Chemetaxonomic profiling of *Penicillium setosum* using high-resolution mass spectrometry (LC-Q-ToF-MS)

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**ABSTRACT**

In the present study, secondary metabolites produced by an endophytic fungus *Penicillium setosum* were extracted using colony agar plug and culture broth extraction methods. High resolution LC-MS was used to explore the chemical nature of the secondary metabolites, as well, compare the reliability of the methods. *P. setosum* was chemotaxonomically distinguished from other members of section *Lanata-divaricata*, by its ability to produce mycotoxin, patulin and also by the presence of certain phenol-derived compounds, like quercetin, dihydroflavonols (dihydroquercetin and dihydromyricetin), kaempferol, luteolin, while some *Penicillium* specific compounds such as, citromycetin and andrastin D reveal its similarity towards section *Lanata-Divaricata* members. For the first time, the presence of dihydroquercetin is remarkably and spectrometrically confirmed from a microbial source. In addition, a few polyketides, anthroquinone compounds, hydrocarbons, and fatty acids were also detected in the culture extract. Being the first report on the production of polyphenolic compounds by an endophytic fungus of *Penicillium* species, the current research is crucial, and moreover the starin itself is a novel species.

**1. Introduction**

Endophytic fungi are diverse polyphyletic groups of microorganisms, and can thrust asymptomatically in different healthy tissues of living plants above and/or under the ground, including stems, leaves, and/or roots (Jia et al., 2016). They establish an irreplaceable plant–microbe interaction entirely inside the plant tissues without affecting them lethally. The close interaction offers metabolic interchanges and support the synthesis of some valuable compounds of the plant and the endophyte. Studies have reported that some isolated endophytes are able to synthesize certain secondary metabolites in culture media, which are typically produced by the plants. The central examples are production of taxol by *Taxomyces andreanae* (Stierle et al., 1993), podophyllotoxin by *Phialocephala fortinii* (Eyberger et al., 2006), deoxypodophyllotoxins by *Aspergillus fumigatus* (Kusari et al., 2009a), camptothecin by *Fusarium solani* (Kusari et al., 2009b), piperine by *Colletotrichum gloeosporioides* (Chithra et al., 2014).

*Withania somnifera* (common name: Ashwagandha, Family: Solanaceae) is a widely-known medicinal plant having various therapeutic qualities. It is an inevitable component in the Ayurvedic medicinal preparations like Rasayana. This plant has not been explored enough for its endophytic occurrence. So, endophytes present in this under-explored medicinal plant may be of great importance, in terms of investigating the biosynthesis of natural products and/or bio-active metabolites. In a previous work, we had isolated a novel *Penicillium* species from the surface sterilized root of *W. somnifera*. The identification of its secondary metabolites is being dealt here.

*Penicillium* is one of the most promising biologically active metabolite producers of the fungal genera among the group of micro-fungi. Under different environmental conditions, almost 429 species have been already reported in this genus (Visagie et al., 2014). Since the discovery of penicillin from *P. notatum* by Alexander Fleming, *Penicillium* species have gained much attention for discovering novel bio-active secondary metabolites/extrolites. *Penicillium* isolated from little studied habitats have shown the ability to synthesize both previously-known and new physiologically-active compounds with diverse structures (Kozlovskii et al., 2013). Novel species have been reported continuously from these genus together with their persuasive biological action.

Filamentous fungi like *Penicillium* are difficult to discriminate using a conventional microbiological approach, so extrolite profiling analysis was considered as an additional assistance in this regard. A profile of secondary metabolites is comprised of all the different compounds...
produced by a fungus on a given substratum. It includes toxins, antibiotics, fatty acids and other outward-directed compounds (Silva et al., 1998; Frisvad et al., 2004). The expression of certain metabolites in fungi is correlated with differentiation and with the formation of either sexual or asexual spores, including cleistothecia and other types of differentiated cells (Avalos et al., 2014). Therefore, the consistent production of secondary metabolites in the mycelium and spores of a fungus (Visagie et al., 2014), differentiates it from other species (Frisvad et al., 2008).

