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Original Research

Azaphilone pigments from *Penicillium maximae*

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Abstract

Azaphilones are an important class of secondary metabolites derived from the polyketide pathway. These metabolites have an intense yellow-orange-red pallet of colour and have been studied as promising natural pigments for use in the food industry. On a screening with 30 fungi isolated from soil and cultivated in different conditions, a fungal species furnished an orange extract containing a very major metabolite, as determined by HPLC analysis. The species was identified *Penicillium maximae* by molecular biology and morphological studies, and it was selected to determine the major secondary metabolites responsible by the colour present in the extract. The dichloromethane extract prepared from *P. maximae* was column chromatographed leading to the isolation of sclerotiorin (1**,** 16.36 % yield), eupenicilazaphilone B (2, 0.77 % yield) and sclerotioramin (3, 1.05 % yield). Sclerotiorin yield is encouraging for industrial production. These azaphilones of orange, yellow and red colour respectively, are the major metabolites produced by *P. maximae* on a culture medium containing peptone as a differential. High concentration of sucrose and the presence of iron ions in the culture medium did not improve the metabolites yield. *P. maximae* is, therefore, a good source of pigments for potential food, pharmaceutical and dyeing applications.

Keywords: Food industry, Microbial metabolites, Natural dye, Screening, Soil fungi

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1. Introduction

Fungi produce small molecules with diverse chemical structures to mediate processes such as intra and inter-species communication and self-defence towards biotic and abiotic adversities present in their natural habitats (Deshmukh et al., 2024; Oliveira et al., 2022). Research in this

area has gained strength over the last decades with the decrease in the cost of genome sequence analysis and the development of novel approaches to mine fungal metabolites for pharmaceutical and food and other industrial applications (Kenshole et al., 2021). The advancement of omics techniques has also sped up the development of research with fungal metabolites (Saini et al., 2024; Takahashi et al., 2024).

One of the most common fungal genera is *Penicillium*, which can be found in different ecosystems, having an important role in pharmaceutical area as well as in the environmental balance as decomposers and bioremediation agents (Martins et al., 2016; Saxena et al., 2024; Wang et al., 2010). *Penicillium* species have been described as outstanding producers of metabolites from diversified biosynthetic pathways (Bazioli et al., 2017) and with several medicinal applications such as antiviral, anticancer, immunosuppressant, and neuroprotective activities (Toghueo & Boyom, 2020). Among 354 species of *Penicillium*, *P. maximae* section *Sclerotiora* can be considered an under-reported taxon (Houbraken et al., 2020). This species is reported to grow well in Czapek Yeast Autolysate (CYA) Agar at 30 °C, Malt Extract Agar (MEA) and Yeast Extract Sucrose (YES) Agar and moderately in Dichloran 18% glycerol (DG18) agar, being distinguishable from closely related species *P. sclerotiorum* by the absence of sclerotia (Visagie et al., 2013). *P. maximae* has already been isolated from marine environment in Korea (Park et al., 2019) and as an endophyte capable of producing azaphilone alkaloids (Koyanagi et al., 2021).

Azaphilones are fungi-exclusive secondary metabolites derived from the polyketide pathway with a pyrone-quinone structure and a chiral quaternary carbon atom (Pimenta et al., 2021). The intense yellow-orange-red pallet of colour of azaphilones makes them promising pigments for food and textile applications to replace synthetic dyes (Morales-Oyervides et al., 2020; Venil et al., 2020). In addition to their application as pigments, azaphilones possess antimicrobial, antifungal, antiviral, antioxidant, cytotoxic/antitumor, nematicide and anti-inflammatory activities, which are likely a consequence of their reactions with amino groups present in amino and nucleic acids (Chen et al., 2020). The bioactivity of these pigments adds an important functional appeal to their incorporation in foods, beyond the dyeing property.

This work describes the screening of terrestrial fungal strains capable of producing coloured pigments and the isolation and identification of three azaphilones from *P. maximae*, an understudied species of *Penicillium*.

2. Materials and methods

Thin layer chromatography (TLC) plates were obtained from Silicycle (Canada); silica gel for column chromatography (230-400 mesh) was purchased from Merck (Germany). Analytical grade solvents, chemicals and culture media were purchased from VETEC and KASVI (Brazil), and HIMEDIA (India). Deuterated solvents were acquired from Sigma Aldrich (USA) and Cambridge Isotope Laboratories (USA).

