ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR
Tawfik et al., 2017, 1 (2), 100-109

Journal of Advanced Pharmacy Research

Metabolomics and Dereplication Study of the Endophytic Fungus *Aspergillus chevalieri* in Search of Bioactive Natural Compounds

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Submitted on: 23-02-2017; Revised on: 03-03-2017; Accepted on: 05-03-2017

ABSTRACT

Objectives: This study aimed to point the significant rule of metabolomics tools to assess the chemistry of the bioactive metabolites produced by endophytic fungus *Aspergillus chevalieri* isolated from *Lagerostromia tomentosa* C. presl. The anticancer of crude extracts, fractions and pure compounds and antimicrobial of pure compounds were investigated as part of this study. **Methods:** An endophytic fungus (*Aspergillus chevalieri*) was isolated from the tissues of the stem of *Lagerostromia tomentosa* C. Presl and identified through molecular biological procedure by DNA isolation, PCR, DNA sequencing and through searching the Gene Bank. Metabolomics profiling and dereplication studies were employed to choose the optimum growth medium and conditions that yield the most significant metabolites. The crude extract of the 30-days rice culture of *Aspergillus chevalieri* was subjected to bioactivity and metabolomics guided isolation approach. The structure of the isolated compounds was determined on the basis of 1D, 2D NMR and mass spectrometry (HR-ESIMS) analysis. **Results:** four fractions were further purified to produce five pure compounds, which are Ergosterol (1), Ergosterol peroxide (2), Campesterol (3), Flavoglaucin (4) and 3-*O*- methyl caffeic acid (5). Multivariate data analysis highlighted the most significant metabolites contributed to the bioactivity. The pure compounds were tested for the anticancer and antimicrobial activity, compound (1) exhibited significant anti-trypanosomal activity, while compounds (2, 3, 4 and 5) effectively inhibited the growth of *Escherichia coli, Staphylococcus aureus and Candida albicans.* **Conclusion:** A combination of metabolomic- and bioassay-guided approaches gives an access to a shorter and faster route to highlight the active metabolites, which are highly correlated to the bioactivity during the first stage of fractionation.

Keywords: *Antitrypanosomal activity; Aspergillus chevalieri; Cytotoxic activity; Dereplication*; *Lageorstroemia tomentosa; Lyceraceae; Metabolomics.*

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INTRODUCTION

Lagerstroemia is an important member of family Lytheraceae, which contains thirty one genera. Many species of genus *Lagerstroemia* are known to have an important medicinal uses as anti-oxidant, antibacterial, antiviral, anti-inflamatory, antinociceptive, anti-diarrhoeal, cytotoxic, xanthine oxidase inhibition and anti-obesity, also the leaf extract shows anti-diabetic activity due to presence of ellagitannins and corosolic acid[1].

Aspergillus is one of the most popular and common endophytic fungi found to be associated with natural sources either marine or terrestrial habitat, it belongs to Ascomycetes fungi[2,3]. Endophytes are a highly diverse group of fungi capable of living

symbiotically inside plant tissue without causing apparent symptoms of diseases[4]. Endophytes might be involved in the biosynthesis of plant products; however, they might also be themselves the producers of many substances of potential use in the modern medicine, agriculture and pharmaceutical industry[5, 6]. An area of major interest is to explore endophytic secondary metabolites as novel anticancer and antimicrobial agents.

Since Human African Trypanosomiasis "HAT" (sleeping sickness) is an endemic disease in thirty African countries with population at risk being about 60 million. It caused around 9000 deaths per year 2010, down from 34000 in 1999, which was motivated us to search for significant antitrypanosomal drugs. The infection of HAT is caused by protozoan parasites belong to the genus *Trypanosoma* which are transmitted to humans by Tsetse fly (*Glossina* genus) bites[7]. It has two stages: the first one is the haemolymphatic stage which lasts for one to three weeks, followed by the chronic stage in which trypanosomes cross the blood–brain barrier to invade the central nervous system resulting in chronic meningo-encephalitis and eventually leads to encephalopathy[8].

Metabolomics is the technique designed to deliver general qualitative and quantitative profiles of metabolites in organisms exposed to various conditions. Plants and microorganisms produce many metabolites with different chemistry and bioactivity under stress conditions. Metabolomics gives access to figure out the complex relationships between the endophytes and their host plants which aids to discover novel bioactive natural components[9]. The metabolome is the complete set of small molecules found in a cell, tissue or organism at a certain point in time. Dereplication is the process of testing sample mixtures which in screening showed activity in order to recognize the novel compounds out of the studied active substances. Dereplication was accomplished by employing differential expression analysis softwares like MZmine which involves dictionary of natural products database (DNP) to aid in compound identification[9]. By using combinations of analytical, statistical and dereplication methods, the bioassay-guided isolation route is getting shorter and rapid dereplication of known activities is rapidly delivered[10].