The analysis of metabolites or their derivative products in the extract can be determined using two-dimensional analytical technique like Gas chromatography-mass spectrometry (GC-MS) and Liquid chromatography-mass spectrometry (LC-MS). Liquid chromatography-mass spectrometry is considered as the most versatile tool for detecting chromatography-mass spectrometry (Visagie et al., 2014). It has a significant role in determining the accurate masses and to estimate the molecular composition of ions of known and unknown compounds. Its high sensitivity and specificity permit the differentiation of isobaric compounds of different elemental composition, even at microgram concentrations (Altemimi et al., 2017). Structural analysis of different bio-active metabolites could be carried out using electrospray ionization (ESI) combined with tandem mass spectrometry (MS/MS) (Cuyckens and Claey s, 2004; Stalikas, 2007), thus permitting direct screening of vast array of natural products without having to resort to laborious isolation procedures. Generally, GC-MS is used to analyze the volatile and semi-volatile secondary metabolites present in the extract. In the case of Penicillium species, it permits the determination of hydrocarbons and fatty acids. Cellular fatty acid profiles have been successfully used to characterize and differentiate a sub groups of Penicillium (Dart et al., 1976; Silva et al., 1998).

The chance of discovering novel metabolites or biologically important compounds from a taxonomic novel species is very high compared to other species (Strobel, 2018). Hence, this study is focused on the identification of secondary metabolites of newly-identified Penicillium setosum using colony agar plugs and culture broth extraction methods. The dissociation pattern and product ions of important metabolites are elucidated using liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (LC-Q-ToF-MS). Hydrocarbons and fatty acid secondary metabolites are determined using GC-MS. This is used to establish the chemotaxonomic distinctiveness of the isolate from other species of Penicillium.

2. Materials and methods

An endophytic Penicillium species was isolated from the surface sterilized roots of Withania somnifera, subsequently introduced as Penicillium setosum sp. nov. based on the distinctive fungal morphology and phylogenetic analyses of partial β-tubulin (Bta), calmodulin (CaM) and RNA-dependent RNA polymerase II (RPB2) gene sequences.

2.1. Extraction of secondary metabolites from P. setosum

Colony agar plug and culture broth extraction methods were implemented to understand the effectiveness of the extraction process, for the identification of the secondary metabolites of P. setosum. For the colony agar plug method, the fungus was inoculated on Czapek yeast autolysate (CYA) agar, and incubated for 7 days at 25 °C in the dark. Five agar plugs from the incubated fungal culture plate were expurgated using a sterile 7 mm cork borer. They were transferred to a glass scintillation vial containing a mixture of dichloromethane, ethyl acetate and methanol (3:2:1 (v/v/v)) with 1% (v/v) formic acid. The vial was vortexed for 30 s and sonicated for 30 min at room temperature (Visagie et al., 2014, 2016). Then the solvent extract was filtered, dried, and dissolved in 1 mL methanol. For the culture broth extraction method, the fungus was inoculated in CYA broth and incubated at 25 °C for 10 days at 100 rpm. After incubation, fungal biomass was discarded using Whatman no. 1 filter paper. Cell free culture broth and an equal volume of ethyl acetate were taken in a separatory funnel, the mixture was shaken vigorously for 30 min and the solvent part was collected. The step was repeated three times, pooled and dried using a rotary drum evaporator at 45 ± 2 °C. The crude extract was reconstituted in 1 mL of methanol. For spectral analyses, samples were filtered through an ultra-membrane filter (pore size 0.45 μm) and collected in 1.5 mL amber HPLC glass vials (Frisvad et al., 2008).