2.1 Isolation and screening of fungal species

Soil samples (1 g) were suspended in aqueous NaCl (0.85 m/v, 4.5 mL) and serially diluted to the final concentration of 10^{-3} gL^{-1}. Aliquots of 100 µL were added to Petri dishes containing bacteriological agar (15.0 gL^{-1}), peptone (50.0 gL^{-1}), yeast extract (50.0 gL^{-1}), and rose bengal (0.033 gL^{-1}). After 24 to 72 h, individual colonies were transferred successively to freshly sterilized agar until recovery of 30 pure cultures that were stored in PDA at -80 °C. A screening was carried out with 30 fungal species, that were cultivated in 100 mL of three different culture

media, as following described. Culture medium 1: Yeast extract (12.0 gL^{-1}), Sucrose (150.0 gL^{-1}), $MgSO_4.7H_2O$ (0.5 gL^{-1}), and Glucose (20.0 gL^{-1}). Culture medium 2: Glucose (20.0 gL^{-1}), Bacterial peptone (5.0 gL^{-1}), Yeast extract (3.0 gL^{-1}), and KH_2PO_4 (5.0 gL^{-1}). Culture medium 3: Glucose (20.0 gL^{-1}), Bacterial peptone (5.0 gL^{-1}), Yeast extract (3.0 gL^{-1}), KH_2PO_4 (5.0 gL^{-1}), Fe^{3+} (2.5 gL^{-1}), and Fe^{2+} (2.5 gL^{-1}).

The culture media were distributed to 500 mL Erlenmeyer flasks; the pH was adjusted to 5.12 and the flasks were autoclaved for 15 minutes at 121 ºC. After cooling, the 30 fungal species were individually inoculated to the media in a laminar flow hood, previously sterilized with 70 % ethyl alcohol. Resting growth was awaited, and three flasks were discarded during the process, as the respective fungi suffered contamination during cultivation. After 14 days, 30 mL of ethyl acetate was added to each Erlenmeyer flask, followed by filtration at reduced pressure to separate the mycelium from the liquid phase containing ethyl acetate and broth. The mycelium was then collected, placed in an Erlenmeyer flask, and left immersed in ethyl acetate for further 24 hours. The extraction procedure was carried out in a separation funnel individually for each of 27 fungi. Vigorous stirring was carried out to remove all secondary metabolites from the broth, collecting the organic phase. The process was repeated twice with 30 mL of extracting solvent and the organic phases were combined. The solvent in which the respective mycelium was immersed was separated from the mycelia and added to the organic phase. Finally, the organic phase was taken to rotary evaporation to remove the solvent and obtain the extracts. The mycelia were autoclaved for 15 minutes at 121 ºC and subsequently discarded. A species presenting white mycelia orange-coloured reverse which presented an outstanding HPLC profile was selected for identification and large-scale study.

2.2 Identification of *P. maximae*

Fungal mycelium was transferred to microtubes containing buffer (Tris-HCl 0.05 M, EDTA 0.005 M pH 8.0, NaCl 0.1 M and sodium dodecyl sulphate 1 %) (400 µL). Stainless steel beads were added to improve the extraction. The material was homogenized for 5 min in a Bullet Blender24 and then incubated (60 °C; 30 min). After that, cetyltrimethylammonium bromide buffer (200 µL) and *β*-mercaptoethanol (4 %; 4 µL) were added and the microtubes were stirred and incubated (65 ºC; 30 min). Then, chloroform:isoamilic alcohol (24:1, 570 µL) were added and the microtubes were gently shaken followed by centrifugation at 13,200 rpm (15 min). The aqueous phase was then transferred to new microtubes containing a solution of sodium acetate (3 M, 10 %). After mixing, solutions were centrifuged (13,200 rpm, 10 min) and the supernatant was transferred to microtubes and followed by precipitation with isopropanol. The tubes were centrifuged (13,200 rpm, 10 min). The supernatant was removed, and the DNA pellet was washed twice in 70 % cold ethanol, dried down, and resuspended in 50 µL of Tris-EDTA (Tris-HCl 0.01 M and EDTA 0.001 M). Pure genomic DNA was assayed in NanoDrop ND 1000 (NanoDrop Technologies) and stored at 20 ºC until amplification of the internal transcribed spacer (ITS) region. After DNA extraction, the ITS region was amplified with the universal primers ITS1 and ITS4 (Abrão et al., 2014; White et al., 1990). Successfully amplified PCR products were purified by precipitation with ethanol/ ethylenediaminetetraacetic acid. The sequencing reactions and quality control of individual sequences were performed (Applied Biosystems). Molecular fungal identification was performed as described in Ferreira-Silva et al. (2021). The consensus sequence was submitted to GenBank and assigned an accession number: MZ683178. Phylogenetic analysis was performed in MEGA version X (Kumar et al., 2018). The ITS sequences were aligned using MUSCLE. The alignments were prepared including sequences of all relevant ex-type strains, from sequences obtained from GenBank to ensure an accurate identification. The Bayesian

information criterion was used to identify the most appropriate model of evolution. The molecular identification was in accordance with macroscopic examination and optical microscopy (Visagie et al., 2013).

