

EPIGENETIC MODIFIERS AND MINERALS AS TOOLS TO DIVERSIFY SECONDARY METABOLITE PRODUCTION IN FUNGI

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ABSTRACT

Secondary metabolite production of fungi can be modified by different approaches, including epigenetic modifiers, culture-dependent methods, and genomic-based methods. In this study, secondary metabolite production was explored in the presence of epigenetic modifiers and minerals using a microscale fermentation approach. Thirteen fungi originally isolated from mushrooms and soils were grown in 96-well microtiter plates (MTPs) using 70% of potato dextrose broth (PDB) with the addition of epigenetic modifiers and minerals in different combinations and concentrations. All cultures were fermented at 10 °C or 28 °C for 2, 3, or 5 weeks and extracted by solid phase extraction. The resulting extracts were subjected to high-performance liquid chromatography (HPLC) and the chromatograms were analyzed on a qualitative and quantitative basis. In addition, major secondary metabolites from four fungi were identified as penicillic acid, patulin, pseurotin A, and javanicin. Epigenetic modifiers and minerals induce significant changes in the profile of the secondary metabolites. Their usage combined with microscale fermentation provides a cost-efficient tool for exploring fungal secondary metabolism.

Key words: Epigenetic modifier, elicitors, secondary metabolites, soil fungi

INTRODUCTION

Fungi offer enormous biodiversity and differ from one another not only in their morphology and development but also in their metabolic potential. The products of these pathways include important pharmaceuticals, including antibacterial, anticancer, and insecticide drugs (Newman, 2019). These products are known as secondary metabolites. However, many putative fungal secondary metabolites remain elusive presumably because the corresponding biosynthetic gene clusters are not transcribed under conventional laboratory conditions (Bentley, 2002; Ochi & Hosaka, 2013, Rutledge *et al.*, 2015). These unexpressed genes are known as sleeping genes; they are present, but silent under standard screening conditions (Haferburg *et al.*, 2009). These silent

metabolic pathways may lead to novel bioactive compounds and methods to awaken sleeping genes are thus of major interest. Methodologies to activate silent biosynthetic pathways can broadly be divided into two types that are molecular approaches (Gross, 2009; Hertweck, 2009; Scherlach & Hertweck, 2009) such as the generation of gene “knockouts” (Chiang *et al.*, 2008), promoter exchange (Chiang *et al.*, 2009), and cultivation-dependent approaches which include One Strain Many Compounds (OSMAC approach) (Bode *et al.*, 2002, Pan *et al.*, 2019). The cultivation-dependent approaches can be further categorized as biotic or abiotic. The biotic approach to activating silent biosynthetic pathways includes co-culture (Yu *et al.*, 2019), while abiotic conditions involve either physical parameters, e.g. agitation and temperature, or chemical elicitation, e.g. AgNO₃ and CdCl₂ (Radman *et al.*, 2003). Chemical elicitors which have the potential to cause chromatin modification, such as inhibitors of

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histone acetyltransferase (HATs) and DNA methyl transferase (DNMTs), have been created as tools to activate these silent genes to optimize the production of existing metabolites or promote the production of new compounds (Tonghuevo *et al.*, 2020). William *et al.* (2008) attempted to directly express silent genes with the help of epigenetic modifiers such as histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA). As a result, new cladochromes were obtained from *Cladosporium cladosporioides*, while cephalosporolides B and cephalosporolides F were isolated from *Isaria tenuipes* using a combination of SAHA and 5-azacytidine, an inhibitor of the DNA methyl transferase. Shi *et al.* (2020) used sodium butyrate on *Trichoderma harzianum* to wake its sleeping genes. Three new terpenoids were isolated from the fungal culture including cleistanthane diterpenoid, harzianolic acid A, harziane diterpenoid, harzianone E, and cyclonerane sesquiterpenoid, 3,7,11-trihydroxy-cycloneran. The objective of this study was to compare elicitors reputed for activating the expression of silent or poorly expressed pathways, i.e. epigenetic modifiers and minerals using a microscale fermentation approach.