MATERIALS AND METHODS

General instruments

^{1}H, ^{13}C and 2D-NMR were recorded at 25°C in DMSO-d6 using Nuclear Magnetic Resonance spectroscopy machine JNM-LA400 model (JEOL, Japan) and the magnet NMR AS400 model EUR0034 (Oxford) Instruments, England at Strathclyde Institute of Pharmacy and Biomedical Science and an AVANCE-III 600 instrument with a 14.1 T Bruker Ultra Shield magnet at Chemistry Department, Faculty of Science, Strathclyde University. ESI-HRMS was measured using FTHRMS-Finnigan LTQ Orbitrap and Exactive mass spectrometer (Thermo Scientific). HPLC analysis was carried out using DionexUltiMate 3000-Thermo Scientific Exactive system instrument, Germany. Crude extracts were initially fractionated using medium pressure liquid chromatography (MPLC) (BÜCHI, Germany), MPLC instrument was the Sepacore Purification System with Versaflash column stand. The Reveleris® Flash Forward system of Grace Davison Discovery Sciences (Illinois, United States) was also used for further isolation, which is characterized of having two detectors, an evaporative light scattering detector (ELSD) and a UV detector (wavelength range: 200-500 nm). The fractions were investigated on normal phase thin layer chromatography plates (TLC silica gel 60 F254), reverse phase TLC plates (TLC silica gel 60 RP-18 F254S) and fractionated using preparative TLC plates (TLC silica gel 60 F254 on 20x20 cm aluminium sheets) (Merck KGaA, Germany). Spots were visualized under UV lamp (short λ nm 254 and long λ nm 380) and after spraying with anisaldehyde and heating till the colour development.

LC-MS spectra were viewed using ThermoXcalibur 2.1 (Thermo Scientific, Germany). To convert the raw data into separate positive and negative ionization files, a program called MS convert from Proteo Wizard was used. The files were then imported to the data mining software MZmine 2.10 for peak picking, deconvolution, deisotoping, alignment and formula prediction[9.] Macro file with built in databases was written in Excel, used to combine positive and negative MS files and for further clean-up of media components[11]. The databases used for the identification of compounds were the Dictionary of Natural Products (DNP) 2015, MestReNova (MNova) 2.10 by Mestrelab Research, S.L, (Santiago de Compostela, Spain) was used to process all NMR data and SIMCA 14 (Umetrics AB, Umeå, Sweden) was used for multivariate data analysis.
Laminar flow hood (BioMAT2) (Medical Air Technology, UK), the stand incubator (Incu-160S) ,used for agar plates, (SciQuip Ltd., Shropshire), the homogenizer (IKA T18 Basic Ultra-Turrax) and handheld homogenizer (Ultra-Turrax T8) (IKA Labortechnik, Germany) were used for microbiological part.

Plant material

Fresh plant (*Lagerstroemia tomentosa*) was collected from El-Zohria Botanical Garden in Giza, Egypt and identified by Dr. Therese L. Yousef, senior taxonomist at Orman Garden and by Engineer Mervat A. Hasan, herbarium curator at Orman Botanical Garden.

Voucher specimens (000021 LC 06-01-03-27) have been deposited in herbarium of Orman Botanical Garden. Fresh plant materials including all arial parts of the plant were collected a day before isolation of fungal strains, kept in zipped plastic bags under 4ºC for the isolation work.

Culture media

Wickerham medium (yeast extract 3.0 gm, malt extract 3.0 gm, peptone 5.0 gm, glucose 10.0 gm and distilled water to 1000 mL with pH adjusted at 7.4) was used as a liquid culture for the endophyte. Whereas solid medium composed of 100 gm Rice and 100 ml distilled water.

Cell lines and culture media for cytotoxic assay

Lung cancer cells (A549), Prostatic cancer cells (PC3), breast cancer (ZR75), ovarian cancer cells (A278O) and normal epithelial cells derived from human prostate (PNT2A cells) were purchased from ECACC (Sigma-Aldrich, Dorset, UK). A549 and PC3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and PNT2A, ZR75 and A2780 cells were cultured in RPMI 1640 media; both were supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine and 50 µ g/mL penicillin/streptomycin solution (all Invitrogen, Paisley, UK) in a humidified incubator at 37 ºC in the presence of 5% CO_2. Cells were routinely passaged at 90%–95% confluence.

Isolation of the endophytes

The leaves and stems of the plant were rinsed with sterilized distilled water twice. Surface sterilization was carried out by immersing leaves and stems in 70% isopropanol for 2 min (twice) followed by rinsing again with sterilized distilled water (twice). That was done in order to eliminate surface contaminating microbes. With a sterile scalpel a small segment of leaves and stem tissue (1 cm in length) was cleaned from outer tissue, the inner tissues were carefully dissected under sterile conditions and placed on malt agar plate containing antibiotic to suppress bacterial growth (medium composition: 15 gm agar (Oxoid), 15 gm malt extract (Oxoid) and chloramphenicol (Acros organics, purity > 98%) in distilled water, pH was adjusted to (7.4-7.8) and incubated at 30ºC. After 3-4 weeks, hyphal tips of the fungi were removed and transferred to fresh malt agar medium. Plates were prepared in duplicates to eliminate the possibility of contamination. Pure strains were isolated by repeated inoculation. The purified fungus was later transferred to the liquid medium for scaling up[12].

Identification of fungal strain

The fungus was isolated as *Asperagellus chevellieri* according to molecular biological procedure by DNA extraction, amplification and sequencing of the ITS region15. BLAST search of the FASTA sequence was performed with the option "nr", including GenBank, Ref Seq Nucleotides, EMBL, DDBJ and PDB sequences on the BLAST homepage, NCBI, Bethesda, USA. The accession number was LT670923.

