained with AO/EtBr and visualised using fluorescent microscopy at a suitable range of fluorescent emissions. The untreated cells displayed green nuclei with an intact structure. Although the initial apoptotic cells contain a bright green nucleus with abridged chromatin, the late apoptotic cells contained abridged and fragmented orange chromatin. This result strongly demonstrates that the IC_{50} concentrations of FChR and ChR hold the ability to induce apoptosis in the MCF-7 breast cancer cell line (Figs. 9b, 10a).

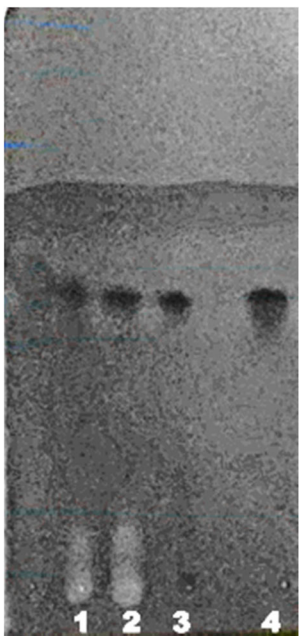

Fig. 4 Thin layer chromatographic (TLC) spots of FChR derived from endophitic fungal isolates. Lanes:*1 A. alternata* PI-1, *2 C. capsici* PI-2, *3 C. taiwanense* PI-3, *4* Standard ChR

Hoechst 33528 staining

After treatment with Hoechst staining for 48 h, MCF-7 cells begin to exhibit characteristics of apoptosis, such as cell shrinkage, nuclear condensation, and fragmentation. In the control group, the cells had normal morphology and contained an intact nuclear architecture (Figs. 9b, 10b).

DPPH scavenging activity

DPPH scavenging activity for ChR and FChR was evaluated at different concentrations ranging from 20 to 100 μg/mL. Both ChR and FChR showed dose-dependent inhibition ranging from 9-27% and 9-26%, respectively (Fig. 11). The observed ChR and FChR activities were lower than the standard BHT at 42% to 83%.

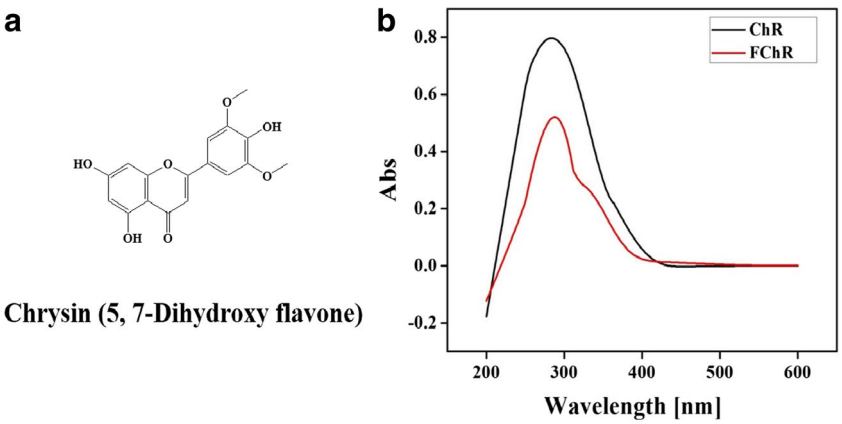

Fig. 5 **a** Structure of chrysin, **b** UV-vis spectrum of ChR and FChR

Discussion

In this study, the ChR-producing endophytic fungal strains *A. alternata* (KT380662), *C. capsici* (KT373967), and *C. taiwanense* PI-3 (KX580307) were isolated from *P. incarnata* leaves. Based on preliminary screening, *A. alternata* (KT380662) was considered as the strain with potential maximum yield and thus studied further. Moreover, *Alternaria* sp. is a mostly dominant diverse taxon in many plants. For example, 174 endophytic fungi (18 taxa) were isolated from *C. acuminate*, with *Alternaria* (12.6%) being the most prominent from all (Lin et al. 2007). Likewise, Fernandes et al. (2009) isolated 22 endophytic fungal strains from *C. arabica* L. The *Alternaria* species uproot from plants that are able to produce pharmaceutically relevant aromatic compounds. Specifically, previous reports revealed that the *A. alternata* have the ability to produce compounds such as paclitaxel (Tian et al. 2006), anthranoids (Phunlap et al. 2013), perylene derivatives (Gao et al. 2009), and anthraquinone derivatives (Chen et al. 2014).