MATERIALS AND METHODS

Biological material

Fungi used for this study were selected from AuRIns' culture collection. *Aspergillus* sp. (D1D1-1), *Geomyces* sp. (A1C-3), *Talaromyces aculeatus* (B1-3), and unidentified B2C-6 were isolated from soils from Svalbard Island, Norway. *Fusarium solani* (SC14a-2), *Penicillium* spp. (TOWB-F2 and SC14b-1), and *Pseudallescheria boydii* (ERS) were isolated from Malaysian soils. *Aspergillus aculeatus* (SC7b), *Fusarium proliferatum* (SC2f), *Penicillium simplicissimum* (SC12a), *Trichoderma harzianum* (SC18c), and *Trichoderma spirale* (SC19b) were endophytes from Malaysian mushrooms.

Microscale fermentation

The steps for culturing the fungi at the microscale in parallel are described below.

Inoculum preparation

Fungi used to prepare hyphal suspension are grown for 2-3 weeks on PDA. For each isolate, three mycelial discs are cut from the culture plate with a 6-mm stainless steel cork borer and transferred into a 15-mL Falcon tube containing 10 mL of PDB. The tubes are incubated at 10 °C (A1C-3, B2C-6, B1-3) for 2 weeks or at 28°C (ERS, SC14a-2, TOWB-F2, SC14b-1, SC12a, SC7b, SC2f, SC18c, SC19b, D1D1-1) for 7 days. The cultures were then homogenized in a Genogrinder® (SPEX SamplePrep, USA) with the

addition of 4-mm steel grinding balls at 1,000 strokes per min (spm) for 5 min.

Simultaneous culture on 96-well plates

Fungi with similar growth rates were grown simultaneously in 2-mL deep-well microtiter plates (MTP, Phenomenex®, USA) covered with Sandwich Covers from System Duetz® (Germany) and clamped using an in-house designed device. In this series of experiments, each MTP contained 12 different media (each dispensed in a column) made of 70 % PDB supplemented with elicitors. Epigenetic modifiers consisted of 5-azacytidine, suberoylanilide hydroxamic acid (SAHA), valproic acid (VA), sodium butyrate, and S-adenosylhomocysteine (SAHC) in two concentrations that were 0.1 mM (high concentration, H) and 1 µM (low concentration, L). Minerals concentration was standardized at 0.1 mM. Each well was added with glass beads, and, after sterilization, inoculated with 100 µL of a fungal suspension obtained as above described. Each plate was inoculated with eight fungi, one fungus per row. The total medium volume in each well was 1000 µL. The cultures were incubated at 10°C or 28°C for 2 weeks (ERS, SC 14a-2, TOWB-F2, SC 14b-1, SC18c, SC19b, D1D1-1), 3 weeks (SC12a, SC7b, SC2f) or 5 weeks (A1C-3, B2C-6, B1-3).

Fungal cultures extraction and HPLC analysis

The cultures were homogenized at 1,000 spm for 5 min and centrifuged at 2,140 g for 15 min. The cultures were extracted by solid phase extraction (SPE; Strata-X 33u Polymeric Reversed Phase, 30 mg/well, Phenomenex®, USA) and the fungal secondary metabolites were eluted with a methanol/ acetonitrile mixture (1:1, v/v). The extracts were subjected to HPLC (Agilent 1200) equipped with a diode array detector (DAD) on a Synergi Hydro-RP 80Å column (150 x 4.6 mM, 4 µM, Phenomenex®, USA) with an acetonitrile-water mobile phase and an elution gradient of 10-100 % acetonitrile over 30 min.

Scale-up fermentation and fractionation

Selected fungi were cultured for 2 or 3 weeks in 1 L of PDB supplemented by elicitors as follows: TOWB-F2 – VA (H) + SAHC (H), 2 weeks; ERS – NaNO₃, 2 weeks; SC 14b-1 – KH₂PO₄, 3 weeks; SC 14a-2 – KH₂PO₄, 3 weeks; SC12a – CuSO₄, 3 weeks. The cultures were then extracted by liquid-liquid extraction with ethyl acetate (EA). The crude extracts were fractionated and compounds purified by preparative HPLC on a PLC 2020 system (Gilson, USA).

