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Metabolomic profiling, biological evaluation of *Aspergillus awamori*, the river Nile-derived fungus using epigenetic and OSMAC approaches†

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LC-HRMS-based metabolomics approach was applied to the river Nile-derived fungus *Aspergillus awamori* after its fermentation on four different media and using four epigenetic modifiers as elicitors. Thereafter, a comprehensive multivariate statistical analysis such as PCA, PLS-DA and OPLS-DA were employed to explain the generated metabolomic data (1587 features). PCA showed that the fungus displayed a unique chemical profile in each medium or elicitor. Additionally, PLS-DA results revealed the upregulated metabolites under each of these conditions. Results indicated that both rice and malt dextrose agar were recognized as the best media in terms of secondary metabolites diversity and showed better profiles than the four applied epigenetic modifiers, of which nicotinamide was the best secondary metabolite elicitor. Testing the antibacterial and cytotoxic effects of all *A. awamori*-derived extracts revealed that using epigenetic modifiers can induce antimicrobial metabolites against *S. aureus* and *E. coli*, whereas using rice, malt dextrose or nicotinamide can induce groups of cytotoxic metabolites. OPLS-DA results assisted in the putative identification of the induced metabolites that could be responsible for these observed inhibitory activities. This study highlighted how powerful the OSMAC approach in maximizing of the chemical diversity of a single organism. Furthermore, it revealed the power of metabolomics in tracing, profiling and categorizing such chemical diversity and even targeting the possible bioactive candidates which require further scaling up studies in the future.

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1. Introduction

Fungi are a distinct singular kingdom that comprises a premium resource of nutrition and therapeutics for the coming centuries.[1] Fungi play an important role in addressing major global challenges. Use of fungal processes and products can lead to increased sustainability through more efficient use of natural resources. Fungi are one of nature's most promising hotspots for finding new drug candidates and antimicrobials. Fungi have interesting potential as the new way of manufacturing biological medicines and a wide spectrum of new value added bio-based products.[2] Nearly, 42% of more than twenty thousand fungal bioactive products are acting as antiviral, antibacterial, antifungal, cytotoxic and immunomodulating agents.[3]

New developments in fungal genomes revealed exciting facts concerning the complexity and capabilities of fungal genes in regulating their secondary metabolites biosynthetic routes.[4] The gene clusters responsible for regulating the majority of secondary metabolites are often silent under normal environmental conditions.[5] The activation of these silent gene clusters requires the development of several approaches.[6] One of such approaches is the OSMAC (One Strain Many Compounds)

approach. The OSMAC strategy has been developed in the early 2000s as a simple and effective way to increase the diversity of fungal secondary metabolites.[7] In this strategy, silent genes were activated by modification of culture conditions, including media composition, UV irradiation, shaking and incubation temperature.[8,9] This strategy assumes that one microbial strain could be a reservoir of genetic potentials for diverse biosynthetic pathways that possess high flexibility in secondary metabolites production depending on the various environmental factors.[10]

A delicate mission is the manipulation of the silent epigenome; this mission can be attained through managing certain proteins responsible for chromatin remodeling. The influence of these proteins on gene expression and the silent biosynthetic pathways is obvious.[10] Histones are among the most important proteins which are subjected to several modifications like acetylation. Histone deacetylase (HDAC) could reverse acetyl group addition to histones which are catalyzed by histone acetyltransferase. On the other hand, DNA methyltransferase (DNMT) catalyzes the transfer of methyl groups from *S*-adenosylmethionine (AdoMet, SAM) to the C-5 position of cytosine. Inhibition of HDAC or DNMT is a promising approach that allows the activation of certain silent biosynthetic gene clusters (BGC).[4] Successfully, this was achieved by various small molecules which promote the production of novel secondary metabolites and affect fungal protein and carbohydrate metabolism.[11]

Previous reports confirmed that the fungal growth and metabolism were affected by the physicochemical parameters (*e.g.* pH, aeration, and incubation temperature) and nutrients (*e.g.* carbon and nitrogen sources).[12] Typically, microorganisms maneuver their metabolism, particularly the secondary metabolite production in response to environmental changes in order to adapt themselves to endure and survive in different condition.[13] The secondary metabolites biosynthesis supports microbial growth and survival through deterring pathogens or acting as extracellular signaling molecules among the competing microbial communities.[13] Furthermore, significant effects on the fungal secondary metabolite profiles were attributed to the variation in the growing environmental factors. In the industrial paradigm, remarkably increased titer of the metabolite of interest could be obtained *via* the optimization of the fermentation parameters. Such optimization is challenging in a drug-discovery programs, since the optimum conditions for one organism may induce poor metabolic diversity for a different organism.[14]

Metabolomics encompass unprejudiced analysis of the overall metabolites in a biological sample under a specific set of parameters, serving as a method to provide the central information of cell, tissue, or whole organism picture.[15] The LC-HRMS-based metabolomics profiles linked to multivariate statistical analysis is considered an efficient appliance that could aid in measuring the effects of different fermentation parameters on the metabolome of a given organism, as well as in the correlation between the biological activities of a given extract with the possible molecular entities responsible for such observed biological effects.[16]

In a previous study, we have investigated the entitled fungus as a source of antimicrobial and anticancer metabolites.[17]

Herein, as a part of our ongoing drug-discovery screening efforts, we aimed at investigating the metabolome of this promising fungus, under various culture conditions (OSMAC) and upon its treatment with a number of epigenetic modifiers to find out the factors that may induce the chemical and biological diversity of this fungus. Additionally, the extracts derived from this fungus in each culture condition were tested for their *in vitro* antibacterial and anticancer activities.