Five different endophytic fungi isolated from different parts of *L. tomentosa* were identified as *Phoma Sp., Chaetomium bostrychodes, Alternaria alternata* (from the leaves), *Medurella fahi* and *Aspergillus chevalieri* (from the stems). *A. chevalieri* was selected as it was the most interesting being of unique chemical nature in the dereplication and metabolomic study among all endophytic extracts as well as being biologically active as anti-cancer against ovarian cancer A278O cell line.

Small-scale extraction for screening, metabolomics profiling and dereplication

A plate of each fungal species was transferred into 250 mL flask, then macerated with ethyl acetate and left overnight followed by homogenization and filtration. The mycelia were macerated rice with 200 ml ethyl acetate and filtered. The filtrate was then dried under vacuum. One mg of each extract was subjected to HRMS analysis and 8-10 mg to NMR analysis for metabolomics profiling and dereplication studies. A sample of 1 mg/mL concentration of each fungal extract was prepared in duplicate and was subjected to bioassay against ovarian cancer (A278O), lung cancer (A549), prostatic cancer (PC3) and breast Cancer (ZR75) cell lines.

Fermentation, extraction and isolation

Fresh fungal cultures were transferred into Erlenmeyer flasks (1L each) containing 100 g rice for solid cultures, then incubated at room temperature as stand cultures for 30 days. That was repeated several times to optimize the perfect growth conditions (culture type and incubation period). Moreover, large-scale cultivation was carried out using 20 one-L Erlenmeyer flasks for rice cultures, then 250 ml EtOAc was added to Erlenmeyer flasks and left overnight to stop cell growth. Culture media and mycelia were then homogenized in the Ultraturrax for 10 min for cell destruction, followed by filtration using a Buchner funnel. The mycelium residue was discarded while EtOAc culture filtrates were collected, pooled, dried under vacuum[13]. The dried ethyl acetate extract (10gm) was dissolved in 10% aqueous MeOH and partitioned in a separating funnel with n-hexane as a defatting step. The n-hexane (8.2gm) soluble layer was concentrated and subjected to further fractionation a Grace flash chromatography instrument using silica gel cartridge 80g with n-hexane and EtOAc gradient elution to yield compound 1, compound 2 and compound 3. The MeOH soluble portion (880 mg) of the EtOAc extract was then dried and applied to a Grace flash chromatography instrument using silica gel

cartridge 24g with n-hexane and EtOAc gradient elution to givr F4-5 and F36-47 which were purified on a Grace silica cartridge 4g employing n-hexane/EtOAc as a gradient elution system started at 30% EtOAc reaching 100% EtOAc in 45 min to give compound 4, compound 5.

Cytotoxic activity

Cells were seeded in clear 96 flat-bottomed plates and allowed to adhere overnight. After that time, metabolite extracts and fractions were added at a final concentration of 30 µg/mL, and at a concentration of 10mM/ml for the pure compounds and allowed to incubate for 48 hours. Viability was determined using Alamar Blue® (Thermo Fisher, Paisley, UK), according to the manufacturer's instructions and incubated for a further 6 h. The resulting fluorescence was measured using a Wallac Victor 2 1420 multi-label counter (Perkin Elmer, Beaconsfield, UK), in fluorescence mode: excitation 560, emission 590.Vehicle treated control cells (media with 0.3% DMSO) were considered 100% viable against which metabolite extract treated cells (at a concentration of 30 µg/mL, at least n = 2) were compared. All results were confirmed microscopically[14].

Antitrypanosomal activity

Antitrypanosomal activity was tested following the protocol of Huber and Koella[15]. *Trypanosoma brucei brucei* strain TC 221 were cultivated in Complete Baltz Medium with concentration of 10^4 Trypanosomes per ml. Trypanosomes were tested in 96-well plate chambers against different concentrations of test substances at 0.25–50 µM in 1% DMSO to a final volume of 200 µL. For controls, 1% DMSO as well as parasites without any test compound were used simultanously in each plate to show that DMSO did not perturb the results. The plates were then incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. After addition of 20 µL of Alamar Blue, the activity was measured after 48 and 72 h by light absorption using an MR 700 Microplate Reader (Dynatech, Chantilly, United States) at a wavelength of 550 nm with a reference wavelength of 650 nm The IC_{50} values of the test compound were quantified by linear interpolation of three independent measurements.

Anti-microbial activity

Evaluation of Antimicrobial activity was carried out using a modified Kirby-Bauer disk diffusion assay[16,17], against pathogenic bacteria (*Staphylococcus aureus* strain 12600 and *Escherichia coli* strain 11775) and Fungi (*Candida albicans* strain 7102). Standard discs of Ampicillin (Antibacterial agent), Amphotericin B (Antifungal agent) served as positive controls for antimicrobial activity and a filter discs impregnated with 10 µl of solvent (DMSO) were used as a negative control.

RESULTS

Compound (1)

White needles (10 mg), ^{1}H-NMR (DMSO, 400 MHz), ^{13}C-NMR (DMSO, 100 MHz) data presented in table 1; ESIHRMS(pos): m/z [M-H]$^-$ 395.3309 (calcd. for $C_{28}H_{44}O$). Thus compound 1 was assigned in accordance to the reported data[18] as **(**22E, 24R)-Ergosta-5,7,22-trien-3β-ol) **ergosterol**.