2.2. Secondary metabolite analyses using high resolution LC-MS

The crude extract of P. setosum was analyzed using LC-Q-ToF-MS (Waters Xevo G2 Q-ToF, USA) system. Chromatographic separation was conducted in a reverse-phased C18 analytical column (50 mm × 2.1 mm and 1.7 μm particle size) and the ultra pressure liquid chromatographic separation achieved on Acquity H class (UPLC, Waters, USA). A syringe pump delivering 5 μL was adjusted by the direct loop injection method. Mobile phase used was 0.1% formic acid with water and methanol, operated at a flow rate of 0.3 mL/min. The chromatographic method was held at the initial mobile phase composition (5% methanol constant for 0.1 min, followed by a linear gradient to 95% methanol for 6 min, then decreased to 5% in 6–8 min, followed by a one min post-run time after each analysis. All samples were eluted using gradient mobile phase of methanol and water over 9 min. The UPLC-separated samples were then analyzed by ToF-MS (mass range: 50–1000 Th). ESI was operated in each of positive and negative ionization mode.

The operating parameters were as follows: the cone voltage was set at 3 kV and the spray was stabilized with a nitrogen sheath gas (900 L/hr). Nitrogen was used as nebulizing gas (50 L/hr), capillary source temperature and desolvation temperature were 135 and 350 °C, respectively, and the capillary voltage was 3 kV for positive while 2.5 kV for negative ESI and collision energy 15–25 eV for MS/MS. METLIN Metabolomics Database was used to find most similar compounds and Masslynx V 4.1 was used to predict the possible elemental compositions of product ions. Then the observed product ions were compared with previously authenticated data available in the literature and MassBank (https://massbank.eu/MassBank).

2.3. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS was carried out using a Shimadzu 2010 plus gas chromatograph is connected to a QP 2020 mass spectrometer with an SH-Rxi-5Sil MS (cross bond to 5% diphenyl/95% dimethyl polysiloxane) 30 m × 0.25 mm × 0.25 μm column. Column oven temperature was set to 70 °C as initial temperature for 5 min, then increased up to 290 °C (at 10 °C/ min) and held for 5 min. The injection port temperature was set to 260 °C and helium was used as the carrier gas at a flow rate of 1.2 mL/min. The ionization voltage was 70 V. Ion source temperature and interface temperature were 220 and 300 °C, respectively. The injection volume was 1 μL done with AOC-20i injector and performed in a split mode as 10:1. The MS started after 3 min, and ended at 35 min with solvent cut time of 3 min. Retention time and ion signal intensity were analyzed by using in-built GC-MS software. NIST Ver. 11 MS data library was used for comparing the spectrum obtained through GC-MS and compounds present in the fungal extract were identified.

3. Results

Morphological and phylogenetic analyses of Penicillium setosum revealed that, it belongs to section Lanato-divaricata and showed close relation to Penicillium javanicum. Detailed phylogenetic analyses with larger number of related strains resolved the identity of this species from P. javanicum as well as from other closely related members (George et al., 2018). Hence, secondary metabolites of P. setosum are studied in order to find out their distinctiveness from other species.
3.1. Secondary metabolites from P. setosum extract

P. setosum culture extract was analyzed using LC-Q-ToF-MS system. Trial runs were conducted to optimize the instrument conditions for the identification of secondary metabolites in the crude extract. The structural interpretation was accomplished with an extensive investigation on the high-resolution mass spectra. Masslynx V 4.1 software was used to calculate mass accuracy, and accurate mass of possible elemental compositions for each analyte, based on the high-resolution mass. Depending on the accurate mass and mass accuracy value, probable elemental composition of the analyte was selected and searched in chemical structure database (chemspider). The obtained match list of compounds in agreement with the searched elemental composition was prudently studied and screened for all the possible compounds related to microbial or plant metabolites. These compounds were further analyzed by MS/MS. The obtained product ions of each analyte were compared with the expected compound product ions, which were retrieved from the MassBank database. The compound was again confirmed on the basis of elemental composition of each product ion and also by comparing with the previously published product ion mass spectra of the same. The significant limit of less than 4 ppm mass error was observed for all the identified compounds.

