

ORIGINAL ARTICLE

Piperine production by endophytic fungus *Periconia* sp. isolated from *Piper longum* L.

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The endophytic fungus *Periconia* sp. produces piperine (5-(3, 4-methylenedioxyphenyl)-1-piperidinopent-2, 4-dien-1-one) under liquid culture. This is the first report of the alternative source for this chemical other than its host, *Piper longum*. The highly functionalized fungus-derived piperine exhibits strong antimycobacterial activity against *Mycobacterium tuberculosis* and *M. smegmatis* with minimum inhibitory concentrations of 1.74 and 2.62 $\mu\text{g ml}^{-1}$, respectively. The compound was crystallized and the structure was elucidated by single-crystal X-ray crystallography. This finding is of significance as piperine is a potential cancer preventative agent. It is reaffirmed by this report that important pharmaceuticals can be produced by endophytic microbes, and these molecules appear to be mimetic to their host origin. Therefore, we can enhance the bioactive principles of medicinal plants by isolating and identifying the endophytes, thereby showing the importance of preserving the biodiversity of these plants.

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INTRODUCTION

With the discovery of taxol from the endophytic fungus *Taxomyces andreanae* in pacific yew (*Taxus brevifolia*) by Stierle *et al.*,¹ a new era of research in endophytic biology was opened. Until that time, leaf material of the pacific yew was the only source of 'Taxol' known. Since then, other authors have established that certain endophytes can produce an array of chemicals similar to those found in their hosts.² This has encouraged researchers to select medicinal plants for endophytic study that have a known therapeutic as well as ethnobotanical history and that contain a well-characterized set of chemicals. Some well-known systems are *Hypericum perforatum*–*Hypericin*, *Catharanthus roseus*–*vincristine/vinblastine* and *Camptotheca foetida*–*camptothecin*, etc.^{3–6} In the light of these works, we have made efforts to isolate endophytic fungi and bacteria from well-known spice pepper (*Piper longum* L.), which contains a very potent alkaloid 'piperine'. The dried unripe fruit contains piperine (5–9%), volatile oil (1–2.5%), pungent resin (6.0%), piperidine and starch (about 30%). The volatile oil contains mainly 1-phyllandrene and caryophyllene. Piperine content is 3–5% (on dry weight basis) in *P. longum* and varies within different species of piper plant. Piperine is a piperidine derivative with multiple pharmacological and physiological activities.^{7–8} The traditional uses include analgesic, antipyretic, antidepressant,⁹ neuroprotective,^{10,11} anti-inflammatory,^{12,13} antioxidant,^{14,15} anticonvulsant, antibacterial, antitumor¹⁶ and hepatoprotective activities.^{17–19} Piperine provides protection against seizures in epilepsy²⁰ and has

been shown to enhance the bioavailability of several drugs, such as sulfadiazine, tetracycline, streptomycin,²¹ rifampicin, pyrazinamide, isoniazid,²² ethambutol²³ and phenytoin.²⁴ The pepper plant has formed the basis of many traditional formulations that have been in existence for thousands of years in the Indian system of medicine called 'Ayurveda'. The pepper and its phyto-constituents have an essential role in healthcare in the several other traditional systems of medicine in many other countries.⁷ Thus, given the importance of piperine and other related alkaloids, we attempted to isolate endophytic fungi from *P. longum* plants with the objective to screen and isolate strains that have the potential to produce piperine and related alkaloids as an alternative source other than their host. To date, a large number of bioactive compounds from endophytic microbes in an array of plants have been extracted, isolated and characterized in details with extensive references in several review articles.^{25–30} However, very few reports on endophytic fungi concern *P. longum* itself and these mainly focus on the potential of some endophytic bacterial strains to show activity against phytopathogens; such as *Phytophthora capsici*.³¹ The only report other than the present one is the isolation and characterization of piperine from an *Ulocladium* sp.,³² but this report does not give details about the biology and ecology of the fungus.

Here, we report for the first time that an endophytic *Periconia* sp. isolated from *P. longum* is able to produce piperine and as a result, the fungus has antimycobacterial activity.

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MATERIALS AND METHODS

Plant samples

A random sample of 10 *P. longum* L. plants were taken from the campus of Banaras Hindu University, Varanasi (25.5° N 82.9° E, elevation 279 ft per 85 m) in December 2009. Fresh plant samples of healthy and symptomless leaves, stem and root were collected. The cut ends were wrapped with parafilm before they were placed into sterile polythene bags for further isolation procedures in the laboratory.