Compound (2)

White needles (24 mg), ^{1}H-NMR (DMSO, 400 MHz), ^{13}C-NMR (DMSO, 100 MHz) data presented in table 1; ESIHRMS (pos): m/z 429.3368 [M+H]+ (calcd. for $C_{28}H_{44}O_3$). Thus compound 2 was assigned in accordance to the reported data[18] as (5,8-Epidioxy-5α, 8α-ergosta-6,22-dien-3β-ol) **ergosterol peroxide**.

Compound (3)

White needles(27 mg), ^{1}H-NMR (DMSO, 400 MHz), ^{13}C-NMR (DMSO, 100 MHz) data presented in table 1; ESIHRMS (pos): m/z 399.3621 [M+H]+ (calcd. for $C_{28}H_{46}O$). Thus compound 3 was assigned in accordance to the reported data[19]as ((24R)-5-Ergosten-3β-ol) **Campesterol**.

Compound (4)

Yellow needles (9mg), ^{1}H-NMR (DMSO, 400 MHz), ^{13}C-NMR (DMSO, 100 MHz) data presented in table 1; ESIHRMS(pos): m/z 304.2027 [M+H]+ (calcd. for $C_{19}H_{28}O_3$). Thus compound 4 was assigned in accordance to the reported data[20]as **Flavoglaucin**.

Compound (5)

A white amorphous powder (9 mg), ^{1}H-NMR (DMSO, 400 MHz), ^{13}C-NMR (DMSO, 100 MHz) data presented in table 1; ESIHRMS (neg): m/z 193.0545 [M-H]-(calcd. for $C_{10}H_{10}O_4$). Thus compound 5 was assigned in accordance to the reported data[21]as **3-O- methyl caffeic acid**.

DISCUSSION

ESI-MS data produced by Excel-macro database file after combining positive and negative modes and removing the media effect, was subjected to R software to apply the heatmap script. The heat map of all extracts showed that *Aspergillus chevalieri* extract and *Alternaria alternate* fungal extracts were the richest in metabolites of different mass range as shown in figure 1**.** The cytotoxicity assay showed that *Aspergillus chevalieri* was active against A278O cell line and non-toxic for the normal cells PNT2A (Figure 2)**,** implying that *Aspergillus chevalieri* could have a unique chemical and biological fingerprints.

A. chevalieri was then cultivated on small scale solid and liquid cultures to test the optimum growth condition producing the highest amount of interesting metabolites. HRESI-MS data of crude extracts of both rice (RC) and liquid (LC) culture media of *A. Chevalieri* have been subjected to a metabolomics work flow starting with data mining by MZmine. The heatmap for the processed ESI-MS data of both RC and LC extracts of *A. Chevalieri* showed more abundance of metabolites in the 30 days RC extract (Figure 3). Moreover, Multivariate data analysis (MVDA) of different culture extracts of *A. chevalieri*, performed by SIMCA-P V.14 software, discriminated 30-days RC extracts from other fungal extracts as shown in the PCA score plot (Figure 4a) which was indicative of the unique nature of the metabolites produced in RC-30 extract. PCA loading plot (Figure 4b) illustrated the metabolites which could be contributed to the variation of 30 days RC extract.

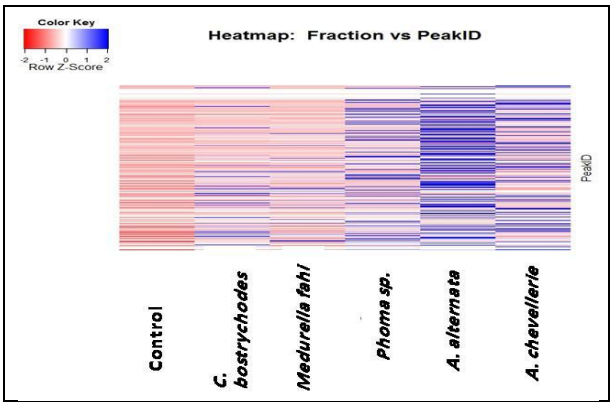

Figure 1. Heatmap of ESI-MS data of all endophytic extracts isolated from *L. tomentosa* (the blue lines represented the produced metabolites).

These metabolites were dereplicated by searching DNP 2015 as shown in table 2. Most of these metabolites were reported previously in the literature however metabolites at m/z (retention time in minutes) 581.562 $[M+H]^+$ (35.62), 615.528 $[M+H]^+$ (35.82), 642.528 $[M+H]^+$ (32.95), 767.537 $[M-H]^-$ (31.83) and 788.549 $[M-H]^-$ (36.02) were not identified in the database. This was motivating for further work on 30 days RC extract, since it was the most active against the selected cancer cell line and showed no toxicity toward normal cells, 30 days RC was chosen for scale up and further isolation work.