Spectroscopy analysis

Pure compounds were analyzed by nuclear magnetic resonance (NMR), and mass spectrometry-time of flight (MS-TOF). UV spectra were derived from the DAD data obtained during HPLC analyses.

RESULTS AND DISCUSSION

Effect of epigenetic modifiers and minerals

The microscale fermentation method allows simultaneous fermentation at the microscale and is suitable and convenient for handling a large number of samples. Such a parallel fermentation process was found much appropriate for a reliable comparison of fungal metabolite production. To test various methods reported in the literature as productive tools for uncovering new metabolites or boosting their production, fermentations were carried out in the presence of different elicitors in each well. As a result, 13 fungi were grown in different medium compositions, amounting to a total of 136 cultures, including 13 control cultures (no elicitor added). This led to contrasting results as shown in Table 1. In some cases, the fungus did not seem affected by its secondary metabolism. For example, *Penicillium* sp. TOWB-F2 did not significantly react to the presence of minerals or combinations of epigenetic modifiers in its growth medium (Figure 1) and consistently produced a compound in large amounts, which was eventually identified as penicillic acid (**1**, Figure 2). In other cases, the stress put on the fungi seemed too strong and they simply do not grow. This is seen for example when combinations of 5-azacytidine (H) and SAHC (H) or SAHA (H) and VA (H) are used (Table 1).

Mixed results are commonly obtained whereby the production of some metabolites is increased while that of others is decreased. This is observed for example with *Penicillium simplicissimum* SC12a. The chromatogram of the culture extract of this fungus grown in the absence of elicitors (Figure 3, Control) showed an important peak at retention time (t_R) of 6.9 min corresponding also to penicillic acid (**1**, Figure 2). When the medium was added with both a low concentration of VA and a high concentration of SAHC, an approximate 80 % decrease in the production of **1** was noted, while that peak was increased fivefold when the medium was supplemented with CuSO_4 . In contrast, a peak at 16.0 min decreased in the presence of VA + SAHC or completely disappears in the presence of CuSO_4 . An interesting yet modest effect was induced by VA + SAHC whereby a new peak appears at 6.5 min that is not observed from other media. The relative concentrations of VA and SAHC would need to be optimized to increase the production of this undetermined metabolite.

These epigenetic modifiers occasionally can alter the production of secondary metabolites to some dramatic extent. The chromatogram of B2C-6 obtained from a control culture (i.e. without any elicitor) showed a series of well-separated peaks

between 4 and 15 min (Figure 4). A combination of SAHA and SAHC led to peaks between 11 and 14 min to disappear completely, while significant peaks appeared at 8.0 and 17.4 min. This result is consistent with the previous literature that stated SAHA is a potent HDAC inhibitor (Cole, 2008). SAHA has been successfully used as an epigenetic chemical in a variety of eukaryotic systems.

The overall results are summarised in Table 2. Out of the 123 cultures carried out in the presence of elicitors, 29 (23.5 %) had their secondary metabolism negatively affected, while 46 (37.3 %) were unaffected. In turn, the extracts of 42 cultures (34 %) had their chromatograms display larger and/or new peaks. It must be pointed out here that neither the choice nor the amount of the elicitor(s) were truly optimized before these experiments, but were rather inspired by the literature. Even though epigenetic manipulation lacks a predictable outcome, it nevertheless has certain advantages, such as the ability to quickly examine potential pools of silent or cryptic fungal natural products in their native hosts (Li, 2020).

A positive effect was observed in one in three cultures with a limited number of tested elicitors. In epigenetic modification, acetylation and methylation are the most recognized and both modifications are stable, inheritable, and reversible (Brosch, 2008). Minerals and epigenetic modifiers had little difference in their success rate in stimulating the fungal secondary metabolism, but neither group showed superiority in this respect. Rather, they were complementary tools for the exploration of fungal metabolism.