Surface treatment, isolation and culture conditions

A total of 45 tissue segments, 5 from each of 3 leaf, stem and root samples from each of 10 selected plants were taken and surface treated by immersion in 70% ethanol for 1 min, 5% NaOCl for 5 min, 96% ethanol for 0.5 min, followed by three rinses in sterile distilled water. All sample tissues were then placed onto separate petri dishes (60 mm) containing potato dextrose agar supplemented with 250 mg l⁻¹ oxytetracycline hydrochloride (Terramycin, Pfizer, Mumbai, India) for isolating endophytic fungi.³³ S-agar medium (containing per litre: 10 g dextrose, 4.0 g casein hydrolysate, 0.5 g K₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, 10 mg ferric citrate, 0.01 mg CoSO₄·7H₂O, 0.1 mg CuSO₄·5H₂O, 1.5 mg H₃BO₃, 0.8 mg MnSO₄·H₂O, 0.2 mg (NH₄)₆Mo₇O₂₄·4H₂O, 0.6 mg ZnSO₄·7H₂O and 6.0 g agar) for isolating actinomycetes³⁴ and nutrient agar for isolating endophytic bacteria. All petri dishes were sealed with sterile parafilm to protect them from contamination during repeated handling while examining endophytes, and from desiccation. The plates were incubated at 26 ± 2°C and 98% relative humidity (under 12 h fluorescent light/12 h dark light), enclosed in translucent white-cover plastic boxes, in a biological oxygen demand cum humidity incubator (Remi, Mumbai, India; VDF-265) for 25 days. Fungi that grew from the tissue fragments were subcultured onto fresh potato dextrose agar plates. Isolates that failed to produce reproductive structures were subcultured onto 2% malt yeast extract (Oxoid, Mumbai, India; malt extract L₃₉ 20 g l⁻¹, and agar 20 g l⁻¹) supplemented with streptomycin 250 mg l⁻¹ in a petri dish (50 mm) containing a thin strip of autoclaved host tissue to encourage the sporulation. Cultures that failed to sporulate even under these conditions were considered sterile.

Identification of endophytic *Periconia* sp.

All microscopic analysis was on the basis of observations with an eclipse E600 research microscope (Nikon, Tokyo, Japan) attached with coolpix 3.34 MP digital camera. Scanning electron microscopy (SEM) was performed on *Periconia* sp. using the techniques described earlier by Ezra *et al.*³⁵ The fungal biomass was dried and a gold coating was done using sputtering technique and examined with a JEOL6100 SEM (JEOL Ltd., Tokyo, Japan). However, because the conidia and conidiophores of this fungus were very fragile and easily disrupted, the fungus was subjected to environmental SEM, which retains the sporophores intact (spores attached), and images were recorded with a Philips XL 30 ESEM FEG (Philips, IL, USA; see Figure 1). A gaseous secondary electron detector was applied with a spot size of 3 at 15 kV. The temperature was 4 °C, with a chamber pressure that ranged from 5 to 6 Torr, providing humidity up to 100% around the sample. Identification of endophytic *Periconia* sp. was performed with the help of the characteristic features and standard taxonomic manuals.^{36–41}

Fermentation, extraction and characterization of fungal piperine

The piperine was isolated from 4-week-old shake cultures of endophytic *Periconia* sp. grown in 2 l of potato dextrose broth flasks at 25 ± 2 °C. The fungal biomass was removed from flasks by filtration and the culture fluid was extracted twice with equal volumes of ethyl acetate. The ethyl acetate fraction was dried by flash evaporation and the yield of crude material was up to 5.4 g. This material was dissolved in a minimal volume of chloroform and was subjected to silica gel (size 60–120 mesh, Ranbaxy, Bangalore, India) column chromatography on a 3 × 30-cm column. The organic solvent extract was applied to the column and eluted using 500 ml of ethyl acetate/methanol/water (10:1:0.1 (v/v)). The first 100 ml eluted from the column was discarded and the next 400 ml eluted from the column (possessing bioactivity) was taken to dryness (1.3 g), redissolved in 2.0 ml of chloroform, and placed on a second silica gel column (identical in size to the first column). It was eluted with *n*-hexane/ethyl-acetate/methanol/water (2:3:2:3 (v/v)). Fractions (20 ml) were collected and the bioactivity appeared at 90–120 ml. The residue was dissolved in a minimum quantity of chloroform. Adding diethyl ether to this solution resulted in immediate appearance