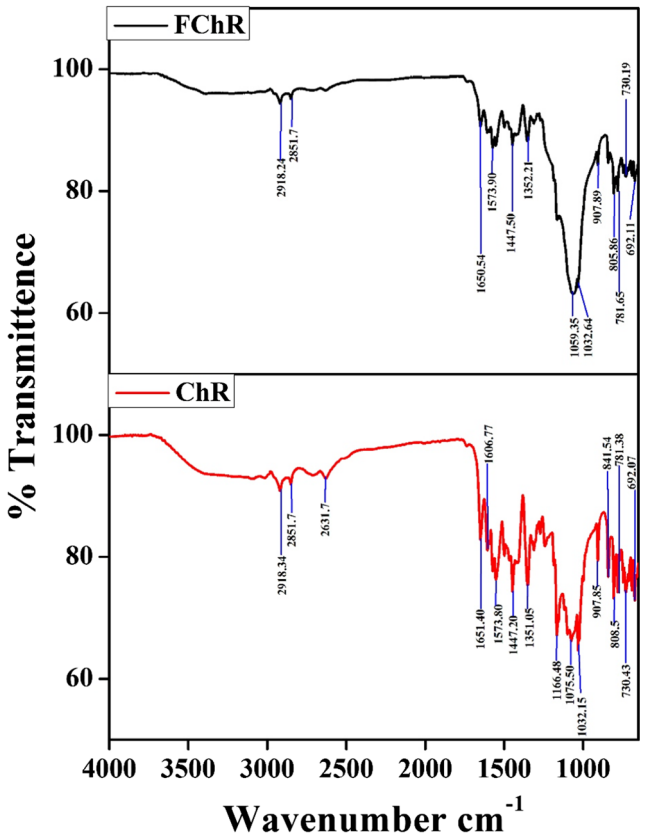

Fig. 6 Fourier transform infrared spectroscopy (FT-IR) spectroscopic results of ChR and FChR, showing the major transmittance corresponding to O–H, C = O and C–C functional groups, which clearly manifests the presence of chrysin

Chrysin is a dietary flavone, which was originally extracted from *P. caerulea* by Wolfman et al. (1994). Later, chrysin was also extracted from *P. incarnata*, *O. indicum*, mushroom, and *P. ostreatus* (Brown et al. 2007; Jayakumar et al. 2009). Brown et al. (2007) extracted ChR from *P. incarnata* and evaluated it for the anxiolytic effect under in vitro conditions. In this study, we have successfully identified novel indigenous fungal strains that produce ChR. Different analyses, such as TLC, HPLC, LC-ESI-MS, FTIR, and $^{1}H_{1}$ NMR, were performed to identify and confirm the presence of FChR as compared to that of standard chrysin. In an earlier report, Saric et al. (2004) optimised TLC conditions for chrysin. In the current study, the Rf values of FChR strongly match the Rf values of authentic chrysin. Further, the molecular mass and structure of chrysin were confirmed by comparison with data from previous literature (Liu et al. 1993, 2001; Larit et al. 2012). The quantitative HPLC analysis revealed that the yield of FChR from *A. alternata* KT380662 was higher when compared with previously reported bioresources. The amount of chrysin extracted from other sources was 2.92–20.4 mg/L in *Oroxylum indicum* root bark (Zaveri et al. 2007); 400 mg/kg in ethanolic extract of the mushroom *Pleurotus ostreatus*; 0.48 mg/kg in a honey sample (Hadjmohammadi and Nazari 2010); and 500 mg/kg in an ethanolic extract of *Cytisus multiflorus* (Pereira et al. 2012). This clearly demonstrates that the endophytic fungus PI-1 is an effective source for the commercial production of chrysin. Moreover, the commercialisation of chrysin needs further work to improve the yield of chrysin