Identification of compounds

After scaling up the fermentation process, secondary metabolites were isolated from *Pseudallescheria boydii* ERS, *Fusarium solani* SC14a-2, *Penicillium* sp. SC14b-1 and TOWB-F2, and purified by PLC. Apart from above mentioned penicillic acid (**1**) isolated from *Penicillium* sp. TOWB-F2 and *Penicillium simplicissimum* SC12a, patulin (**2**), pseurotin A (**3**), and javanicin (**4**) were identified from the cultures of *Penicillium* sp. SC14b-1, *Pseudallescheria boydii* ERS, and *Fusarium solani* SC14a-2, respectively. A previous study stated that penicillic acid and patulin were small lactone compounds produced by *Penicillium* sp. (Frisvad, 2018). The most common fungus that produced patulin is *Penicillium expansum* (Moake *et al.*, 2005). Pseurotin A is a secondary metabolite isolated from *Pseudoeurotium ovalis* (Bloch, 1976) and several species of *Aspergillus* sp. (Shaaban *et al.*, 2014). Another known compound, which is javanicin, was previously isolated from *Fusarium javanicum* (Arnstein *et al.*, 1946) while in this study, this compound was produced by *Fusarium solani* SC14a-2.

Table 1. Effect of epigenetic modifiers and minerals on secondary metabolite production of selected fungi

Fungus	SAHA (H) + SAHC (H)	SAHA (L) + SAHC (H)	VA (H) + SAHC (H)	VA (L) + SAHC (H)	Na butyrate (H) + SAHC (H)	5-azacytidine (H) + SAHC (H)	MgSO ₄ (H)	FeSO ₄ (H)	KH ₂ PO ₄ (H)	CuSO ₄ (H)	NaNO ₃ (H)
<i>Pseudallescheria boydii</i> ERS	-	+	+	+	+	+	+	+	+	+	+
<i>Fusarium solani</i> SC14a-2	-	+	+	+	+	-	-	-	+	+	+
<i>Penicillium</i> sp 1 TOWB-F2	0	0	0	0	0	0	0	0	0	0	0
<i>Penicillium</i> sp 2 SC14b-12	0	0	0	0	0	0	0	0	0	0	0
<i>Geomyces</i> sp A1C3	-	+	+	0	0	+	nt	nt	nt	nt	nt
B2C-6 (unidentified)	-	+	-	+	-	ng	nt	nt	nt	nt	nt
<i>Talaromyces aculeatus</i> B1-3	+	+	0	-	+	-	nt	nt	nt	nt	nt
<i>Aspergillus</i> sp D1D1-1	+	+	0	+	-	+	nt	nt	nt	nt	nt
<i>Penicillium simplicissimum</i> SC12a	-	-	-	-	-	ng	+	-	0	+	-
<i>Aspergillus aculeatus</i> SC7b	-	-	-	0	+	ng	0	+	0	-	0
<i>Fusarium proliferatum</i> SC2f	0	+	-	0	0	ng	-	-	-	+	-
<i>Trichoderma harzianum</i> SC18c	-	0	0	+	+	ng	+	0	0	+	0
<i>Trichoderma spirale</i> (SC19b)	+	+	0	0	0	ng	0	0	0	+	0

+: overall increase of SM production; -: overall decrease of SM production; 0: no major difference in SM production; ng: no growth; nt: not tested