2. Material and methods

2.1. Solid state fermentation with different growing condition

To study the effect of changing fermentation media parameters on secondary metabolite profile, *A. awamori* was initially revived in malt extract agar for 3 days. Then, inoculation of a single colony from malt agar in 150 mL malt extract broth (malt extract 15 g L^{-1}) was performed, this seed culture was allowed to grow statically for 3 days at 30 °C. Four forms of solid media including malt extract agar, nutrient agar, tryptone soya agar and solid rice media were prepared. These four media were made as follows: malt extract agar (15 g malt extract and deionized water to 1 L), nutrient agar medium (3 g beef extract, 5 g peptone, 20 g agar and deionized water to 1 L), trypticase soya agar (15 g pancreatic digest of casein, 5 g peptic digest of soybean, 5 g NaCl, 15 g agar and deionized water to 1 L), and rice medium (100 g commercially available shelled rice were immersed in 100 mL of deionized water and kept overnight prior to autoclaving).

2.2. Extraction of fungal culture

Each Petri dish from each treatment was extracted with EtOAc in a 1 : 4 (agar : solvent) ratio. Firstly, agar cultures were sliced and homogenized with an ultra-turrax in the extracting solvent in order to achieve the highest extraction efficiency. Subsequently, each extract was evaporated under vacuum to obtain dry extracts. The obtained residues were re-dissolved in MeOH for the LC-HRMS analysis. About 1 mg aliquots were transferred into separate vials for LC-HRMS profiling and bioactivity testing.

2.3. Metabolomics analysis and statistical analysis

Extracts of 1 mg mL^{-1} in MeOH were analyzed in triplicates on an Accela HPLC (Thermo Fisher Scientific, Bremen, Germany), using a UV detector at 280 and 360 nm with and an Exactive-Orbitrap high resolution mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Media blanks were prepared for comparison reasons. The HPLC column was an ACE (Hichrom Limited, Reading, UK) C$_{18}$, 75 mm × 3.0 mm, 5 μm column. The mobile phase consisted of purified water (A) and acetonitrile (B) with 0.1% formic acid in each solvent. The gradient program started with 10% B and B was increased linearly in 30 min to 100% B at a flow rate of 300 μL min^{-1} and remained isocratic for 5 min before linearly decreasing in 1 min to 10% B. The column was then re-equilibrated with 10% B for 9 min before the next injection. The total analysis time for each sample was 45 min. The injection volume was 10 μL and the tray

temperature was maintained at 12 °C. High resolution mass spectrometry was carried out in both positive and negative ESI ionization modes with a spray voltage of 4.5 kV and capillary temperature of 320 °C. The mass range was acquired from m/z 150 to 1500. Multi-fragmentation (MSn), The RAW data were then processed on Mzmine.[18,19] PCA and PLS-DA are powerful tools for qualitative data analysis and discriminant data classification.[20] Moreover, OPLS-DA is a robust regression technique used for the investigation of the relationship between two data sets.[21]

2.4. Antimicrobial activity of *A. awamori* extracts

To measure the antibacterial activity of the 8 crude extracts, Gram-positive bacteria (*Staphylococcus aureus* ATCC9144 and *Bacillus subtilis* ATCC29212), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC27853 and *Escherichia coli* ATCC25922) were used as test organisms and antibacterial tests were performed as in ref. 22. The testing was performed in 96-well flat polystyrene plates. 10 μL of test extracts (1 mg mL^{-1} final concentration) were added to 80 μL of lysogeny broth (LB broth) followed by inoculation of 10 μL of bacterial culture suspension at (log phase). The plates were then incubated overnight at 37 °C. After incubation, the positive antibacterial effect of the tested compound observed as clearance in the wells, while compounds that didn't influence the bacteria, the growth media appeared opaque in wells. The absorbance was measured after about 20 h at OD$_{600}$ in a Spectrostar Nano Microplate Reader (BMG LABTECH GmbH, Allmendgrun, Germany). The positive control (bacteria plus distilled water), while, negative control (growth media plus distilled water).

2.5. Cytotoxic assay

2.5.1. Cell line. Human lung fibroblast normal cell line (WI38), colorectal carcinoma Colon cancer (HCT-116), mammary gland breast cancer (MCF-7) and hepatocellular carcinoma (HePG-2). The cell lines were obtained from ATCC *via* VACSERA, Cairo, Egypt.

2.5.2. Chemical reagents. RPMI-1640 medium, MTT and DMSO (Sigma Co., St. Louis, USA), fetal bovine serum (GIBCO, UK). Doxorubicin was used as a standard anticancer drug for comparison.

2.5.3. MTT assay.[23] The MTT assay is a colorimetric assay for assessing cell metabolic activity. MTT assay was carried out to determine the inhibitory effects of compounds on cell growth of the above-mentioned cell lines. The conversion of the yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells is the basis of this colorimetric assay. Cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum. Penicillin (100 units per mL) and streptomycin (100 μg mL^{-1}) antibiotics were added at 37 °C in a 5% CO$_2$ incubator. The cell lines were seeded in a 96-well plate at a density of 1.0×10^4 cells per well at 37 °C for 48 h under 5% CO$_2$. After incubation, different compounds concentrations were added to the cells and then incubated for 24 h. After 24 h of drug treatment, 20 μL of MTT solution at 5 mg mL^{-1} was added

and incubated for 4 h. In each well, 100 μL dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan formed. The colorimetric assay was measured and recorded at absorbance of 570 nm using a plate reader (EXL 800, USA). The relative cell viability in percentage was calculated as (A_{570} of treated samples/A_{570} of untreated sample) × 100.