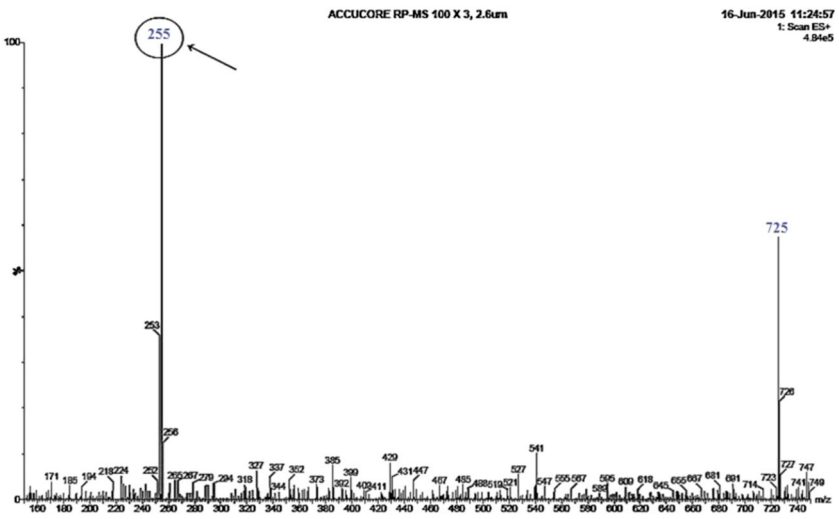

Fig. 7 LC-ESI-MS analysis of FChR displays the molecular ion at m/z = 255, which clearly confirms the separated biocompound to be chrysin

from milligrams to grams by studying its biosynthetic pathway and the genetic details of *A. alternata* PI-1.

The culture was stored in an artificial medium, which resulting in a reduction in the production of the compound. Consequently, we need to understand the mechanism involved in the production of compounds by endophytes. Thus, based on the results, we concluded that we have successfully isolated from the *P. incaranata* plant an endophytic fungus, *A. alternata* KT380662, that produces chrysin at a concentration of 846 mg/g. The extracted FChR has similar properties to plant-derived chrysin.

The DPPH scavenging activity method is a standard, quick, and simple technique with which to evaluate antioxidant activity. The principle of DPPH activity is that antioxidants react with stable free radicals. Through the free radical response, DPPH (a,a-diphenyl-b-picrylhydrazyl) is converted into a,a-diphenyl-b-picryl hydrazine by means of a colour change. When antioxidant potentials are specified, the pink colour gradually transforms to colourless. De Martino et al. (2012) demonstrated that chrysin is a complex compound that can have either inhibitory or stimulating action on the radical development of radish. The initial dose stimulates radical growth, yet, when the concentration increases, the chrysin becomes inhibitory in nature. This result was more consistent with our results.

Flavonoids are a special group of phenolic products that originate from plants, honey, and mushrooms. These compounds are safe and have low toxicity, which leads to their use as anticancer agents. Chrysin is a member of the flavonoid group. Nowadays, researchers are paying significant attention to the chrysin compound. In our study, we demonstrated that chrysin triggers cytotoxicity and

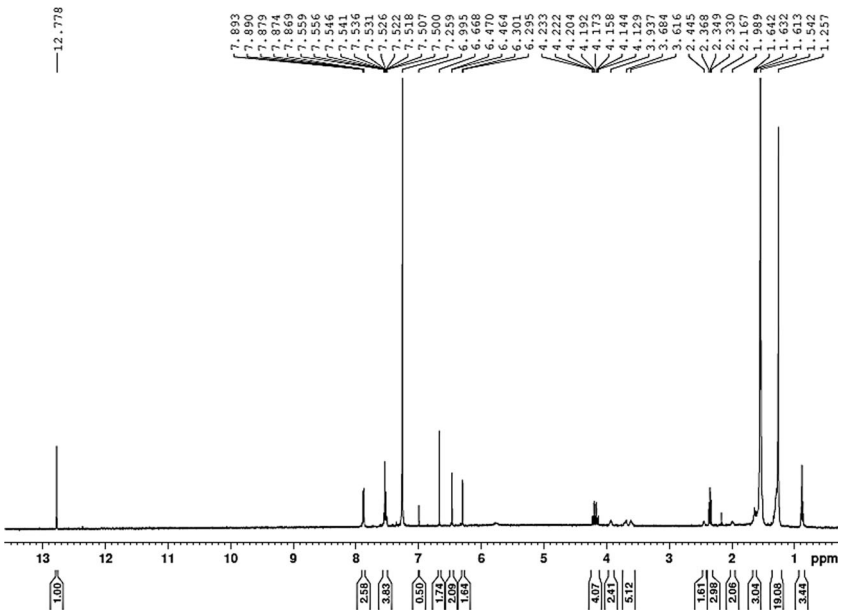

Fig. 8 $^{1}H_{1}$ NMR spectrum of FChR showing signals at 6.995 (1H, s), 6.470 (1H, s), and 6.295 ppm (1H, s), chemical shifts at 7.893 (d, H2, H6'), 7.559 (m, H3', H4', H5'), and signals at d 12.778 ppm, d 3.8 ppm, revealing that the extracted fungal entity is indeed the FChR skeleton system