The thirty-day liquid culture extract of *A. chevalieri* was subjected to fractionation using MPLC, the resulted fractions were imported into SIMCA for MVDA. The bioassay guided MPLC fractionation of the 30-days rice culture extract sorted the active from the inactive fractions according to their activity toward

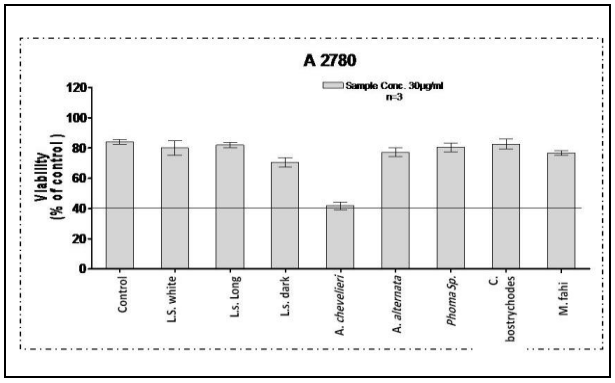

Figure 2. Cytotoxic activity of all endophytic extract isolated from *L. tomentosa*.

cancer cell line A278O (Figure 7). Fractions have been classified into active and inactive in OPLS-DA analysis. OPLS-DA score plot (Figure 5a) displayed a clustering of fractions 4-5 and 6-24 in the active side. The respective OPLS-DA loading plot (Figure 5b) showed that fraction 4-5 and 6-24 were characterized by these metabolites m/z 153.055 $[M+H]^+$, 291.124 $[M-H]^-$, 301.181 $[M-H]^-$ (which were identified in DNP as 2- or 4-hydroxyphenylacetic acid, Curvularin and Auroglaucin; 1',2',3',4'-Tetrahydro respectively. The S-plot of active versus inactive fractions showed the most significant metabolites highly correlated to the cytotoxicity of active fractions (Figure 5c). These metabolites were dereplicated as shown in table 3. The bioactivity reported for these metabolites revealed that metabolites at m/z (retention time in minutes) 153.05 $[M+H]^+$ (5.99), 291.124 $[M-H]^-$ (12.04), 301.181 $[M-H]^-$ (24.54) equivalent for $C_8H_8O_3$, $C_{16}H_{20}O_5$ and $C_{19}H_{26}O_3$ respectively, had cytotoxic and/ or antioxidant activity against different types of cancer cell lines[22,23,24]. This confirmed the efficiency of metabolomics in predicting the bioactive metabolites at first stage of fractionation. However, the rest of significant metabolites in table 3 were not reported to have anticancer activity hence further purification of the active fractions was fundamental to confirm the structure of the previously reported bioactive compound and test the cytotoxicity for the unreported metabolites.

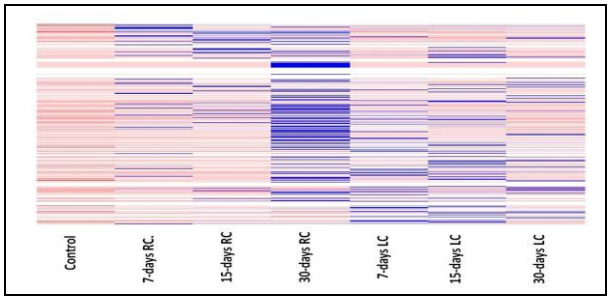

Figure 3. Heatmap for liquid and rice culture extracts of *A. chevalieri* (the blue lines represented the produced metabolites).

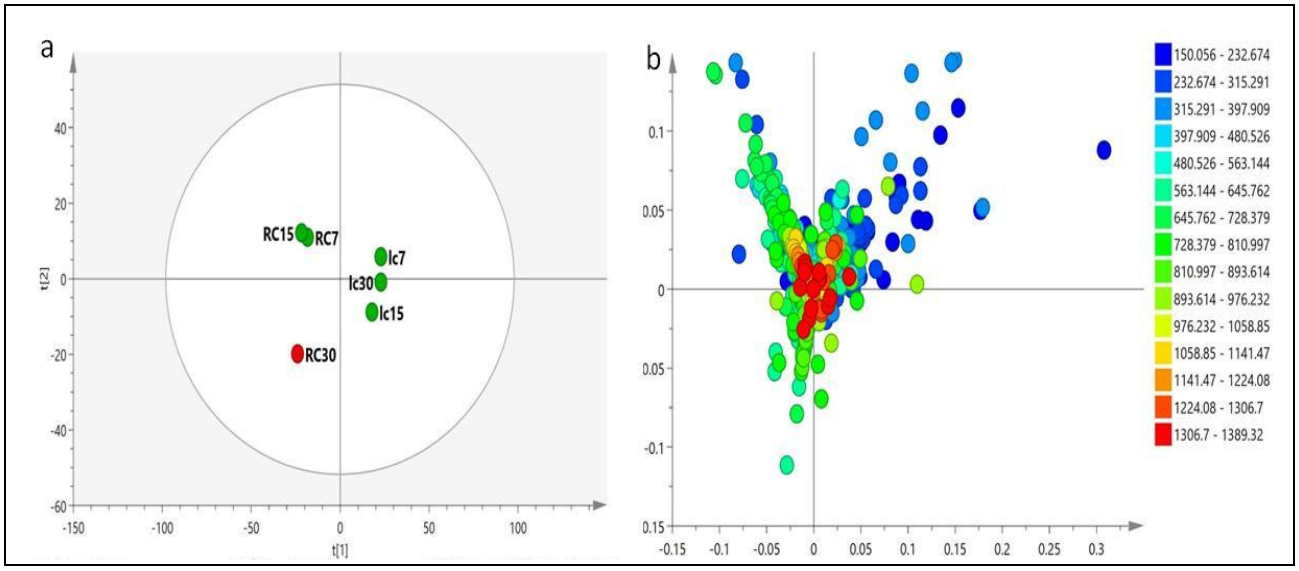

Figure 4. a: PCA score plot of different extracts from solid and liquid fungal culture of *A. chevalieri*., b: PCA loading plot showing metabolites contributed in 30 days rice culture of *A. chevalieri*.