2.3 Fermentation, extraction, and isolation of secondary metabolites

P. maximae was grown (10 × 1 L) using (gL^{-1}) glucose (20.0), peptone (5.0), yeast extract (3.0), KH_2PO_4 (5.0) at pH 5.65 ± 0.07, at room temperature (25 ± 4 °C). To maximize pigments recovery, the growth was allowed for 35 days. After this period, the mycelia were filtered under vacuum and the broth was exhaustively extracted with dichloromethane (3 × 300 mL CH_2Cl_2/L broth), which promoted higher yield of extract than EtOAc. The solvent was removed to furnish the broth extract. The mycelia were soaked overnight with CH_2Cl_2, the organic fraction was recovered by filtration and the solvent was removed, furnishing the mycelia extract. Broth and mycelia extracts were combined (8.56 g total) and subjected to silica gel column chromatography using hexane, ethyl acetate and methanol gradient. Fractions were combined according to their TLC profiles into nine groups (F1–F9). Group F2 (1.40 g) was constituted by a pure metabolite (1). Groups F7 (0.23 g), and F8 (0.53 g) were further chromatographed to furnish 2 (66.0 mg) and 3 (90 mg), respectively.

2.4 Nuclear Magnetic Resonance (NMR) Analysis

NMR spectra were determined on a Bruker Avance DRX 400 MHz Spectrometer with tetramethylsilane (TMS) as an internal standard. Samples (10 mg) were solubilized in 0.7 mL of chloroform-*d* (1, 3) or methanol-d_4 (2). Chemical shifts (δ) are given in parts per million (ppm) relative to the internal standard TMS. Coupling constants (*J*) are given in hertz (Hz).

3. Results and discussion

Thirty fungal species isolated from soil were cultivated in three different culture media. Culture medium 1 was distinguished by its high sucrose content (150 gL^{-1}) as an additional carbon source. Sucrose is associated with the production of azaphilones (Pimenta et al., 2021). Culture medium 2, in turn, contained peptone, as an additional nitrogen source in its composition. Peptone is an ingredient classically present in culture media aimed at producing pigments, also favoring fungal growth and biomass production (Gomes, 2024; Lucas et al., 2007). Culture medium 3 contained the same ingredients as culture medium 2, with the addition of iron ions. The presence of metals in the culture medium causes abiotic stress, which interferes with metabolic pathways, intensifying or reducing metabolic production (Takahashi et al., 2013). The profiles of the extracts, analyzed by HPLC, showed, in general, low metabolic production, however one species stood out for the production of colored metabolites, which was suppressed in the presence of iron (Fig. 1).

This species was identified and selected for large scale cultivation. The identification was based on the analysis of the ITS region. The nucleotide sequence showed 100 % alignment and 99 % identity with *P. maximae* NRRL 2060 (CBS 134565) type species deposited in GenBank [NR_121343]. The phylogenetic analysis confirmed the distinct clustering with bootstrap support (99 %) of the isolate studied with NRRL 2060 strain (Fig. 2). The identification was confirmed morphologically by the absence of sclerotia, lack of sporulation on CYA, and lack of growth in CYA at 37 °C. *P. maximae* presented white and orange mycelia with orange reverse pigmentation on PDA, fading to orange in the center (Visagie et al., 2013) (Fig. 2).