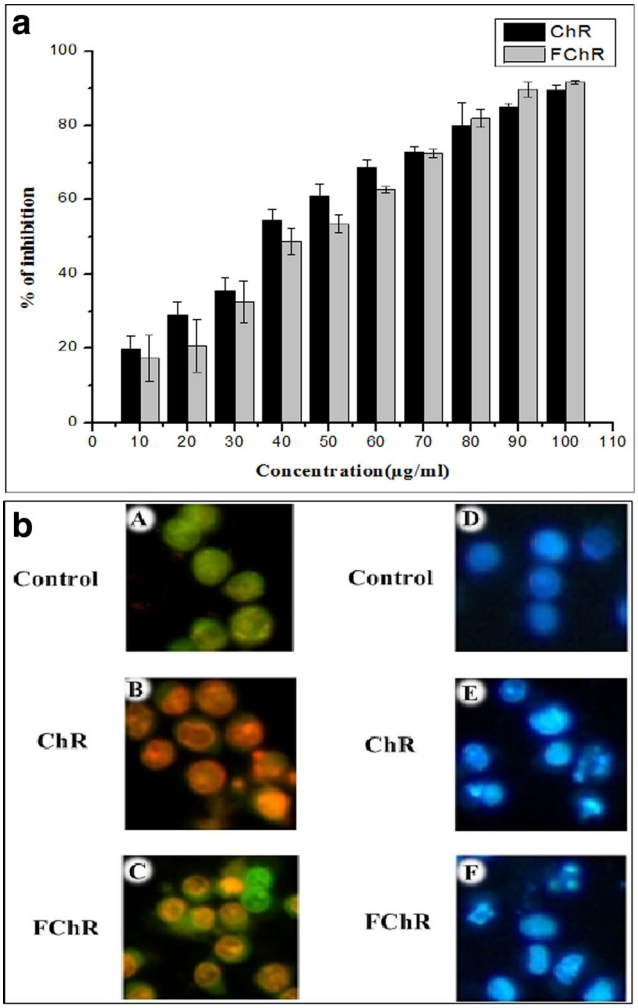

Fig. 9 **a** Cell viability assay (MTT) of ChR and FChR. **b** Results of AO/EB staining; *A* Control (untreated cells); *B* treated with ChR; *C* treated with FChR. Results of Hoechst staining: *D* Control (untreated cells), *E* treated with ChR; and *F* treated with FChR

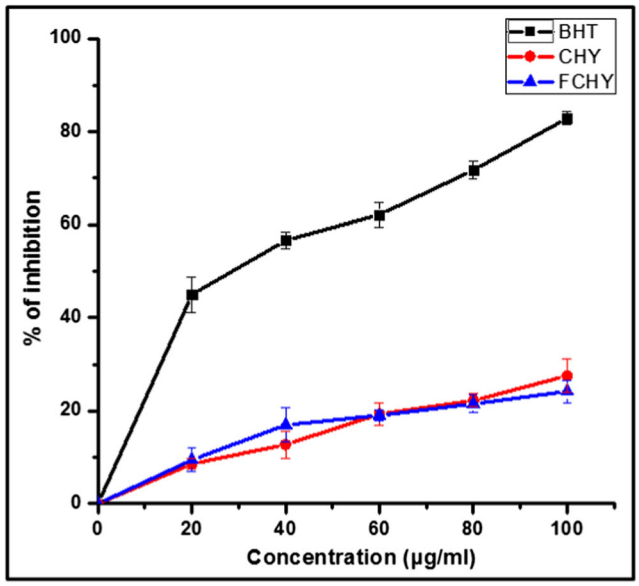

Fig. 11 DPPH scavenging activity of BHT (positive control), ChR and FChR. The assay was performed in triplicate and the results are presented as mean ± SD

apoptosis against the MCF-7 breast cancer cell line. A similar phenomenon was reported previously with the chrysin-organo germanium (IV) complex in MCF-7 cells (Yang et al. 2013). Apoptosis is a natural program of cell mortality categorised by structural changes, genetic obliteration, and condensation. In this study, the morphological changes associated with apoptosis were well established by utilising AO/EB and Hoechst 33258 staining methods. In addition, apoptosis can be measured by observing the activation of chromatin aggregation, as well as partitioning of the cytoplasm and nucleus into membrane-bound vesicles (apoptotic bodies) that contain ribosomes, morphologically intact mitochondria, and nuclear material (Xiang et al. 2009). In this study, FChR treated