3. Results

3.1. Metabolomic analysis

LC-HRMS analysis of *A. awamori*-derived extracts at different fermentation conditions (*i.e.* four different fermentation media and four different epigenetic modifiers treatments) demonstrated a huge diversity of secondary metabolites. A total of 1587 peaks were found for all extracts under study, from which the known metabolites in the extracts were dereplicated (Table S1†) using The Dictionary of Natural Products (DNP), Metlin, Human Metabolome Database (HMDB) and Yeast Metabolome Database (YMDB) databases through the MZmine software.[24] Due to the fact that the metabolites content in a given extract varying in their physical nature and ionization potential, both the positive and negative ionization modes were applied, so that detection of the maximum possible metabolites was accomplished.

3.1.1. Metabolomic profiling of *A. awamori* under different culture media. The secondary metabolite productivity of *A. awamori* was investigated upon its fermentation on four different solid media; nutrient agar (NA), malt-dextrose agar (MDA), tryptone soya agar (TSA) and rice grains (RG). Extraction of the fungal mycelia grown on each medium was done using EtOAc and high-resolution tandem mass spectrometry (LC-HRMS/MS) was used for analysis. The principal component analysis (PCA) scores plot of the HRMS data (Fig. 1A) revealed a variation in the chemical profiles of *A. awamori* under the selected fermentation media, particularly RG and MDA, where they distributed separately from each other and from both NA and TSA indicating a different chemical makeup, and unlike TSA and NA that were to some extent close to each other indicating some similarity in their chemical constituents (Fig. 1A). To identify metabolites that could discriminate between the culture media groups, the HRESIMS data were further subjected to partial least square-discriminant analysis (PLS-DA). The resulted highly predictive PLS-DA model (R^2: 0.95 and Q^2: 0.91) along with its score plots (Fig. 1B) made it possible to resolve the four culture media into four distinct clusters. The endogenous metabolites that have a major contribution to the separation were found through determination of VIP scores after PLS-DA. Metabolites were considered as potential biomarkers if they satisfied the criteria VIP-value > 2 and *p*-value < 0.05. According to these statistical criteria, the unique up-regulated or induced metabolites in each culture media were putatively identified which indicated that RG was the best medium in terms of induction of secondary metabolites diversity, followed by MDA (Table S2† and Fig. 2). Additionally, there were other commonly induced metabolites that were exclusively detected in these two media, and thus they should be selected in future secondary metabolites large-scale production and isolation from *A.*

awamori. As illustrated in Fig. 3, the most induced metabolites in RG and MDA media belonged to the polyketide class indicating that such media may be associated with the activation of polyketide BTG in *A. awamori*.

3.1.2. Metabolomic profiling of *A. awamori* under different epigenetic modifier treatments. To further explore its secondary metabolite productivity, *A. awamori* was fermented in MDA medium in the presence of different epigenetic modifiers. We have selected valproic acid (Valp), nicotinamide (Nic) and trichostatin A (Tric) as HDAC inhibitors and 5-azacytidine (Aza) as DNMT inhibitor. Different reports suggested HDAC and DNMT inhibitors as efficient tools to activate the possibly cryptic BGC in a given fungus.[25] Similarly, it was found that the metabolomic profiles of *A. awamori* in each epigenetic modifier treatment were unique, where each treatment group was clustered separately in the PCA scores plot of the HRESIMS data (Fig. 1C). Regarding, the characteristic up-regulated metabolites to each treatment, the generated PLS-DA model (R^2: 0.99 and Q^2: 0.95) illustrated that the epigenetic modifiers were less effective than RG medium, and to

some extent similar to MDA media in their ability to induce diverse metabolites (Fig. 1D). Nic was found to be the most metabolite inducer among the other epigenetic modifiers. Moreover, Aza, Tric, and Valp inhibited the production of some metabolites that were normally produced in MDA media.