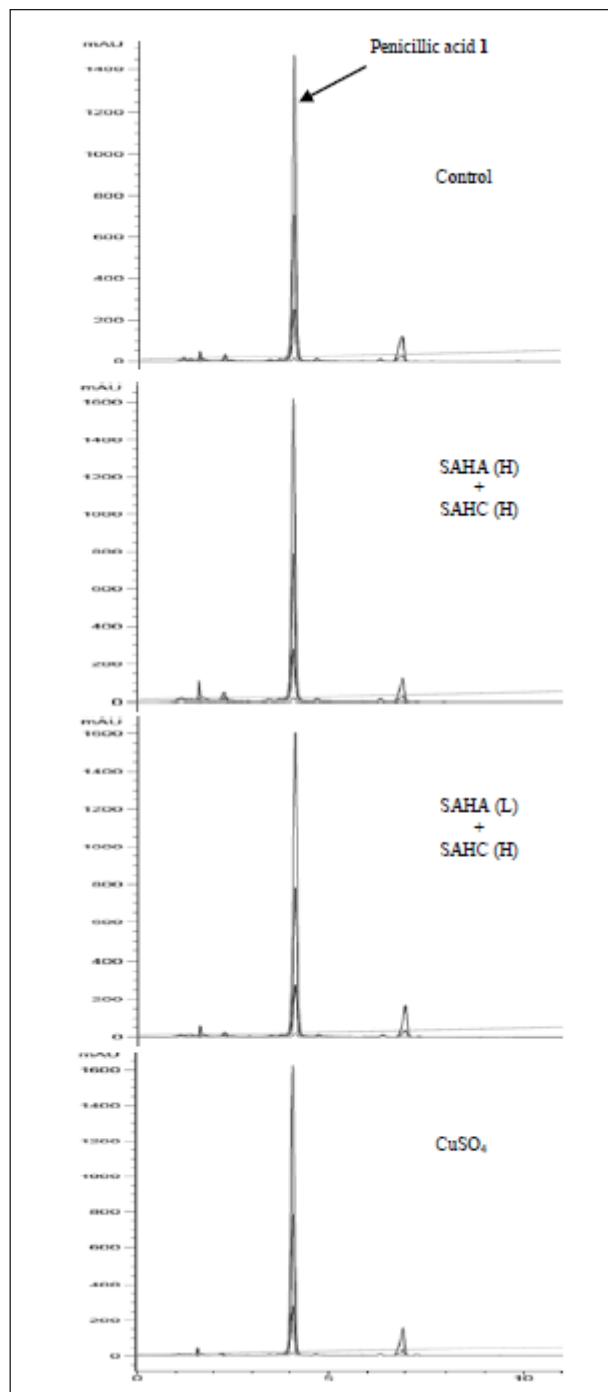


Fig. 1. Chromatograms of extracts from *Penicillium* sp. TOWB-F2 cultures.

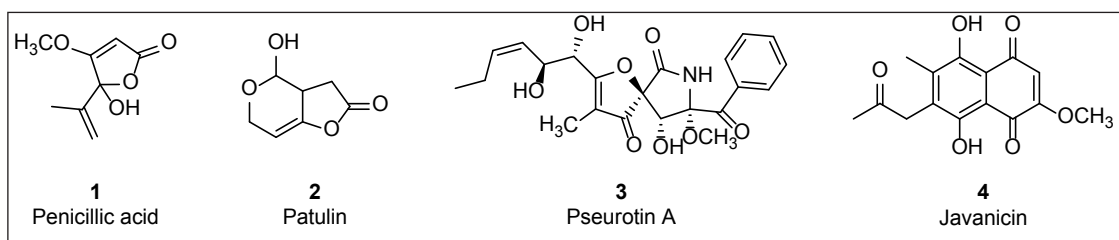


Fig. 2. Chemical structures of isolated compounds.

Table 2. Number of cultures affected by the addition of epigenetic modifiers and minerals with respect to control cultures (PDB 70 % strength)

Elicitors	+	-	0	ng
SAHA (H) + SAHC (H)	3	7	3	0
SAHA (L) + SAHC (H)	8	2	3	0
VA (H) + SAHC (H)	3	4	6	0
VA (L) + SAHC (H)	5	2	6	0
Na butyrate (H) + SAHC (H)	5	3	5	0
5-azacytidine (H) + SAHC (H)	3	2	2	6
MgSO ₄	3	2	4	0
FeSO ₄	2	3	4	0
KH ₂ PO ₄	2	1	6	0
CuSO ₄	6	1	2	0
NaNO ₃	2	2	5	0
Total	42	29	46	6

+: overall increase of SM production; -: overall decrease of SM production; 0: no major difference in SM production; ng: no growth.