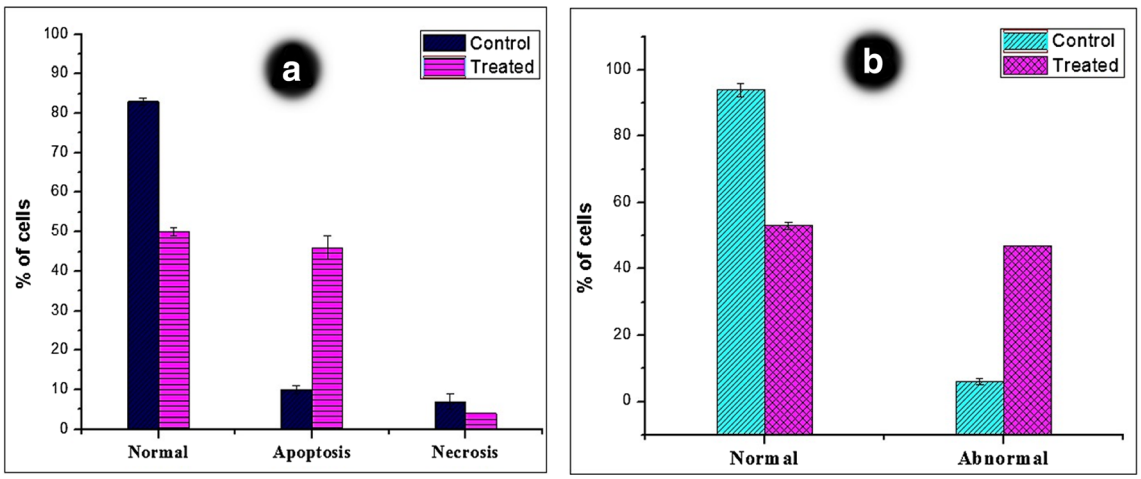

Fig. 10 **a** AO/EB fluorescent study of apoptosis in HeLa cells. Graph shows the manual count of apoptotic cells (%) (data are mean percentage ± SD of triplicate). **b** Hoechst 33258 staining study on HeLa cells. Graph shows the manual count of apoptotic cells (%) (data are mean percentage ± SD of triplicate)

HepG-2 cells loses their viability in a time- and dose-dependent manner. Formation of condensed nuclei, membrane, blebbing, and apoptotic bodies clearly indicate that FChR triggers immediate cellular responses and apoptotic cell death against HepG-2 cells.

Conclusions

In conclusion, we have been successful in identifying an alternative biological resource for the production of the natural anticancer flavone ChR. From isolated fungal strains, *A. alternata* KT380662 was selected as the most potent ChR-producing strain. As a result, the ChR production rate of 846 mg/g was greater than that of previously explored bioresources of plant, honey, and mushroom origin. In addition, the production of pure chrysin was cost-effective compared with conventional synthetic and plant-based production methods. FChR possesses important biological activities, particularly anticancer and antioxidant properties.

Acknowledgements The authors thank the Department of Science and Technology Promotion of University Research and Scientific Excellence (DST-PURSE; DST Sanction Order No- SR/FT/LS- 113/2009) for providing confocal microscopy images, Central Drug Research Institute Sophisticated Analytical Instrument Facilities (CDRI-SAIF), Lucknow for the LC-ESI-MS facility, and the Indian Institute of Science (IISc), Bangalore for the NMR facility.