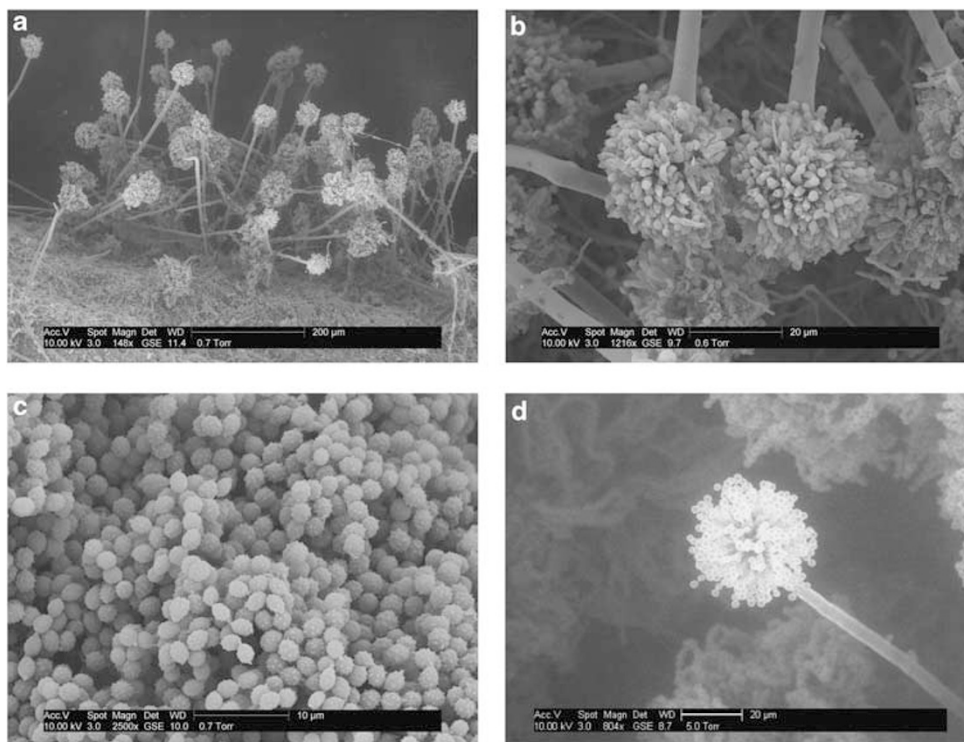


Figure 1 SEM image of *Periconia* sp. showing conidiophores and hyphae (a) and conidiophores with attached conidiogenous cells (b), warty conidia (c) and single conidiophore with an assemblage of spores (s) as taken by environmental SEM (d). Magnifications are shown by the bars on each illustration.

and separation of needle-shaped crystals of piperine (yield ~750 mg). The compound was purified by repeated crystallization to obtain shining yellow needles of piperine. The thin-layer chromatogram of the isolated compound showed a single peak at R_f 0.33 and its UV absorption spectrum exactly overlapped that of the standard piperine. Furthermore, detection of piperine was made by its fluorescence at 340 nm and by spraying the plate with vanillin sulfuric acid spray reagent followed by gentle heating, resulting in a yellowish color spot.⁴² The calculated best-fit empirical formula of this fungal compound 'piperine' is $C_{17}H_{19}NO_3$ (5-(3,4-Methylenedioxyphenyl)-1-piperidinopent-2,4-dien-1-one) and the calculated MW was $285.33 \text{ g mol}^{-1}$.

Single-crystal X-ray crystallography of piperine

The purified extract when dissolved in minimum chloroform, and by adding diethyl ether to this solution, resulted in the formation of numerous small needle-shaped yellowish crystals of piperine. Using a nylon loop, a suitable specimen (crystal of size $0.60 \times 0.50 \times 0.40 \text{ mm}^3$) was chosen and mounted on a Bruker X8 APEX II diffractometer (Bruker AXS, Madison, WI, USA) (MoK α radiation) and cooled to -100°C . Data collection and reduction were done using Bruker APEX2 (v. 1.0–22 user manual, Bruker AXS, 2004) and SAINT + (v. 6.02 user manual, Bruker AXS, 1999) software packages. An empirical absorption correction was applied with SADABS (GM Scheldrick, Program for Empirical Absorption Correction of Area Detector Data, University of Göttingen, 1996). Structures were solved by direct methods and refined on F2 by full-matrix least-squares techniques using the SHELXTL (G.M. Scheldrick, v. 5.10 Bruker AXS, 1999d) software package. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were found in a difference fourier map and refined isotropically. Overall, 15 940 reflections were collected, 4430 of which were symmetry independent $R_{\text{int}}=0.0275$; and final R factor was 4.13% with 3305 'strong' reflections ($F_o > 4\sigma(F_o)$). The crystal belongs to monoclinic space group P 2(1)/n, with $a=8.6712(6)$, $b=13.4428(8)$ and $c=12.9744(9) \text{ \AA}$. The molecular structure was determined by single-crystal X-ray diffraction (see Figure 2 and supplementary data).