3.2. *In vitro* biological activity testing

3.2.1. Antibacterial activity. Eight *A. awamori*-derived extracts were evaluated for their antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa* (Table 1). It was found that all extract derived from epigenetic modifiers treatment were active against *S. aureus* with MIC value of 62.5 μg mL^{-1}, whereas, only Valp and Nic had the ability to inhibit *E. coli* growth with the same MIC value of 62.5 μg mL^{-1}. No extract displayed inhibitory effect against the pathogenic bacterium *P. aeruginosa* (Table 1). These results indicated that a number of specialized metabolites were induced by the epigenetic modifiers which contributed to the observed antibacterial activity toward *S. aureus* and *E. coli*.
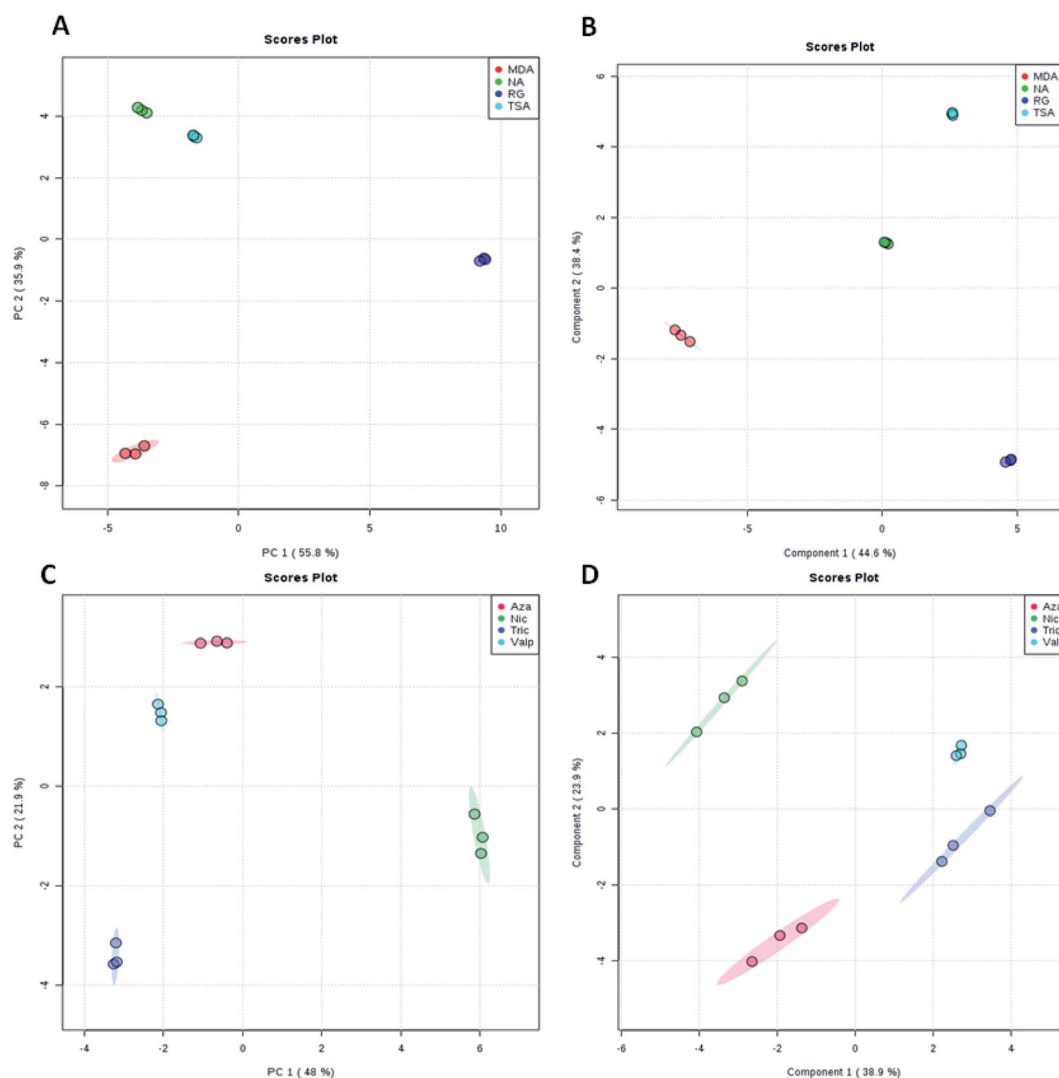

Fig. 1 Score plots representing PCA and PLS-DA results based on the HRMS data obtained for both the four fermentation media (A and B) and the four epigenetic modifiers (C and D) under study.
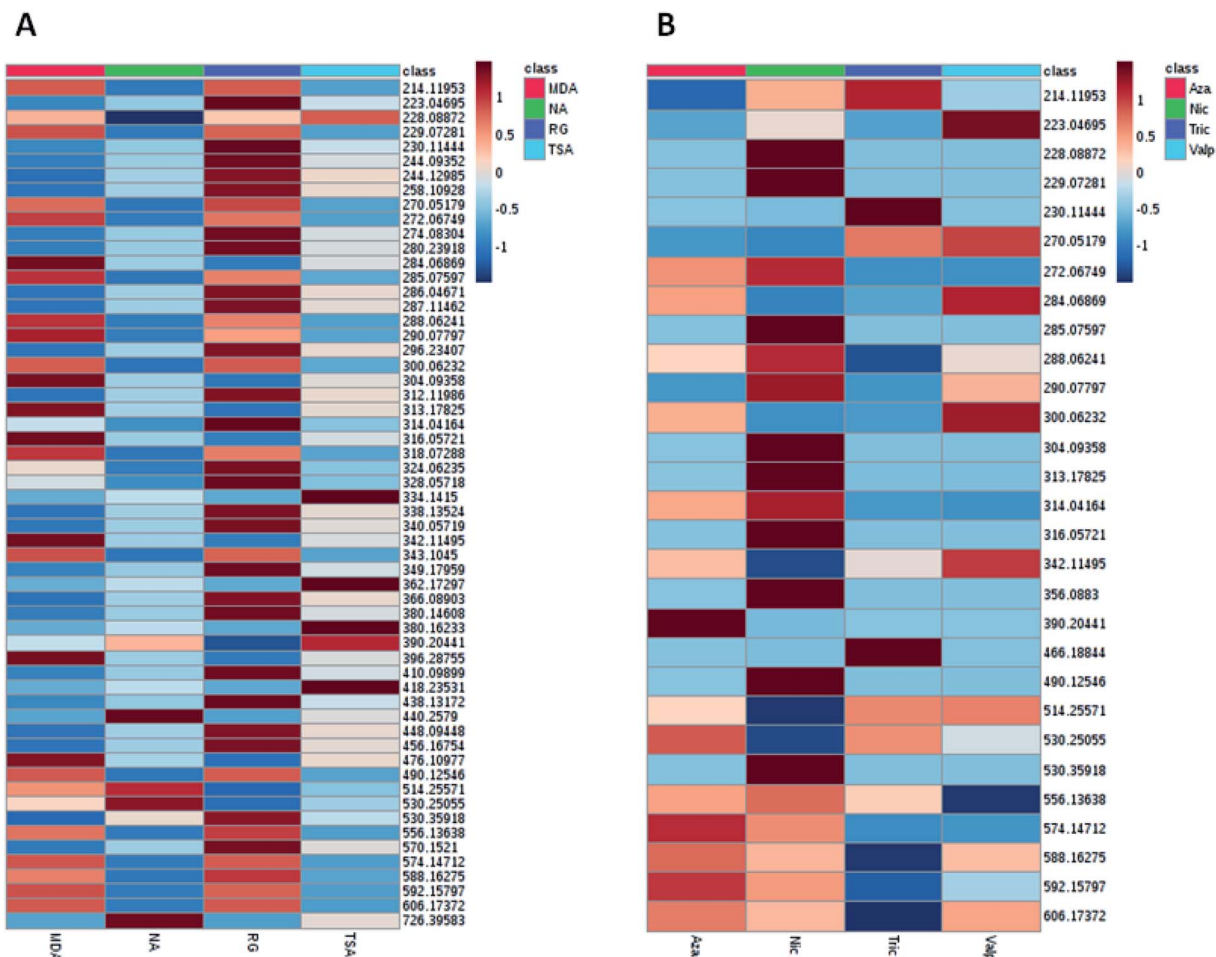
Fig. 2 Heat map generated from LC-HRMS data of *A. awamori*'s extracts displaying distinct metabolic profiles amongst the extracts in each tested culture media (A) and epigenetic modifier (B).