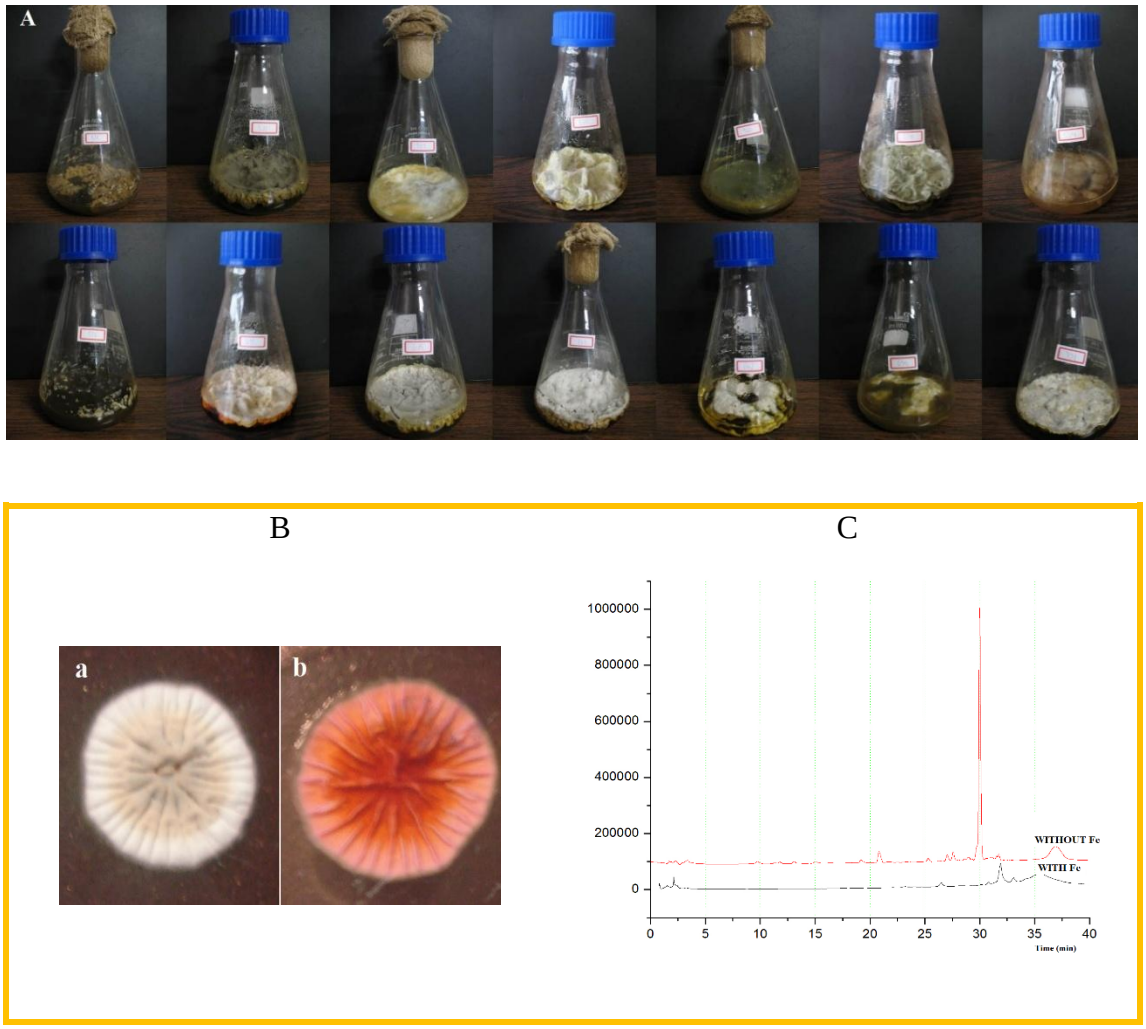

Fig. 1 - A. Overview of the screening step. B. Appearance of *P. maximae* on the surface (a) and back (b) of the Petri dish. C. Chromatogram showing the presence of a major metabolite produced by *P. maximae* grown without iron supplementation.

For the large-scale cultivation, *P. maximae* was grown in liquid media (10 L) generating a dark-orange brownish broth. Three metabolites were isolated from *P. maximae* extract and identified by NMR spectrometry. Compound 1 was isolated as an amorphous solid that furnished an orange solution in chloroform. The ^{1}H NMR spectrum of **1,** identified as sclerotiorin (Lucas et al., 2007), showed characteristic methyl and methylene groups (0.86–2.18 ppm), conjugated olefinic hydrogen atoms (δ_H 5.71–7.94 ppm), and signals attributed to H9 and H10 (δ_H 6.08 and 7.08 ppm respectively, J=15.6 Hz). The ^{13}C NMR spectrum presented 21 signals, including three carbonyl groups at δ_C 186.0 (C6), 191.8 (C8) and 170.1 ppm (C19) and one carbinolic carbon at δ_C 84.6 ppm (C7). Sclerotiorin is a natural pigment previously isolated from *P. sclerotiorum*, a closely related species. Antibacterial properties and potential application of sclerotiorin as dye in the food industry have been reported (Gomes and Takahashi, 2016; Lucas et al., 2007). Sclerotiorin (**1,** 16.37 % yield): orange solid; ^{13}C NMR (chloroform-*d*): 152.7 (C1), 158.1 (C3), 106.4 (C4), 138.7 (C4a), 110.8 (C5), 186.0 (C6), 84.6 (C7), 191.8 (C8), 114.5 (C8a), 115.7 (C9), 142.9 (C10), 132.0 (C11), 148.9 (C12), 35.1 (C13), 30.1 (C14), 12.0 (C15), 20.2 (C16), 12.4 (C17), 22.5 (C18), 170.1 (C19), 20.1 (C20).