The total ion chromatograms (TIC) obtained in each of positive and negative ionization modes of both agar plug and culture broth extraction methods are depicted in Fig. 1. Chromatograms of both the extraction procedures were found to be virtually similar. Number of peaks in the colony agar plug and culture extract method were observed 23 and 24 in positive ionization mode and 20 and 18 in negative ionization mode respectively. Among these, fifteen compounds were identified from the product ion mass spectra.

The positive and negative ionization modes were determined for the analysis, however the later parts incorporated only one type of ionization mode, which demonstrated the precise mass similarity. The identified compounds, retention time, elemental composition, precursor, and ionization mode obtained from both colony plug and culture broth extraction methods are presented in Table 1. Identified secondary metabolites comprised of a few taxonomically relevant compounds of Penicillium species belonging to the class of anthraquinone and polyketides as well other compounds of flavonoids, coumarins etc. Both the colony extract and culture broth extract contained a majority of phenolic/flavonoid compounds. Accurate masses of the protonated or deprotonated molecule, product ions, isotopic distribution, and retention time factors permit the prediction of a compound structure (Vijlder et al., 2018). So, the MS/MS spectra of the suspected secondary metabolites were analyzed.

3.2. Fragmentation pattern of secondary metabolites

3.2.1. Product ion mass spectra of patulin

The primary focus was to screen the culture extract of Penicillium species for its species-specific metabolites. Patulin (molecular weight 154), a mycotoxin generally produced by Penicillium spp was detected in the culture extract. The dissociation pattern of protonated patulin ([M + H]+) molecule consists essentially of four peaks and is shown in Scheme 1A. Protonated patulin molecules give more informative and more intense peaks than do deprotonated patulin molecules. The loss of an H2O molecule from the protonated molecule yields m/z 137 [C7H5O3]+, while consecutive releases of CO, H2O and CO from the protonated molecule yield m/z 127 [C6H4O3]+, 109 [C6H4O2]+ and 81 [C5H5O]+, respectively.

![Fig. 1. Total ion chromatogram of culture extract of Penicillium setosum (A) and (B) positive and negative mode of colony agar plug method (C) and (D) positive and negative mode of culture broth extract method.](image-url)
3.2.2. Product ion mass spectra of flavonoid compounds

The general structure of flavonoids has a C15 skeleton consisting of two phenyl rings (A and B) and a heterocyclic ring (C) containing oxygen (Scheme 2). Based on the position of B ring and degree of unsaturation and oxidation of the C ring, flavonoids are grouped into several sub-classes, such as flavones, isoflavones, flavonols, flavanones, catechins, and anthocyanins. Depending on the structure of each flavonoid, the product ion fragmentation pattern should be different. In this study, the unique product ions of each flavonoid was obtained and identified through MS/MS spectra. The principle fragmentation of the flavonoid involved the C-ring carbon-carbon bond breakage, resulting in the product ions i,j A and i,j B termed as retro Diels-Alder (RDA). The product ions contain the intact A or B ring and part of the C ring. The most important RDA fragmentations considered in flavones and flavonols are the cleavage of C ring at the positions 0/2, 0/4, 1/2 and 1/3 (Hughes et al., 2001). According to a low-energy CID study, certain pathways are