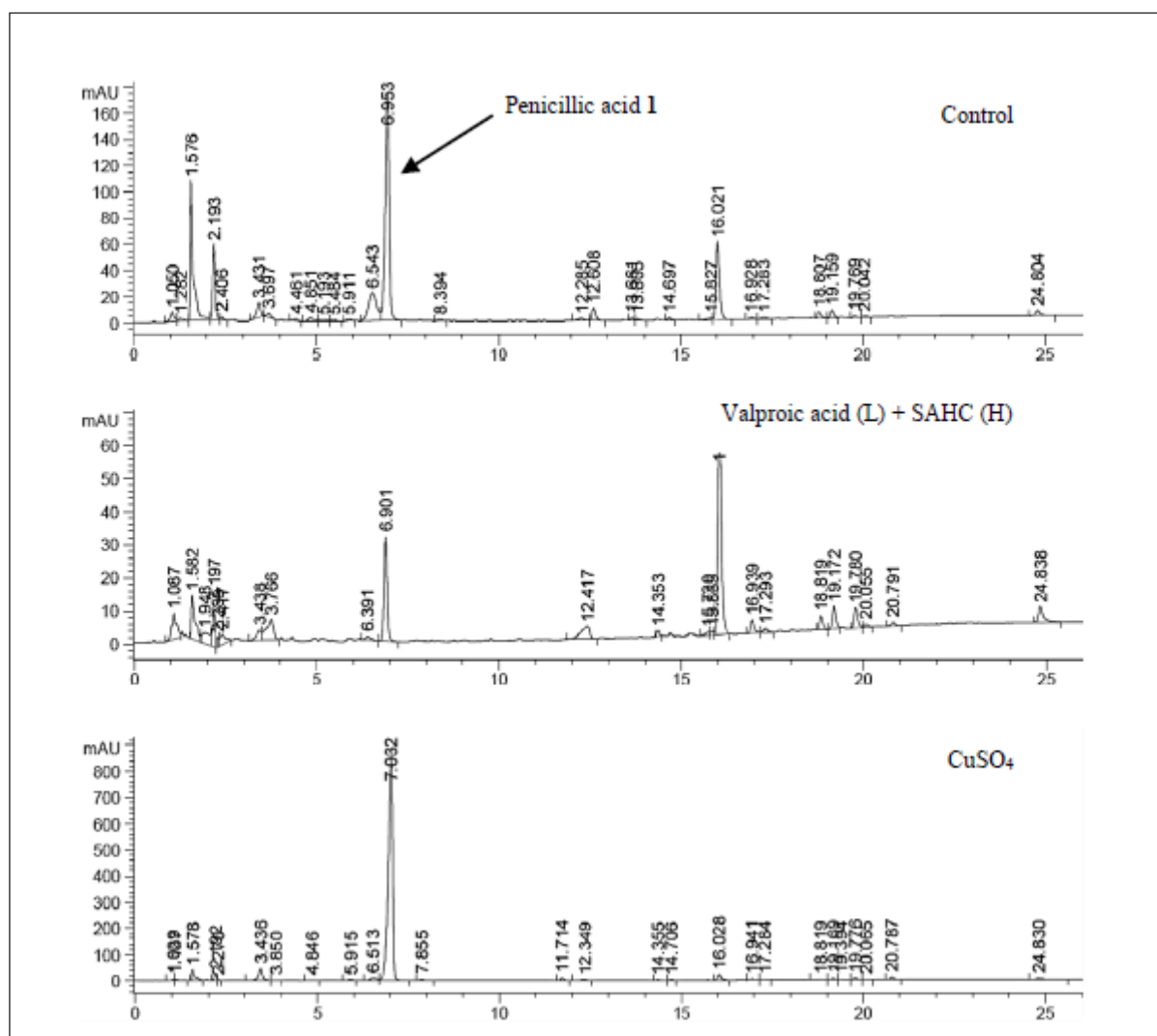


Fig. 3. Chromatograms of extracts from *Penicillium simplicissimum* SC12a (220 nM).