3.2.2. Cytotoxic activity. Similarly, all prepared extracts were tested for their cytotoxicity towards a panel of human tumor cell lines. Table 2 showed that extracts derived from both MDA and RG media and that from Nic treatment were the most active among all extracts (Table 2). Interestingly, RG-derived extract demonstrated strong inhibitory activity against all tested cancer cell lines and with IC_{50} values comparable with the positive control, doxorubicin, indicating that this extract could be investigated further for a potential source of potent anticancer compounds.

3.2.3. Metabolites-bioactivity correlation. Orthogonal projections to latent structures discriminant analysis (OPLS-DA)
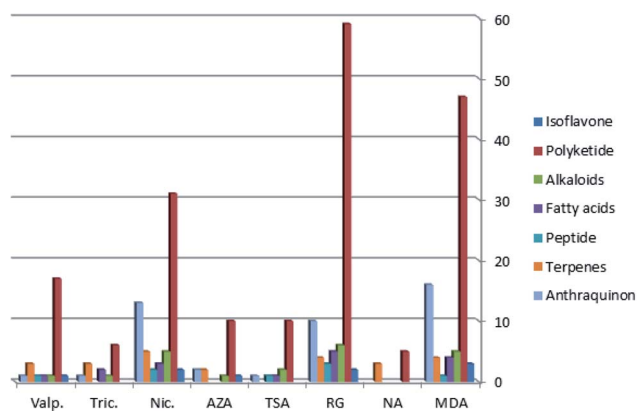

Fig. 3 Chemical diversity of metabolites produced by *A. awamori* in each treatment (different fermentation media and different epigenetic modifiers).

Table 1 Antibacterial activity of *A. awamori*-derived extracts from both fermentation media and epigenetic modifiers treatment[a]

	MIC (μg mL^{-1})		
Tested extract	*S. aureus*	*E. coli*	*P. aeruginosa*
MDA	625	312.5	312.5
NA	312.5	1.25×10^3	1.25×10^3
RG	312.5	312.5	625
TSA	312.5	625	625
Valp	**62.5**	**62.5**	625
Nic	**62.5**	**62.5**	312.5
Tric	**62.5**	1.25×10^3	625
Aza	**62.5**	625	625

[a] NA: no activity.

Table 2 *In vitro* cytotoxicity of *A. awamori*-derived extracts from different fermentation media and epigenetic modifiers treatments

Tested extract	IC$_{50}$ (µg mL^{-1})			
	WI38	HCT116	HePG-2	MCF7
MDA	**25.89 ± 2.9**	**24.10 ± 2.3**	**28.76 ± 2.1**	**29.70 ± 2.9**
NA	83.05 ± 4.1	57.37 ± 3.3	64.16 ± 3.3	73.61 ± 4.1
RG	**19.26 ± 2.5**	**9.39 ± 0.8**	**7.84 ± 0.6**	**11.48 ± 1.0**
TSA	94.26 ± 5.1	38.57 ± 2.6	30.49 ± 2.3	42.65 ± 3.2
Valp	88.15 ± 4.5	53.42 ± 3.1	41.27 ± 2.6	64.73 ± 3.9
Nic	**28.52 ± 2.1**	**17.49 ± 1.5**	**13.78 ± 1.1**	**19.18 ± 1.5**
Tric	86.38 ± 4.3	49.01 ± 2.9	51.35 ± 3.0	62.79 ± 3.7
Aza	77.50 ± 3.8	73.83 ± 3.7	65.84 ± 3.6	87.22 ± 4.6
Dox[a]	6.72 ± 0.5	5.23 ± 0.3	4.50 ± 0.2	4.17 ± 0.2

[a] Doxorubicin.