Table 1

| Sl. no. | Name             | Retention Time | Precursor ion (m/z) | Elemental composition | Polarity | Previous report from fungal origin |
|--------|------------------|----------------|---------------------|-----------------------|----------|-----------------------------------|
|        |                  | Culture extract | Colony plug extract | Culture extract        | Colony plug extract |                                    |
| 1      | Kaempferol       | 4.5            | 4.6                 | 287.0548              | 287.0565  | C15H11O6 [M + H]+                 | (Huang et al., 2014) |
| 2      | Quercetin        | 5.4*           | —                   | 301.0370              | —         | C15H9O7 [M + H]+                  | (Ebada et al., 2016) |
| 3      | Quercetin acetate| 5.1            | 3.7                 | 345.0574              | 345.0590  | C17H13O6 [M + H]+                 | No reports           |
| 4      | Luteolin         | 4.3            | 4.35                | 287.0567              | 287.0557  | C15H11O6 [M – H]                  | (Zhao et al., 2014)  |
| 5      | Dihydroquercetin  | 4.5            | 3.7                 | 305.0651              | 305.0667  | C12H13O7 [M + H]+                 | No reports on these compounds |
| 6      | Dihydromyricetin  | 4.4            | —                   | 321.0519              | —         | C13H13O6 [M + H]+                 | No reports           |
| 7      | Quinalizarin      | 3.0            | 3.62                | 273.0389              | 273.0406  | C16H14O5 [M + H]+                 | (Fouillaud et al., 2016) |
| 8      | Altersolanol A    | —              | 4.4                 | —                     | 337.0932  | C16H14O5 [M – H]                  | (Teiten et al., 2013) |
| 9      | Isorhamnetin      | 2.6            | 2.7                 | 223.0573              | 223.0593  | C11H10O5 [M + H]+                 | No reports           |
| 10     | Andrastin D       | 7.5            | 7.9                 | 429.2634              | 429.2636  | C20H22O5 [M + H]+                 | (Visagie et al., 2016) |
| 11     | Citromycetin      | 3.09           | 3.4                 | 291.0497              | 291.0509  | C14H11O7 [M + H]+                 | (Visagie et al., 2016) |
| 12     | Patulin           | 2.6            | 2.9                 | 153.0186              | 153.0181  | C6H9O4 [M – H]                    | (Capon et al., 2007) |
|        |                  |                |                     |                       |           | Houbraken et al., 2012            | Others               |
| 13     | 6-deoxy erythronolide B | 9.06     | —                   | 367.2772              | —         | C22H20O5 [M – H]                  | No reports on these compounds |
| 14     | Vanillic acid     | 2.5            | 2.7                 | 169.0491              | 169.0506  | C8H6O4 [M + H]+                   | No reports           |
| 15     | 2-Dehydro-3-deoxy-Darabino-heptonate 7-phosphate (DAHP) | 8.2 | — | 289.0327 | — | C7H13O10P [M + H]+ | No reports |

* fraction.

Scheme 1. A. Product ion mass spectra of patulin ([M + H]+) molecule from P. setosum culture extract, obtained at a collision energy of 17 eV (B) GC-MS spectrum of patulin.

3.2.2. Product ion mass spectra of flavonoid compounds

The general structure of flavonoids has a C15 skeleton consisting of two phenyl rings (A and B) and a heterocyclic ring (C) containing oxygen (Scheme 2). Based on the position of B ring and degree of unsaturation and oxidation of the C ring, flavonoids are grouped into several sub-classes, such as flavones, isoflavones, flavonols, flavanones, catechins, and anthocyanins. Depending on the structure of each flavonoid, the product ion fragmentation pattern should be different. In this study, the unique product ions of each flavonoid was obtained and identified through MS/MS spectra. The principle fragmentation of the flavonoid involved the C-ring carbon-carbon bond breakage, resulting in the product ions i,j A and i,j B termed as retro Diels-Alder (RDA). The product ions contain the intact A or B ring and part of the C ring. The most important RDA fragmentations considered in flavones and flavonols are the cleavage of C ring at the positions 0/2, 0/4, 1/2 and 1/3 (Hughes et al., 2001). According to a low-energy CID study, certain pathways are
prominently found in some groups of flavonoids, thereby different classes can be distinguished from each other with respect to the RDA pathway (Cuyckens and Claeys, 2004; Pinheiro and Justino, 2010). This information is essential for identifying the flavonoids in different biological material like plant extract, microbial culture extract, effluents, juices etc. Flavonoids identified from P. setosum represents four subclasses, flavanol, flavone, dihydroflavonol and anthocyanidin. Among these compounds, quercetin and dihydroquercetin attract much attention because of their high biological activity.