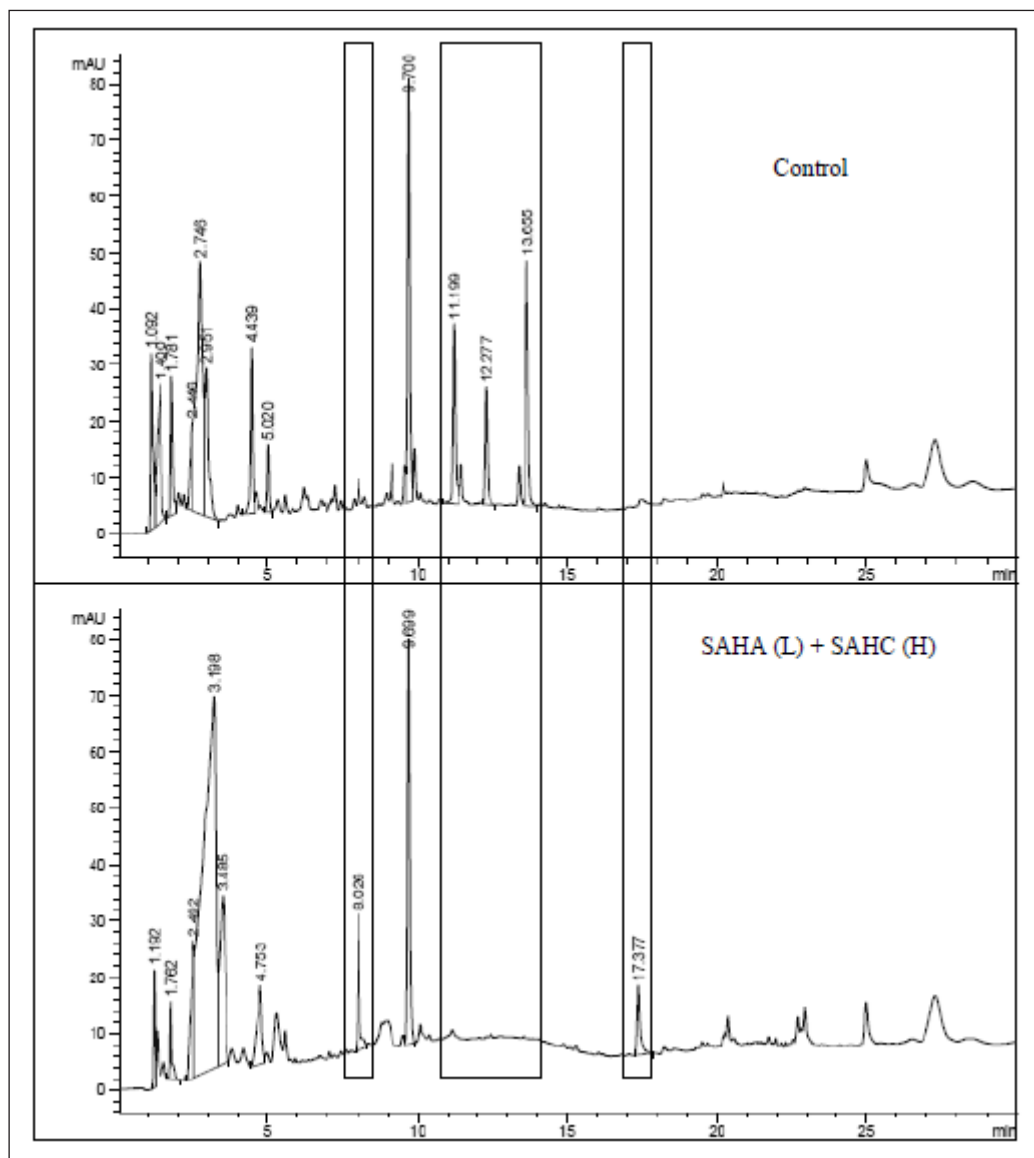


Fig. 4. Chromatograms of extracts from B2C-6 cultures (220 nM).

CONCLUSION

In conclusion, this work demonstrated the usefulness of the above-described approaches. Epigenetic modifiers in various combinations were compared to more traditional mineral elicitors. The above results demonstrated the above-tested compounds may have either a positive, neutral, or negative effect on the secondary metabolism of a given fungal species. Since it is unlikely that we would be able to predict these effects in any foreseeable future, the most practical approach remains to test as many different conditions as possible to explore the silent metabolic pathways. The microscale fermentation method allows the screening of a large number of fungi in various growth conditions for studying the production of secondary metabolites in short times and at a relatively low cost.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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