Antimycobacterial bioassay

Bioassays using the disc diffusion method were performed against two different strains of mycobacteria, a clinical multidrug resistant strain of *Mycobacterium tuberculosis* and an atypical mycobacterium, *M. smegmetis*. A uniform lawn for each of strains was prepared by streaking on molten middle brook B7H11 agar medium (Himedia, Mumbai, India) for *Mycobacterium* in fresh sterilized petri

plates (9 mm, Borosil, Bonn, Germany). An aliquot of 2.0 ml fungal piperine of four different concentrations (2, 5, 10, 15 mg ml^{-1}) was placed onto a sterile disc and the solvent (that is, chloroform) allowed to evaporate to dryness on the surface in a decontaminated laminar hood for 15 min. The zone of inhibition surrounding the disc on a bacterial plate was recorded in three replicates by incubating the plates of *M. tuberculosis* for 20 days and those of *M. smegmetis* for 5–7 days. Microplate alamar blue bioassay was performed with the purified fungal piperine to determine the MICs. A culture suspension of test strains was diluted upto 10^5 cfu ml^{-1} in 1:25 MB7H9 GC broth supplemented with glycerol and casitone. To determine the MIC values, different piperine concentrations were prepared from 0.05 to 6 mg ml^{-1} . 100 μl test strains of *M. tuberculosis* and *M. smegmetis* inoculums were added into a 96-well microtiter plate. After incubation for about 7 days, 50 μl of freshly prepared 1:1 mixture of alamar blue reagent and 10% tween 80 were added to each control well and incubated at 37°C .⁴³ If the well turned pink, indicating growth, the reagent mixture was added to all wells. The MIC was defined as the lowest sample concentration that prevented a color change from blue to pink.

Statistical analysis and data reduction

The inhibition experiments were performed using plates as replicates and values given are means of these. Data were tested for normality, and a one-factor analysis of variance was conducted to determine whether the amount of inhibition differed between piperine concentration treatments. Means were separated by the Tukey HSD test.

RESULTS AND DISCUSSION

A total of 42 isolates of filamentous fungi, 17 isolates of bacteria and 5 actinomycetes were isolated and identified as microbial endophytes residing in the living tissues of *P. longum* plants. Among the dominant fungal isolates were species of *Pestalotiopsis*, *Fusarium*, *Colletotrichum*, *Arthobotrys*, *Arthrinium*, *Cladosporium* and *Aspergillus*, whereas among bacteria and actinomycetes, dominant isolates recovered were species of *Streptomyces*, *Burkholderia*, *Pseudomonas*, *Nocardiopsis*, *Bacillus*, *Acetobacter*, *Enterobacter*, *Serratia* and *Paenibacillus*, etc. (unpublished data, manuscript in preparation). After isolating endophytic microbes from *P. longum* plant, we initiated a comprehensive screening program to find potential endophytes having activity against *Mycobacterium* spp. in a disc diffusion agar bioassay, and those that did were selected for further study to identify and characterize the bioactive compounds. One isolate of endophytic *Periconia* sp. showed very significant activity against *Mycobacterium* spp. This particular strain was isolated from leaf tissues of *P. longum* and was recorded as an incidental rare strain (1/42 isolates). Members of *Periconia* have a very wide host range as an endophyte and have been reported from several other pharmaceutically important plants, such as *Azadirachta indica*,³³ *Eucalyptus citriodora*,⁴⁴ *Torriya grandifolia* etc.⁴⁵ The SEM study of this strain showed very prominent characteristic long conidiophores, dark, long, stout and shortly branched near the apex, bearing attached groups of conidiogenous cells at their tips (Figures 1a and b). As the chains of conidia are very fragile, only a few conidia are visibly attached on conidiogenous cells (Figure 1b), which is why the environmental SEM was performed to scan the head of conidiophores with attached conidia (Figure 1d). The individual conidia were warty (Figure 1c) and commonly bore a tubular segment of the conidiophores attached to them. This isolated *Periconia* sp. was deposited in the fungal type culture collection (f-TCC), Banaras Hindu University, India (f-TCCPERI-2894). A biologically active fraction with very significant antimycobacterial activity was screened and identified as 'piperine', a well-known alkaloid of the pepper plant and the chemical responsible for the pungency of the pepper spices. This was surprising as the only source known for this alkaloid is the host plant itself, but this is the first report of this compound being produced by an