References

Aly AH, Debbab A, Kjer J, Proksch P (2010) Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers 41:1–16

Anand KV, Mohamed Jaabir MS, Thomas PA, Geraldine P (2012) Protective role of chrysin against oxidative stress in D-galactose-induced aging in an experimental rat model. Geriatr Gerontol Int 12:741–750

Blumenthal M, Goldberg A, Brinckmann J (eds) (2000) Herbal medicine: expanded Comission E monographs. Integrative Medicine. Newton, MA, pp 293–296

Brand-Williams W, Cuvelier M, Berset C (1995) Use of free radical method to evaluate antioxidant activity. LWT-Food Sci Technol 28:25–30

Brown E, Hurd NS, McCall S, Ceremuga TE (2007) Evaluation of the anxiolytic effects of chrysin, a Passiflora incarnata extract, in the laboratory rat. AANA J 75:333–337

Chen B, Shen Q, Zhu X, Lin Y (2014) The Anthraquinone Derivatives from the Fungus Alternaria sp. XZSBG-1 from the Saline Lake in Bange, Tibet, China. Molecules 19:16529–16542

Cui JL, Guo SX, Dong HL, Xiao PG (2011) Endophytic fungi from dragon's blood specimen: isolation, identification, phylogenetic diversity and bioactivity. Phytother Res 25:1189–1195

De Martino L, Mencherini T, Mancini E, Aquino RP, De Almeida LF, De Feo V (2012) In vitro phytotoxicity and antioxidant activity of selected flavonoids. Int J Mol Sci 13:5406–5419

Dhawan K, Kumar S, Sharma A (2001) Anti-anxiety studies on extracts of *Passiflora incarnata* Linneaus. J Ethnopharmacol 78(2–3):165–170

Fernandes MDRV, Silva TAC, Pfenning LH, Aparecida DLM, Miguel DCC, Heinrich TA, Alencar SM, Lima MA, Ikegaki M (2009) Biological activities of the fermentation extract of the endophytic fungus *Alternaria alternata* isolated from *Coffea arabica* L. Braz J Pharm Sci 45:677–685

Gao SS, Li XM, Wang BG (2009) Perylene derivatives produced by *Alternaria alternata*, an endophytic fungus isolated from *Laurencia* species. Nat Prod Commun 4(11):1477–1480

Gresa-Arribas N, Serratosa J, Saura J, Sola C (2010) Inhibition of CCAAT/enhancer binding protein expression by chrysin in microglial cells results in anti-inflammatory and neuroprotective effects. J Neurochem 115:526–536

Hadjmohammadi MR, Nazari SS (2010) Separation optimization of quercetin, hesperetin and chrysin in honey by micellar liquid chromatography and experimental design. J Sep Sci 33:3144–3151

Jayakumar T, Thomas PA, Geraldine P (2009) In vitro antioxidant activities of an ethanolic extract of the mushroom *Pleurotus ostreatus*. Innov Food Sci Emerg Technol 10:228–234

Khoo BY, Chua SL, Balaram P (2010) Apoptotic effects of chrysin in human cancer cell lines. Int J Mol Sci 11:2188–2199

Lacap DC, Hyde KD, Liew ECY (2003) An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. Fungal Divers 12:53–66

Larit F, Benyahia S, Benayache S, Benayache F, Leon F, Brouard I, Bermijo J (2012) Flavonoïds from *Calycotome spinosa* (L.). Lamk. Int J Med Arom Plants 2(1):34–37

Liang YC, Tsai SH, Tsai DC, Lin-Shiau SY, Lin JK (2001) Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-gamma by flavonoids in mouse macrophages. FEBS Lett 496:12–18

Lin X, Lu C, Huang Y, Zheng Z, Su W, Shen Y (2007) Endophytic fungi from a pharmaceutical plant, *Camptotheca acuminate*: isolation, identification and bioactivity. World J Microbiol Biotechnol 23:1037–1040

Liu Q, Dixon RA, Mabry TJ (1993) Additional flavonoids from elicitor-treated cell cultures of *Cephalocerus senilis*. Phytochemistry 34:167–170

Liu IX, Durham DG, Richards ME (2001) Vancomycin resistance reversal in enterococci by flavonoids. J Pharm Pharmacol 53:129–132

Medina JH, Paladini AC, Wolfman C, de Stein ML, Calvo D, Diaz LE, Pena C (1990) Chrysin (5,7-di-OH-flavone), a naturally-occurring ligand for benzodiazepine receptors, with anticonvulsant properties. Biochem Pharmacol 40:2227–2231

Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol 65:55–63

Nadeem M, Ram M, Alam P, Ahmad MM, Mohammad A, Qurainy FA, Khan S, Abdin MZ (2012) Fusarium solani, P1, a new endophytic podophyllotoxin-producing fungus from roots of *Podophyllum hexandrum*. Afr J Microbiol Res 6(10):2493–2499