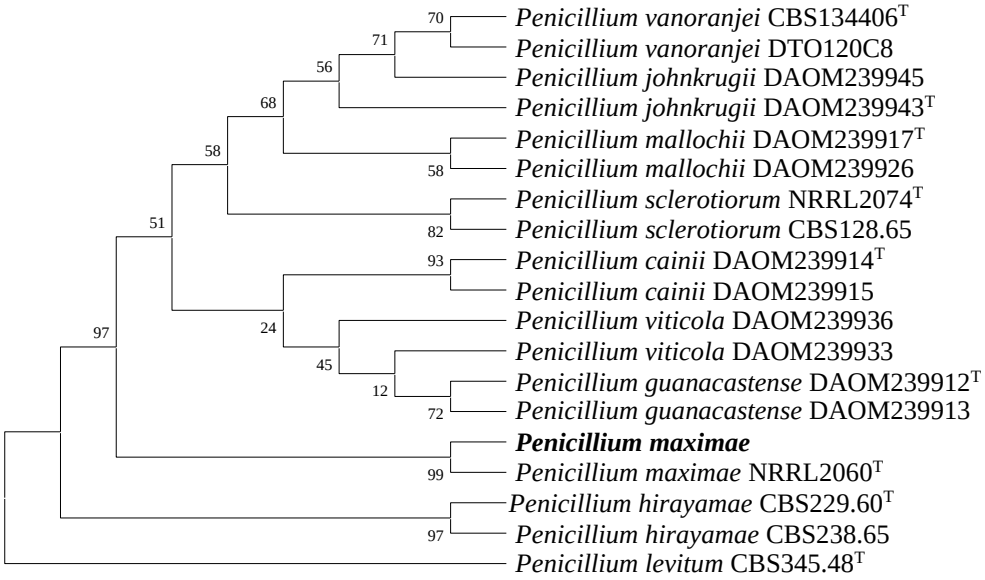

Fig. 2 - Maximum-likelihood tree based on ITS sequences showing the placement of the soil isolate *P. maximae* (in bold, GenBank accession MZ683178) relative to the type strain and closely related species in *Penicillium* section *Sclerotiora*.The tree was rooted with *P. levitum* CBS345.48. Numbers at branches are bootstrap values of 1,000 replications. The final dataset contained 543 aligned positions. The tree was constructed using MEGA software with Tamura 3-parameter model. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter = 0.0500)].

Compound 2**,** isolated as a yellow amorphous solid, was identified as eupenicilazaphilone B (**2**) (Gu et al., 2018), according to the signals of methyl and methylene groups (δ_H 0.86-1.71 ppm) and olefinic hydrogen atoms at δ_H 6.10 (H4), 6.27 and 6.77 ppm (H9 and H10, J = 15.6 Hz). The ^{13}C NMR spectrum (19 signals) comprised signals of carbonyl (δ_C 191.52 ppm, C6), and carbinolic carbon atoms at δ_C 76.8 (C7), 74.5 (C8), 76.6 (C11) and 80.9 ppm (C12). Eupenicilazaphilone B (2**,** 0.77 % yield): yellow amorphous solid; ^{13}C NMR (methanol-d_4): 70.1 (C1), 164.1 (C3), 102.0 (C4), 148.1 (C4a), 120.0 (C5), 191.5 (C6), 76.8 (C7), 74.5 (C8), 38.4 (C8a), 122.6 (C9), 145.2 (C10), 76.6 (C11), 80.9 (C12), 36.4 (C13), 30.0 (C14), 12.2 (C15), 14.3 (C16), 26.4 (C17), 20.1 (C18).