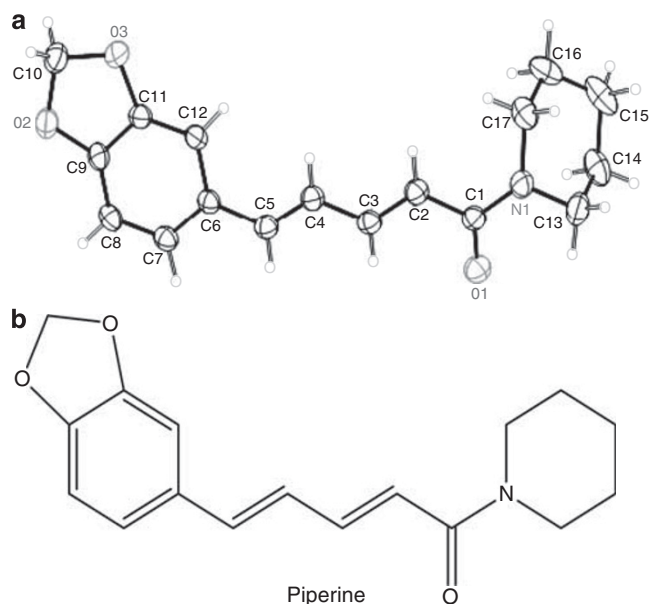


Figure 2 Single-crystal structure of piperine (a), and the molecular structural formula (b) on the basis of single-crystal X-ray crystallography. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

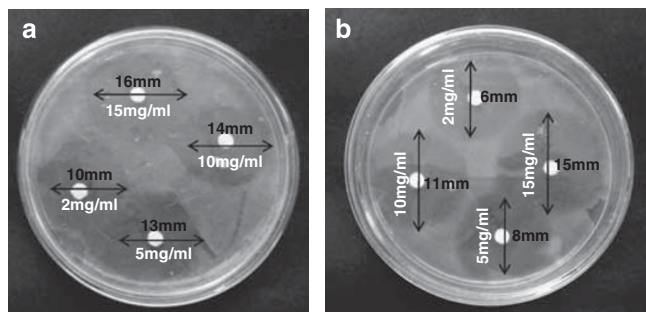


Figure 3 The disc diffusion bioassay against the *Mycobacterium tuberculosis* (a) and *M. smegmatis* (b). Four different concentrations (2, 5, 10, 15 mg ml⁻¹) of purified fungal piperine show different activity and showed that as the concentration increased, the inhibition zones were also widened.

endophytic fungus from the pepper plant. The piperine is an alkaloid and a piperidine derivative of the vanilloid family of compounds and is weakly basic. It is essentially tasteless at first but has a burning aftertaste. The compound is believed to have the ability to enhance activity for some nutrients and drugs and has alleged anti-inflammatory capability and several other pharmacological activities.⁷ Additionally, there are several other reports available that confirm the activity of piperine against *Mycobacterium* spp.; such as its role in inhibition of Rv1258c, a putative multidrug efflux pump of *M. tuberculosis*.⁴⁶ The piperine we obtained from the endophytic *Periconia* sp. was naturally functional and the spectroscopic study of this compound revealed 99% similarity with piperine from the host itself. Although the yield of the purified piperine is very low (~750 mg), when dissolved in a mixture of chloroform/diethyl ether, the yellowish needle-shaped crystals were obtained. Often, crystals were formed only when the extracts were highly purified and no additional components were mixed with them. These crystals were then subjected to single-crystal X-ray crystallography (Figure 2a), which were in close proximity to the natural/parent piperine from the host plant. The molecular structure was obtained with the details of parameters as obtained from crystallography (Figure 2b, supplementary data). This fungal piperine shows very promising potential as an antimycobacterial agent as it showed prominent growth inhibitory activity against two strains of *Mycobacterium* spp. (Figure 3). In *M. tuberculosis*, all treatments differed from each other and there was a clear trend of increasing inhibition with increased fungal piperine concentration ($F_{3,8}=248.3, P<0.001$). A very similar pattern was seen with *M. smegmatis* ($F_{3,8}=452.6, P<0.001$). In general, stronger inhibition was observed with *M. tuberculosis* (Figure 4a) than with *M. smegmatis* (Figure 4b). This general trend was also confirmed by the determination of MICs against these two test strains that ranged from 1.74 for *M. tuberculosis* to 2.62 $\mu\text{g ml}^{-1}$ for *M. smegmatis* (Figure 3). The mycobacterial bioassay established by us and the MICs by the alamar blue assay provided a very clear indication of antimycobacterial activity with purified fungal piperine. These bioactivities confirm that the fungal piperine is as active and functional as piperine from the host plant.