was applied to explore the correlations between the observed antimicrobial and cytotoxic activities of the eight *A. awamori*-derived extracts, and their metabolites constituent.[26] The generated models showed very good performance (goodness of models, $R^2 = 0.73$–0.92) and prediction (predictive power of models, $Q^2 = 0.75$–0.9). Regarding the antibacterial activity, extracts with MIC values ≤125 µg mL^{-1} were considered active as antibacterial, and the higher values considered inactive. Fig. 4 showed clear separations between active and inactive extracts against both *S. aureus* and *E. coli*. Additionally, active extracts in each case were closely clustered together indicating

that there were common metabolites in them that could contribute to their observed antibacterial activity. The OPLS-DA-derived S-plots (Fig. 4) for the antibacterial activity against *S. aureus* and *E. coli* were used to determine the bioactive discriminating metabolites against both *S. aureus* and *E. coli*. Metabolites with accurate mass of 284.0686 (dereplicated as isoviocristin, compound **11**), 300.0623 (dereplicated as methyl-8-hydroxy-3-methoxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate, compound **16**), 270.0517 (dereplicated as hydroxyviocristine, compound **8**) and 530.2505 (dereplicated as iso-citreohybridone I, compound **138**) were selected to be the most discriminating features (*p*-value ≤ 0.1) in the active groups against *S. aureus*. Hence, these identified metabolites were considered the most influential ones as antibacterial agent against *S. aureus*. Similarly, metabolites with accurate mass of 223.0469 (dereplicated as pyranonigrin A, compound **2**), 290.0779 (dereplicated as altenusin, compound **76**) and 418.2353 (dereplicated as 1-hydroxyyanuthone A, compound **126**) were the most correlated ones (*p*-value ≤ 0.1) to the anti-bacterial activity against *E. coli* (Table 3). It worth noting that the metabolites identified as the most possible antibacterial agents in the active extracts were induced by MDA media or the four used epigenetic modifiers (Fig. 2). On the other hand, extracts exhibited cytotoxic activity with IC$_{50}$ values ≤30 µg mL^{-1} were considered active, and the remaining values as inactive. Similarly, the S-plot (Fig. 4) of the cytotoxic activity indicated the following metabolites (induced by Nic) tensidol A (**4**) (mass = 229.0728), 1,3-bis(2,4-dihydroxyphenyl)-2-propen-1-one,
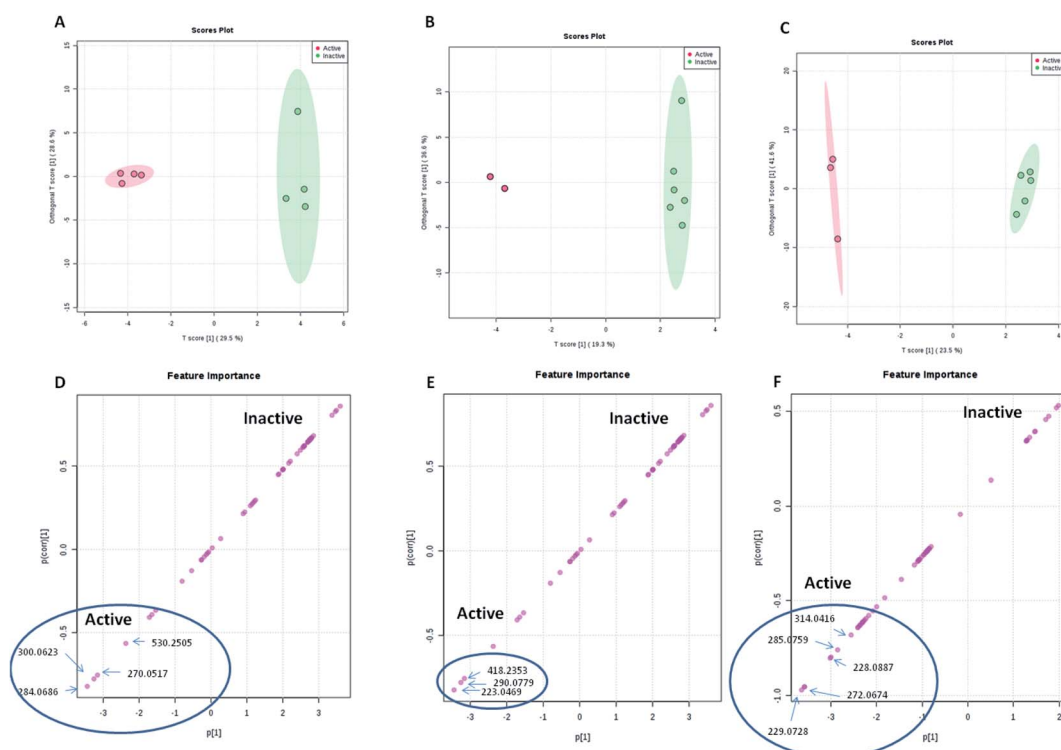

Fig. 4 OPLS score and S-plots of LC-HRMS data of the 8 *A. awamori*-derived extracts tested against *S. aureus* (A and D), *E. coli* (B and E) and human cancer cell lines (C and F).

Table 3 Identified metabolites that showed a possible correlation with the observed biological activity of *A. awamori*-derived extracts