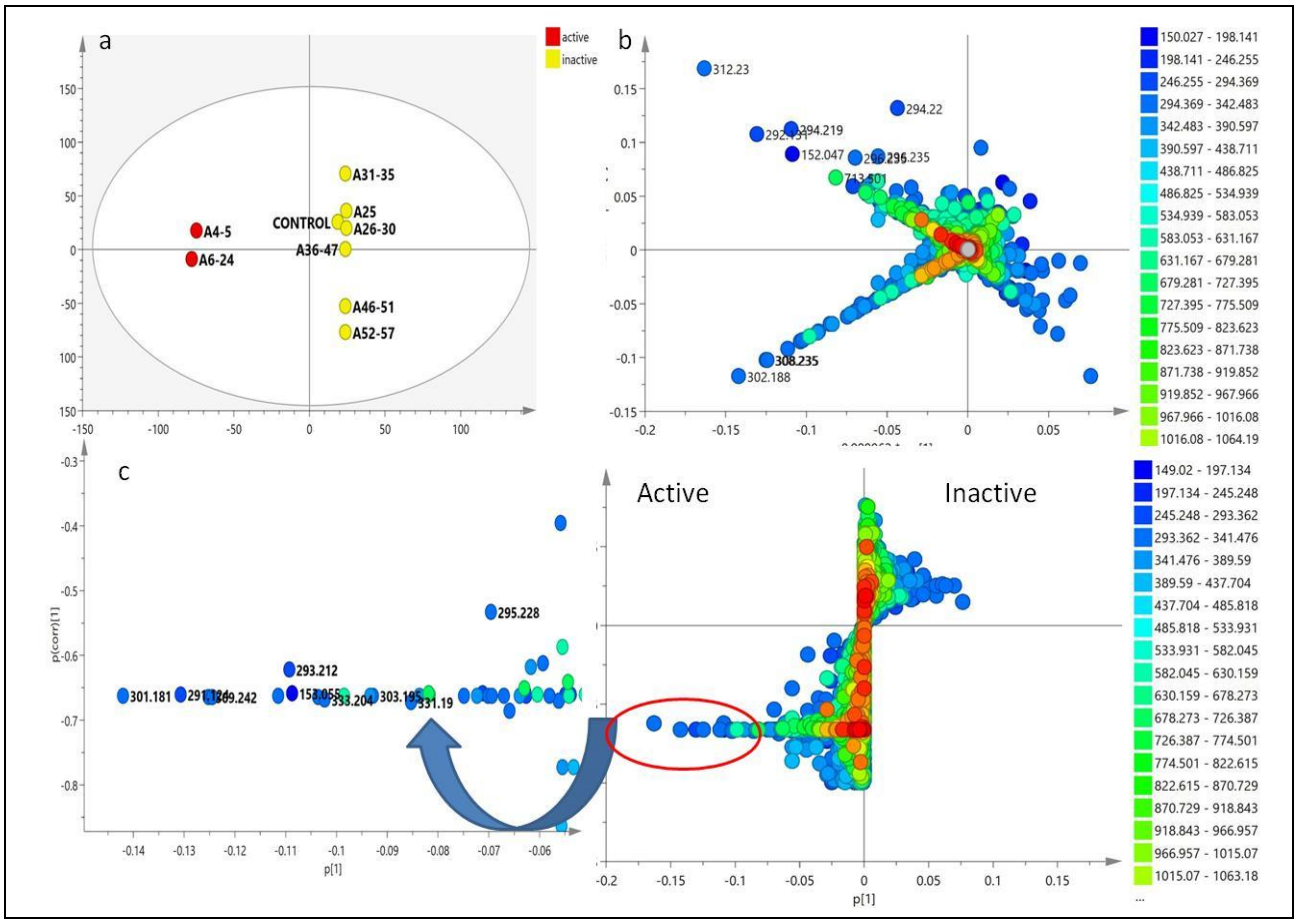

Figure 5. a: OPLS-DA score plot of active versus inactive fractions from 30 days rice culture extract of *A. chevalieri*, b: OPLS-DA loading plot highlighting the features corresponding to the active fractions, c: S-plot of active versus inactive fractions showing the metabolites highly correlated the anticancer activity of *A. chevalieri*.