Periconia spp. isolated from *Torriya grandifolia* was also reported to produce taxol,⁴⁵ thus it seems that member of this genus would be well worth considering in 'bio-prospecting' for novel pharmaceuticals. However, the ability of endophytes to produce these chemicals raises several intriguing questions that have yet to be answered, including, whether the compound is first synthesized by the plant or by the fungus and whether there is a transfer of genetic information between the two. Furthermore, we need to determine whether microbes are

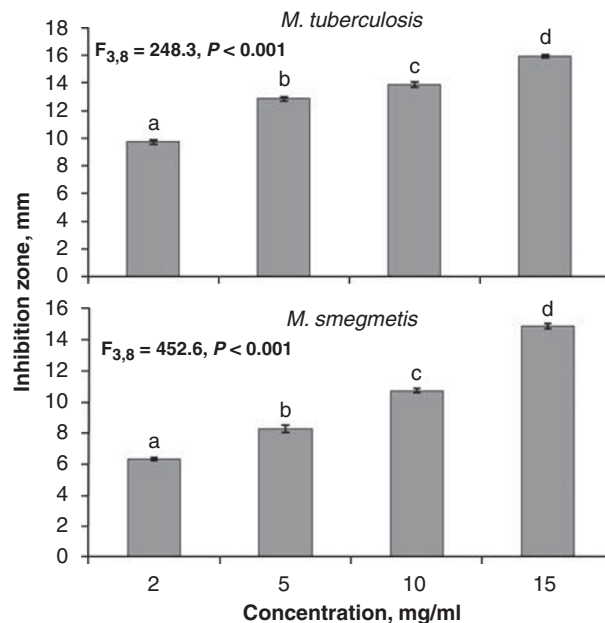


Figure 4 The inhibition pattern of the two strains caused by different concentrations of piperine.

capable of communicating with each other within the plant and whether chemical production occurs as a result of interaction between microbes. It remains a question of further research as to how and why this particular strain of *Periconia* sp. produces an alkaloid identical to that derived from its host plant. As suggested by Tan and Zou,²⁵ there may be the possibility of genetic recombination between endophyte and the host in the course of evolutionary symbiosis, and if this happens, then the exact mechanism should be resolved urgently.

The potential of endophytic microbes in synthesizing bioactive compounds within their host plants is a very significant aspect of the host endophyte interaction.^{1,29,47-49} The discovery of fungal endophytes with piperine-producing potential may have significant scientific and industrial applications. However, there are several factors associated in its industrial implications; one major factor is the metabolic regulation of fungus in axenic culture. It is observed that after second or third successive generations, this potential was substantially attenuated, and the production of interested molecule dropped below the limit of detection.⁶ This might be assumed due to lack of host stimuli in axenic cultures, or rapid inactivation or transformation of the interested molecule. Meanwhile, if endophytes can produce the same rare and important bioactive compounds as their host plants, this would not only reduce the need to harvest slow-growing and possibly rare plants, but also help to preserve the world's ever-diminishing biodiversity. Furthermore, it is recognized that a microbial source of a high-value product may be easier and more economical to produce effectively, thereby reducing its market price.

Thus, in summary, our phytochemical findings provide a basis for exploiting endophytic *Periconia* sp. as a new alternative source for piperine and other related analogs from *P. longum* plants to meet the growing challenge of drug resistance against mycobacteria and other potent human pathogens. We may suggest that these results could be exploited to use piperine alone and/or in combination with other available drugs in an attempt to overcome drug resistance in *Mycobacterium* spp. specifically.

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