The product ion mass spectrum of deprotonated quercetin [M - H]^− consists essentially of four peaks and is shown in Scheme 3. The base peak, m/z 151 (C_{16}H_{12}O_{6}), is formed by fragmentation of $^{1,2}_A$. The ion of m/z 107 (C_{16}H_{12}O_{6}), occurs by fragmentation of $^{0,4}_A$. The ions of m/z 121 and 179 are complementary and may be formed by cleavage of the C2 to C3 bond in the parent ion. The ion of m/z 121 contains B ring whereas that of m/z 179 contains A ring. The product ion m/z 273 arise due to the loss of CO from quercetin m/z 301. Furthermore, the loss of CO and CO$_2$ from product ion m/z 273 generates product ions m/z 245 and m/z 229 respectively (Hughes et al., 2001; March et al., 2006). No other product ions were observed from other fragmentation processes.

In the full scan spectrum of the positive ionization mode, a peak at m/z 305.0651 at a retention time of 3.7 was obtained and subsequent MS/MS spectrum of this peak was analyzed. Based on the product ion mass, the compound was identified as dihydroquercetin (5,7,3',4'-tetrahydroxyflavanol/taxifolin), which is a dihydroflavonol and it was confirmed by comparing the product ion mass reported by

**Scheme 2.** Structure and numbering schemes for carbon atoms and C-ring bonds for flavonoids.

**Scheme 3.** Product ion mass spectra of [M-H]^− of quercetin (m/z 301) from P. setosum culture extract.
Abad-García et al. (2009). Frequently, protonated molecule showed more informative peak of mass product fragmentation pattern. Herewith, dihydroquercetin [M + H]$^+$ consists principally of two peaks and is shown in Scheme 4. The base peak, m/z 153 (C7H4O4), is formed by fragmentation of $^{13}A^+$. The product ion m/z 122 (C6H6O2), arises due to the fragmentation of $^{12}B^-$. These two product ions are more informative and reflected the presence of dihydroflavonols in the P. setosum culture extract. The product ions m/z 286, 256, 212, 185 arise due to the subsequent release of H2O, CH2O, CO2, and CO respectively from m/z 305. The product ion m/z 242 arises due to a loss of CO2 from m/z 286. No other product ions were observed from other fragmentation processes.

Major mass fragments of other flavonoids (luteolin, kaempferol and dihydromyricetin) and other compounds (Quinalizarin, Citromycetin, vanillic acid) were identified from Penicillium setosum culture extract as shown in Scheme 4. The base peak, m/z 153 (C7H4O4), is formed by fragmentation of $^{13}A^+$. The product ion m/z 122 (C6H6O2), arises due to the fragmentation of $^{12}B^-$. These two product ions are more informative and reflected the presence of dihydroflavonols in the P. setosum culture extract. The product ions m/z 286, 256, 212, 185 arise due to the subsequent release of H2O, CH2O, CO2, and CO respectively from m/z 305. The product ion m/z 242 arises due to a loss of CO2 from m/z 286. No other product ions were observed from other fragmentation processes.

3.3. GC-MS analysis

Methyl esters of fatty acid secondary metabolites of P. setosum extracted from colony agar plug and culture broth extraction method were identified using GC-MS. Chromatographically separated peaks were analyzed and the presence of seven fatty acids and hydrocarbon constituents were determined. In addition, the presence of patulin which was identified by LC-MS was once again confirmed. Each identified fatty acid compound in concern with their extraction method, retention time (RT) and structure are presented in Table 2 (Fig. 3). The results revealed that methyl ester of tetradecanoate and hexadecenoate were obtained from both extraction procedure and methyl esters of lauric acid, pentadecanoate, heptadecanoate and 13-docosenoate were recovered from either one of the extraction procedure. Here also the presence of patulin was indisputably identified from both extraction procedure (Scheme 1B).

4. Discussion

The endophytic fungus Penicillium setosum was isolated from a narrowly studied habitat, i.e. the microbiome of Withania somnifera (L) Dunal. This plant has great importance in the Ayurveda system because of its noticeable pharmacological functions contributed by the secondary metabolites. Endophytes also seem to synthesize these secondary metabolites. Environmental conditions and plant physiological conditions together enhance the growth of microbial inhabitants. So, much significance have been given in the exploration of such microorganisms (endophytes) which produce the secondary metabolites that resemble the plant based compounds. P. setosum was found to be a competent biosynthesizer of the secondary metabolites, which fall under the group of flavonoids, as well as some species-specific compounds.