Table 1. ^{1}HNMR and ^{13}CNMR data of isolated compounds (1-5)

Atom No.	Compound (1)		Compound (2)		Compound (3)		Compound (4)		Compound (5)	
	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C
1		29.7	1.57, 1.72 (m)	37.1 (CH_2)		37.3 (CH_2)		118.02		126.3
2		32.0	1.62, 1.23 (m)	30.5 (CH_2)		29.8 (CH_2)		153.6	7.28(d, 1.87Hz)	111.5
3	3.57 (m)	70.2	3.57 (m)	65.2 (CH)	3.51 (m)	71.9 (CH)		127.2		148.7
4		40.8	1.83, 1.80 (m)	37.5 (CH_2)		42.3 (CH_2)	6.98(s)	125.6		149.6
5		141.3		82.16 (C)		140.8 (C)		147.3	6.79(d, 8.26 Hz)	116.0
6	5.34 (brd)	119.6	6.22 (d, 8.5 Hz)	136.1 (CH)	5.34 (d, 4.7 Hz)	121.8 (CH)		129.4	7.08(dd, 1.9, 8.27Hz)	123.3
7	5.52 (brd)	116.3	6.44 (d, 8.5 Hz)	130.7 (CH)		31.6 (CH_2)	2.86 (t, 2H)	23.7	7.49 (d, 15.86Hz)	145.1
8		140.1		79.4 (C)		24.4 (CH)	1.28 (2H)	29.1	6.37 (d, 15.88 Hz)	116.2
9		46.3	1.41 (m)	51.17 (CH)		50.2 (CH)	1.28 (2H)	29.3		168.5
10		37.1		36.9 (C)		36.6 (C)	1.28 (2H)	31.8	3.81(s, 3H)	56.3
11		21.2	1.5, 2.3 (m)	23.4 (CH_2)		21.1 (CH_2)	1.28 (2H)	22.5	-	-
12		39.1	1.16, 1.84 (m)	39.4 (CH_2)		39.8 (CH_2)	1.46 (p, 2H)	31.9	-	-
13		42.9		44.6 (C)		42.4 (C)	0.85 (t, 3H)	14.4	-	-
14		54.6	1.34 (m)	51.6 (CH)		56.8 (CH)	3.9 (d, 2H, J=7.4)	27.0	-	-
15		23.0	1.28, 1.30 (m)	20.8 (CH_2)		28.3 (CH_2)	5.23(t, 1H, J=7.4)	122.1	-	-
16		28.3	1.66, 1.69 (m)	28.8 (CH_2)		26.1 (CH_2)		132.9	-	-
17		55.8	1.19 (m)	56.19 (CH)		56.1 (CH)	1.66 (s, 3H)	18.0	-	-
18	0.58 (s)	12.1	0.76 (s)	13.1 (CH_3)	0.66 (s)	11.9 (CH_3)	1.71(s, 3H)	25.9	-	-
19	0.89 (s)	16.4	0.79 (s)	18.5 (CH_3)	0.99 (s)	18.8 (CH_3)	10.25(s)	197.5	-	-
20		40.3	2.0 m	39.34 (CH)		33.1 (CH)	-	-	-	-
21	0.99 (d, 6.5 Hz)	19.7	0.96 (d, 6.55 Hz)	21.3 (CH_3)	0.90 (d, 6.4 Hz)	19.1 (CH_3)	-	-	-	-
22	5.16 (2H, m)	135.6	5.20 (dd, 7.2, 15.2 Hz)	135.19 (CH)		34.1 (CH_2)	-	-	-	-
23		132.0	5.18 (dd, 8.03, 15.29 Hz)	132.2 (CH)		33.9 (CH_2)	-	-	-	-
24		40.5	1.84 (m)	42.98 (CH)		36.2 (CH)	-	-	-	-
25		33.1	1.46 (m)	33.1 (CH)		31.9 (CH)	-	-	-	-
26	0.79 (d, 6.3 Hz)	21.2	0.78 (d, 6.28 Hz)	20.0 (CH_3)	0.85 (d, 6.6 Hz)	19.9 (CH_3)	-	-	-	-
27	0.77 (d, 6.3 Hz)	20.0	0.81 (d, 6.2 Hz)	20.3 (CH_3)	0.83 (d, 6.6 Hz)	19.4 (CH_3)	-	-	-	-
28	0.87 (d, 6.8 Hz)	17.7	0.88 (d, 6.86 Hz)	17.9 (CH_3)	0.79 (d, 7.1 Hz)	14.2 (CH_3)	-	-	-	-

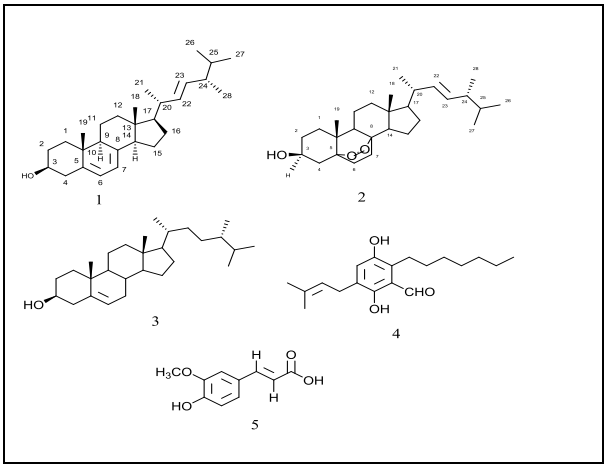

Figure 6. Structure of isolated compounds (1-5) from *A. chevalieri* extract.