Parthasarathy R, Sathiyabama M (2015) Lovastatin-producing endophytic fungus isolated from a medicinal plant *Solanum xanthocarpum*. Nat Prod Res 29(24):2282–2286

Pereira OR, Silva AM, Domingues MR, Cardoso SM (2012) Identification of phenolic constituents of *Cytisus multiflorus*. Food Chem 131:652–659

Phunlap P, Sommit D, Sriubolmas N, Ngamrojanavanich N, Matsubara K, Pudhom K (2013) Antiangiogenetic effects of anthranoids from *Alternaria* sp., an endophytic fungus in a Thai medicinal plant *Erythrina variegata*. Phytomedicine 20:918–922

Prabukumar S, Rajkuberan C, Ravindran K, Sivaramakrishnan S (2015) Isolation and characterization of endophytic fungi from medicinal

plant *Crescentia Cujete* L. and their antibacterial, antioxidant and anticancer properties. IJPPS 7:11

Promputtha I, Jeewon R, Lumyong S, McKenzie EHC, Hyde KD (2005) Ribosomal DNA fingerprinting in the identification of non-sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). Fungal Divers 20:167–186

Pu X, Qu X, Chen F, Bao J, Zhang G, Luo Y (2013) Camptothecin-producing endophytic fungus *Trichoderma atroviride* LY357: isolation, identification, and fermentation conditions optimization for camptothecin production. Appl Microbiol Biotechnol 97:9365–9375

Pushpavalli G, Veeramani C, Pugalendi KV (2010) Influence of chrysin on hepatic marker enzymes and lipid profile against D-galactosamine-induced hepatotoxicity rats. Food Chem Toxicol 48:1654–1659

Saric MM, Jasprica I, Bubalo AS, Mornar A (2004) Optimization of chromatographic conditions in thin layer chromatography of flavonoids and phenolic acids. Croat Chem Acta 77(1–2):361–366

Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260:214–216

Tian R, Yang Q, Zhou G, Tan J, Zhang L, Fang C (2006) Taxonomic study on a taxol producing fungus isolated from bark of *Taxus chinensis* var. mairei. J Wuhan Botan Res 24:541–545

Torres-Piedra M, Ortiz-Andrade R, Villalobos-Molina R, Singh N, Medina-Franco JL, Webster SP, Binnie M, Navarrete-Vázquez G, Estrada-Soto S (2010) A comparative study of flavonoid analogues on streptozotocin-nicotinamide induced diabetic rats: quercetin as a potential antidiabetic agent acting via beta-hydroxysteroid dehydrogenase type 1 inhibition. Eur J Med Chem 45:2606–2612

Wang J, Qiu J, Dong J, Li H, Luo M, Dai X, Zhang Y, Leng B, Niu X, Zhao S, Deng X (2011) Chrysin protects mice from *Staphylococcus aureus* pneumonia. J Appl Microbiol 111:1551–1558

Wolfman C, Viola H, Paladini A, Dajas F, Medina JH (1994) Possible anxiolytic effects of chrysin a central benzodiazepine receptor ligand isolated from *Passiflora coerulea*. Pharmacol Biochem Behav 47(1):1–4

Xiang W, Ke Z, Zhang Y, Cheng GH, Irwan ID, Sulochana KN, Potturi P, Wang Z, Yang H, Wang J, Zhuo L, Kini RM, Ge R (2009) Isthmian, is a novel secreted angiogenesis inhibitor that inhibits tumor growth in mice. J Cell Mol Med 15(2):359–374

Xiong ZQ, Yang YY, Na Z, Wang Y (2013) Diversity of endophytic fungi and screening of fungal paclitaxel producer from *Anglojap yew*, Taxus x media. BMC Microbiol 13:7

Yang F, Jin H, Pi J, Jiang JH, Liu L, Bai HH, Yang PH, Cai JY (2013) Anti-tumor activity evaluation of novel chrysin-organogermanium(IV) complex in MCF-7 cells. Bioorg Med Chem Lett 23:5544–5555

Zaveri M, Sunita J (2007) Gastroprotective effects of root bark of *Oroxylum indicum*. J Nat Rem 7:269–277

Zhao J, Shan T, Mou Y, Zhou L (2011) Plant-derived bioactive compounds produced by endophytic fungi. Mini-Rev Med Chem 11:159–168