Depending on the asexual and sexual reproductive state, fungi develop certain specialized structures like mycelia, cleistothecia, conidia on their cultivation media. Despite of their ultrastructural morphology, different relevance has seen in their ribosome, protein, and lipid composition (Silva et al., 1998). This may obviously affect the secondary metabolite pattern of a fungi. In this study, colony agar plug and culture broth extraction method were adopted and both were found to be feasible to understand the extrolite profiling of Penicillium spp. Major chemotaxonomic related compounds were detected in both analyses with a negligible difference. However, colony agar plug method was found to be more suitable to scrutinize the chemical nature of secondary metabolites as a preliminary approach. This is because it is inexpensive, less intensive, less time consuming, and versatile to discover many extrolites even from five agar plugs of fungal colony. According to the requisite of further study, massive production and functional characterization of metabolites can be proceeded with culture broth extraction method.

The uniqueness of P. setosum from other members of section Lanata-divaricata was chemotaxonomically confirmed by analyzing the secondary metabolite pattern obtained using high resolution LC-MS. The presence of andrastin D and anthraquinone (quinizarin) in the culture extract define the relation of this isolate to the other members of Penicillium section Lanata-divaricata (Taniwaki et al., 2015; Visagie et al., 2015, 2016), while the discrepancy is shown by the presence of patulin, which is mostly produced by Penicillium spp. of other sections (Frisvad, 2018). So far Penicillium brefeldianum, Eupenicillium javanicum (=Penicillium javanicum), Eupenicillium sp. 1 and sp.2, Penicillium simplicissimum are reported from section Lanata-divaricata for patulin production (Okeke et al., 1993). But these strains are not presently included in the revised list of patulin producers, since, above mentioned strains are not available to the scientific community (Frisvad, 2018). Based on the phylogenetic analyses of our study, P. setosum is most closely related to P. javanicum (CBS 341.487), which is not reported for the production of patulin. So this result characteristically distinguishes P. setosum from P. javanicum and also with other members of section Lanata-divaricata. Patulin is both a mycotoxin and an antibiotic. It can also act as a phytoalexin (Ismaiel et al., 2015), and is reported to possess activity against plant pathogenic fungi and bacteria (Gilliver, 1946). According to Houbraken et al. (2016) the phylogenetic relationships of interspecies
patulin-producing *Penicillium* need to be stated from further in-depth phylogenetic analysis.

Liquid chromatography coupled with mass spectrometry offers the rapid study of flavonoids, their ion chemistry, and the elucidation of structure even at low concentration in the extract (Kumar, 2017). The biosynthesizing ability of flavonoids is prominently restricted to the plants, with a few exceptions in animals and fungi (Cuyckens and Claeys, 2004). In this study, the occurrence of potent biologically active compounds such as quercetin, dihydroquercetin and other related flavonoid compounds were identified through mass spectrometry analysis. Quercetin was first reported from an endophytic fungus, *Nigrospora oryzae* isolated from *Loranthus micranthus* (Ebada et al., 2016). As far as our knowledge, this is the first time quercetin is reported to be produced from a *Pencillium* sp. as well as dihydroquercetin from fungal endophytes.

Structurally quercetin and dihydroquercetin differ only at the double bond between the C2 and C3 carbons on the center (C) ring, but show a significant discrepancy in the biological functions. Quercetin, which is a most conspicuous compound among flavonoids, is fat soluble in nature. While taxifolin is a water-soluble compound, which is getting more attention than quercetin in the health care system. This is because, it is less toxic and non-mutagenic compared to quercetin (Makena et al., 2016).