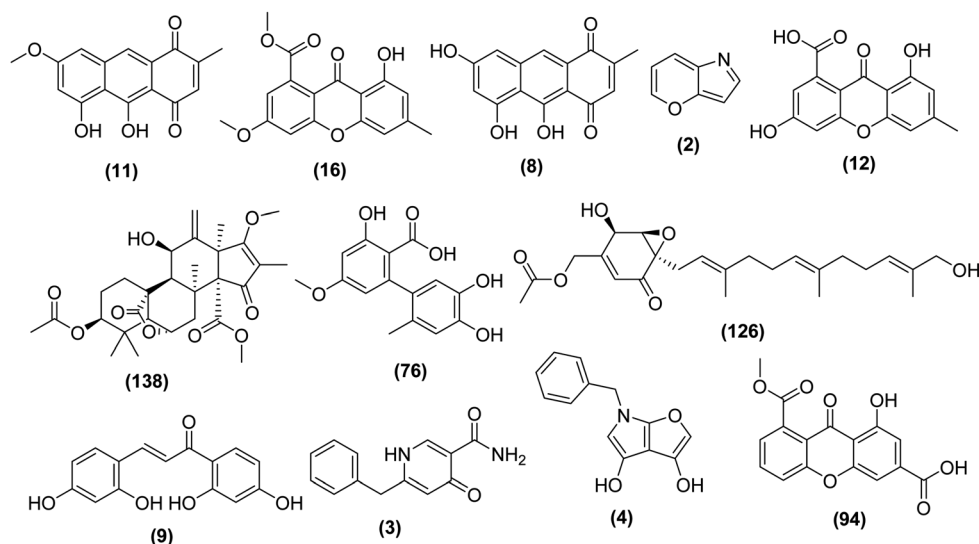| RT	Accurate mass	Molecular formula	Putative identification	Correlated bioactivity	Inducing media or epigenetic modifier
9.50	284.0686	$C_{16}H_{12}O_5$	Isoviocristin	Antibacterial (*S. aureus*)	MDA
3.87	300.0623	$C_{16}H_{12}O_6$	Methyl-8-hydroxy-3-methoxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylate	Antibacterial (*S. aureus*)	Valp
7.74	270.0517	$C_{15}H_{10}O_5$	Hydroxyviocristine	Antibacterial (*S. aureus*)	Valp
6.64	530.2505	$C_{29}H_{38}O_9$	Isocitreohybridone I	Antibacterial (*S. aureus*)	ND
2.68	223.0469	$C_{10}H_9NO_5$	Pyranonigrin A	Antibacterial (*E. coli*)	RG & Valp
4.26	290.0779	$C_{15}H_{14}O_6$	Altenusin	Antibacterial (*E. coli*)	Nic
12.67	418.2353	$C_{24}H_{34}O_6$	1-Hydroxyyanuthone A	Antibacterial (*E. coli*)	TSA
5.4	229.0728	$C_{13}H_{11}NO_3$	Tensidol A	Cytotoxic	Tric
5.34	272.0674	$C_{15}H_{12}O_5$	2,2′,4,4′-Tetrahydroxychalcone	Cytotoxic	MDA & Nic
4.31	228.0887	$C_{13}H_{12}N_2O_2$	Aspernigrin A	Cytotoxic	TAS
5.37	285.0759	$C_{15}H_{10}O_6$	3,8-Dihydroxy-6-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid	Cytotoxic	Nic
4.96	314.0416	$C_{16}H_{10}O_7$	8-(Methoxycarbonyl)-1-hydroxy-9-oxo-9*H*-xanthene-3-carboxylic acid	Cytotoxic	RG

2,2′,4,4′-tetrahydroxychalcone (**9**) (mass = 272.0674), aspernigrin A (**3**) (mass = 228.0887), 3,8-dihydroxy-6-methyl-9*H*-xanthene-1-carboxylic acid (**12**) (mass = 285.0759), and 8-(methoxycarbonyl)-1-hydroxy-9-oxo-9*H*-xanthene-3-carboxylic acid (**94**) (mass = 314.0416) as the most contributing ones to the cytotoxic activities of the active extracts (Table 3 and Fig. 5). Further, the isolation of these bioactive metabolites will need a future scale-up experiment.

4. Discussion

The genus *Aspergillus* comprises a huge number of species that are capable of producing plentiful metabolites with varied structural features and diverse biological activities.[27] The bioactive constituents of *Aspergillus* species are still under-explored, despite being widely distributed. A variety of natural products with intriguing biological activities such as alkaloids, polyketides, peptides, terpenes, and lignans have been isolated from *Aspergillus* sp.[28]

Searching for new bioactive metabolites from microbial sources constitutes a track for many natural products laboratories. So far, as an initial step of the natural products discovery process, most or all microbial strains are passing through a standard culture procedure, the output and success of a natural product screening program would mainly depend on the optimization of this initial step and the microbial culture.[29]

Fig. 5 Structures of identified metabolites that showed a possible correlation with the observed biological activity of *A. awamori*-derived extracts.

Furthermore, it has been confirmed that fermentation parameters have varying effects on the fungal secondary metabolites production,[30] such as the yields of a specific bioactive metabolite in a microorganism which could be optimized through variation of culture conditions.[31]

On the other hand, biotic and abiotic environmental factors affect expression of secondary metabolites associated genes.[32] Under standard laboratory parameters, Most of gene clusters that control secondary metabolism in fungi are often silent. Several strategies have been developed in order to activate such cryptic gene clusters in fungi.[33] Histone modification is one of the ubiquitous epigenetic mechanisms inducing gene transcription through histone proteins manipulation that maintains chromatin structure either in an accessible or inaccessible state to different transcriptional activators for their binding to gene promoters.[34] The chromatin became more open and accessible for transcription factors when histones are acetylated by histone acetyltransferases (HATs), which in turn increase the expression of secondary metabolites producing genes. Conversely, chromatin condensation and gene inactivation obtained by histone deacetylation which is performed by histone deacetylases (HDACs).[35] On the other hand, DNA methyltransferase (DNMT) catalyzes the transfer of methyl groups from S-adenosylmethionine (AdoMet, SAM) to the C-5 position of cytosine. So, inhibiting DNMT has an anti-proliferative effect, so far DNMT inhibition leads to the activation of certain silent biosynthetic clusters.[36] Recently, the ability of small molecules to induce secondary metabolites expression through inhibition of HDACs and DNMTs has been revealed in fungi.[37]