Compound 3 was isolated as an amorphous solid with intense red coloration and showed strong structural similarity with compound 1, according to the NMR data related to the signals assigned to hydrogen atoms from methyl and methylene groups (δ_H 0.85 to 2.47 ppm), to olefine hydrogen atoms (δ_H 5.74 to 8.07 ppm), and to H9 and H10 at δ_H 6.24 and 7.20 ppm, respectively (J = 16.08 Hz). A signal at δ_H 12.07 ppm indicated the presence of a secondary amine in the chemicals structure of compound 3. The ^{13}C NMR spectrum (21 signals) revealed the presence of three carbonyl groups [δ_C 183.6 (C6), 193.4 (C8) and 170.7 ppm (C19)]. A signal at δ_C 85.2 ppm, corresponding to a carbinolic carbon was assigned to C7. The data obtained were compared to those in the literature and the compound was identified as sclerothioramin (3) (Wang et al., 2010). Sclerotioramin (3**,** 1.05 % yield): red amorphous solid; ^{13}C NMR (chloroform-*d*): 138.8 (C1), 147.0 (C3), 110.8 (C4), 148.0 (C4a), 101.1 (C5), 183.6 (C6), 85.2 (C7), 193.4 (C8), 114.7 (C8a), 116.6 (C9), 143.5 (C10), 132.2 (C11), 149.0 (C12),

35.2 (C13), 30.2 (C14), 12.1 (C15), 20.3 (C16), 12.5 (C17), 23.8 (C18), 170.7 (C19), 20.6 (C20).

Sclerotioramin is an antibacterial and antifungal agent previously isolated from *P. citreonigrum* (Ferreira-Silva et al., 2021; Wang et al., 2010). Sclerotioramin (3) contains a nitrogen atom in the heterocyclic ring, instead of oxygen, present in sclerotiorin (1). This replacement is common in azaphilones due to their affinity for amine groups present in proteins, amino acids and nucleic acids (Svilar et al., 2012). Chemical structures of compounds 1–3 are shown in Fig. 3.

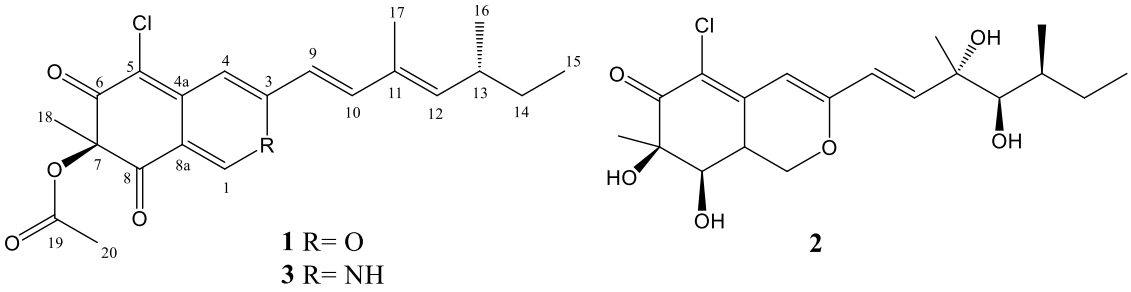

Fig. 3 - Chemical structure of compounds 1–3 from *P. maximae*.

The production of azaphilones by *P. maximae* in a culture medium using glucose, peptone and yeast extract is relevant, as these compounds have broad potential for industrial applications and their production using solid state fermentation was not detected (Rengifo et al., 2023). Sclerotiorin (1) inhibits Hsp90, a therapeutic target for cancer treatment, while the analogue with nitrogen (3) was inactive (Kabbaj et al., 2015), reflecting the role of the heteroatom in the biological activity. Azaphilones with nitrogen in the heterocycle are red while oxygen leads to yellow/orange pigments due to the changes in the chromophore provoked by the heteroatom (Yuliana et al., 2017). Azaphilones were also reported as a promising chemotherapeutic agent to avoid proliferation of cancer-cells (Matsumoto et al., 2023), as well as a natural pigment for several applications in food industry (Dufossé, 2025). *P. maximae* has also been studied due to the high protein content of its biomass, which can be a sustainable source of alternative proteins for human consumption (Moura et al., 2022; Takahashi et al., 2020).

4. Conclusion

P. maximae proved to be a good source of azaphilone dyes, mainly sclerotiorin, produced with a yield of 16.36 % yield. This yield is relevant for large-scale production, given that the initial yield of secondary metabolites by fungi is not always encouraging enough to envisage industrial applications. On the other hand, the use of azaphilones as industrial dyes still needs to be authorized by regulatory bodies, which may occur in the near future, due to the significant increase in cases of allergies, cancer and other diseases directly related to the consume of foods containing some artificial dyes.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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