### Table 2

Identified metabolites from the culture extract of *P. setosum* by GC-MS analysis.

| Sl. no | Compounds* | m/z   | RT (min) | Culture broth | Agar plug extraction |
|-------|-------------|-------|----------|---------------|----------------------|
| 1     | Lauric acid methyl ester (Dodecanoic) | 214.30 | 13.30 | -             |                      |
| 2     | Hexadecanoic (Palmitic) acid methyl ester | 256.40 | -      | 17.53         |                      |
| 3     | Patulin     | 154.02 | 14.44 | 11.93        |                      |
| 4     | Methyl isomyristate (tetradecanoic acid) methyl ester | 242.24 | 17.53 | 20.27        |                      |
| 5     | Methyl (7E)-7-hexadecenoate | 268.42 | 23.00 | 19.85        |                      |
| 6     | Methyl 14-methylpentadecanoate | 270.45 | 21.42 | -            |                      |
| 7     | Methyl isostearate (16-methylheptadecanoic acid) methyl ester | 298.50 | - | 19.83        |                      |
| 8     | 13-Docosenamide | 337.55 | - | 25.61       |                      |

* Compound structures represented in Fig. 3.
2009). It can be effectively used in cancer therapy to prevent the development and proliferation of cancer (Zhang et al., 2013). Conversely in nature, dihydroquercetin is less abundant than quercetin as well, there are no reports on their microbial origin.

According to Hardoim et al. (2015), plant mutualists are largely enriched with diverse genes that are involved in the biosynthetic processes of the secondary metabolites and these compounds function in relation to the plants. The identified secondary metabolite pattern of P. setosum is in agreement with the plant-mutualistic relation. This particular fungus-host interaction definitely causes alterations in the phytochemical profiling of both partners (Ludwig-Müller, 2015).

Some of the endophytic fungi isolated from medicinal plants are reported to produce phenolic and flavonoid compounds (Lunardelli Negreiros de Carvalho et al., 2016). The role of flavonoids in endophytic fungi has not yet been revealed, however the probable explanation for this would be, the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the internal compartments of plants, making them unfavorable niches for the survival of endophytic microorganisms (Hardoim et al., 2015). To stand against this situation, many of the plant mutualists metabolize plant stress related compounds. The mutualistic and/or symbiotic relationships between the fungus and host plant habitually alter the gene expressions of both partners. So further in-depth studies on these compounds in detail is very important to resolve their role in the mutualistic interaction and also in relation with their ambient environmental condition.

Generally, fatty acids and hydrocarbons are produced by Penicillium species. Fatty acid and hydrocarbon secondary metabolites were also considered as an increasingly useful tool for the identification and characterization of a fungi. However, a few reports from genus Penicillium are only available in this concept (Silva et al., 1998; Ghanbari et al., 2014). Identified fatty acids and hydrocarbons found in P. setosum were similar with the findings of Silva et al. (1998). The methyl esters of hydrocarbons, such as hexadecenoate, pentadecanoate and fatty acids such as lauric, palmitic, tetradecanoic, heptadecanoic acids were produced by Penicillium setosum.

Upon the large necessity of flavonoid compounds, plants were found as insufficient to meet the prerequisite amount of specific flavonoids. To cope up with the needling amount of flavonoids, researches are enforced to find an alternate or better source for flavonoid production through metabolic engineering and synthetic biology methods. In this scenario, preliminary identified polyphenol producing Penicillium setosum can be considered as a prospective isolate and its applications can be extensively imparted in different fields such as cuisines, cosmetics, and pharmaceutical firms.

5. Conclusion

Colony agar plug and culture broth extraction method remarkably validate the metabolic capabilities of Penicillium setosum for the production of polyphenolic compounds. This again proved the ability of the endophyte to produce similar compounds as present in plants. Further study on gene expression is needed to confirm the flavonoid production pathway in the fungus.

Declarations

Author contribution statement

Tijith K George: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed re-agents, materials, analysis tools or data; Wrote the paper.

Dineep Devadasan: Contributed re-agents, materials, analysis tools or data.

Jisha M. S: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study has been deposited at GenBank under the accession number KT852579.

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