Metabolomics is a complete investigation of the small molecules with molecular weight < 1000 Da in a biological system at a specific time and under a certain set of conditions.[38] LC-HRMS based metabolomics is an effective analytical tool to check compound mixtures or extracts in a biological system to prioritize in the discovery of new natural products.[39] Statistical approaches like supervised and unsupervised multivariate data analysis have been used to examine the variations between different sample groups in terms of their m/z ratio in HRMS.[40] Optimization of the best fermentation conditions for microbial isolates to yield their bioactive secondary metabolites were performed using metabolomic tools.[41] Additionally, metabolomics is used to investigate and compare intra- and extracellular produced secondary metabolites.[42]

In this study, LC-HRMS based metabolomics as well as bioassay data coupled with multivariate statistical analysis were utilized to evaluate the effect of four different fermentation media and four different epigenetic modifiers treatments on the production of secondary metabolite patterns by the river Nile derived fungus *A. awamori* and to find correlations between the bioactivity of *A. awamori* extracts and their associated constituents. Results revealed that RG was found to be the best medium in terms of diverse secondary metabolites induction, followed by MDA. Additionally, there were common induced metabolites that were detected exclusively in these two media. Such results agreed with published reports which indicated that rice would serve well as a standard/efficient fungal culture media.[43]

Concerning the epigenetic modifiers treatments, Nic was found to be the best metabolite inducer among the other epigenetic modifiers. These results coincided with previous reports that investigated the production of bioactive secondary metabolites by the sponge-derived fungus *Penicillium brevicompactum* under the effect of two different histone deacetylase (HDAC) inhibitors; nicotinamide and sodium butyrate, and revealed the potential impact of nicotinamide in the induction of cryptic fungal genes for new secondary metabolites production.[10]

On the subject of DNMT inhibitor, 5-azacytidine treatment was not up to the level of metabolites diversity, in contrast to what expected from previous reports which pointed out its significance in secondary metabolites production in *Aspergillus oryzae*. Moreover, 5-azacytidine inhibited the production of some metabolites that were normally produced in MDA media.

Regarding the bioactivity, all extract derived from epigenetic modifiers treatment were active against *S. aureus* with MIC value of 62.5 µg mL^{-1}, whereas, only Valp and Nic were able to inhibit *E. coli* growth with MIC value of 62.5 µg mL^{-1}. These results indicated that there were a number of specific metabolites that were induced by the epigenetic modifiers which contributed to the observed antibacterial activity toward *S. aureus* and *E. coli*. OPLS-DA score and S-plots of LC-HRMS data of the *A. awamori*-derived extracts tested against *S. aureus* and *E. coli* were used to determine the bioactive discriminating metabolites, among these metabolites; altenusin which has been reported to display broad antimicrobial activity against several additional multidrug-resistant bacterial and fungal strains.[44]

On the other hand, extracts derived from both MDA and RG media and that from Nic treatment were the most active ones against cancer cell lines. Interestingly, RG-derived extract demonstrated strong inhibitory activity against all tested cell lines with IC$_{50}$ values comparable with the positive control doxorubicin. Similarly, the S-plot of the cytotoxic activity indicated that few metabolites are the most contributing ones to the cytotoxic activities of the active extracts. However, nothing could be traced about the cytotoxic activity of these metabolites, and hence, further optimization and isolation of these cytotoxic metabolite candidates would need another scale-up in a future study.

Multivariate data analysis (MVDA) is applied to summarize the large amount of results revealed by metabolomics study. MVDA can be applied either as supervised or unsupervised analysis method. The unsupervised method is illustrated by principal component analysis (PCA) while the supervised method is demonstrated by partial least square (PLS) projection to latent structures (alternatively, orthogonal partial least square projection to latent structures differential analysis (OPLS-DA)). PCA is an efficient tool of unbiased reduction of dimensionality that reveals cluster structures or grouping where variability between groups is higher than within groups. PLS algorithms allow visualizing the separation between groups. The application of PCA provides fair conclusions on data variations and similarities, which could be confirmed on PLS plots. PCA and PLS results are presented in score and loading plots.[42]

Here, the OPLS-DA loading S-plot was used to predict the bioactive discriminating metabolites against both *S. aureus* and *E. coli*. Similarly, the S-plot of the cytotoxic activity indicated the few metabolites (induced by Nic) as the most contributing ones to the cytotoxic activities of the active extracts. Targeted isolation work of these predicted bioactive metabolites is considered intriguing subject in future studies.

5. Conclusion

In our efforts to find optimum conditions for scaling up the culture of the river Nile-derived fungus *A. awamori*, metabolomics-based LC-HRESIMS analysis of *A. awamori*-derived extracts under different fermentation conditions, coupled with extensive multivariate analysis were utilized to investigate the induced metabolites in each fermentation condition. A total of 1587 peaks were detected for all extracts under study, from which the known metabolites in the extracts were dereplicated using different natural products databases. Multivariate analysis of the LC-HRESIMS data indicated that RG was the best medium in terms of diverse secondary metabolites induction. Meanwhile, metabolomic profiles of *A. awamori* in each epigenetic modifier treatment were less effective than RG medium, and comparable to MDA medium in their ability to induce diverse metabolites. Nicotinamide was found to be the best metabolite elicitor among the studied epigenetic modifiers. Additionally, *in vitro* antibacterial and anticancer screening of these extracts was performed. The OPLS-DA-derived S-plots were used to determine the bioactive discriminating metabolites which considered the most contributing ones to the cytotoxic and antibacterial activities of the active extracts. Further optimization and isolation of suspected new metabolites candidates from RG medium and the bioactive ones from Nic treated medium is recommended as a promising future study.

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Conflicts of interest

The authors declare no conflict of interest.

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