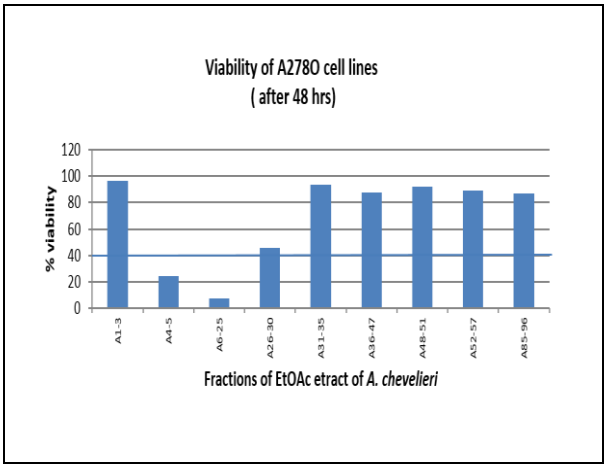

Figure 7. Cytotoxic activity of the MPLC fractions of 30 days Rice culture of *A. chevalieri*

Table 2. Dereplication of the metabolites contributed to the variation of 30 days RC extract of *A. chevalieri*

m/z	Retention time	M.wt	Name	Molecular formula	Source
581.562	35.62	580.55	Unknown		
544.363	23.42	543.35	Ergokonin B; 3-O-(2-Amino-2-methylpropanoyl)	$C_{32}H_{49}NO_6$	*Tolypocladiuminflatum and Sesquicillopsisrosariensis*
613.409	25.50	614.42	Lanosta-8,24-diene-3,21-diol; 3-form, 21-Carboxylic acid, 3-O-(3-hydroxy-4-methoxycarbonyl-3-methylbutanoyl)	$C_{37}H_{58}O_7$	*Fomitopsispinicola*
615.528	35.82	616.54	Unknown		
642.528	32.95	643.54	Unknown		
685.436	30.78	684.43	Icosalide A3	$C_{34}H_{60}N_4O_{10}$	The fungus *OSI 74159*
767.537	31.83	768.54	Unknown		
788.549	36.02	789.56	Unknown		

Metabolomics- and bioactivity guided studies were greatly focused on the anticancer activity due to the significant inhibition demonstrated by the crude extracts and fractions from 30 day RC fungal extract of *A. chevalieri*. Since the putatively identified metabolites, which were highly correlated to this activity, were produced in a very small amount, it was not possible to purify them from the active fractions. The major compounds (1-5) obtained from the purification of the fractions of 30-days rice culture were identified as Ergosterol(1), Ergosterol peroxide (2),Campesterol(3), Flavoglaucin(4) and 3-*O*- methyl caffeic acid (5) (Figure 6), showed no activity against the tested cancer cell lines. Consequently, they were investigated for their antitrypanosomaland antimicrobial activity.

Compounds 2 (ergosterol peroxide), 3 (campesterol), 4 (flavoglaucin) and 5 (3-*O*- methyl caffeic acid) showed antimicrobial activity against *E. coli* and *S. aureus* with zone of inhibition ((11,11), (9,10), (13,12), (9,9) mm) respectively. While, Compounds 4 showed antifungal activity against *C. albicans* with zone of inhibition (11mm).

Moreover**,** Testing the compounds for antitripanosomal activity illustrated that compound 2 (ergosterol peroxide) showed a significant activity against *T. bruceibrucei*with IC_{50} 3.96 µM (48 hrs) and 4.10 µM (72 hrs). Compound 2 was structurally related to steroidal compounds ergosterol peroxide, which has been reported before having antimicrobial activity. Compound 5 showed a moderate anti-trypanosomal activity with IC_{50} 25.3 µM. The antitrypanosomal activity of these compounds was reported for the first time in this study**.**

Table 3. Dereplication of the metabolites highly correlated to the activity of fractions from 30 days RC extract of *A. chevalieri*

m/z	Retention time	Molecular weight	Name	Molecular formula	Source
153.055	5.99	152.04	2- or 4-hydroxyphenylacetic acid	$C_8H_8O_3$	*Gibberellafujikuroi*
291.124	12.2	292.13	Curvularin	$C_{16}H_{20}O_5$	*Curvularia spp., Penicillium sp.*
343.248	14.51	344.256	8-Hydroxy-9,12-octadecadienoic acid	$C_{18}H_{32}O_3$	*Aspergillus nidulans and Laetisariaarvalis*
301.181	24.54	302.19	Auroglaucin; 1',2',3',4'-Tetrahydro	$C_{19}H_{26}O_3$	*Aspergillus ruber and Eurotium rubrum*
435.275	16.01	436.283	Gibberellin A1	$C_{19}H_{24}O_6$	*Gibberella fujikuroi*
425.215	24.48	424.20	Unknown	-	-

CONCLUSION

A combination of metabolomic- and bioassay-guided approaches gave an access to a shorter and faster route to highlight active metabolites highly correlated to the bioactivity during the first stage of fractionation, Metabolomics can detect the bioactive metabolites at micro or nano-gram level which supports the hypothesis that the bioactivity doesn't rely on the yield of the bioactive compounds.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

Acknowledgment

The authers would like to thank toLouise C. Young for providing cells and her assistance in lab culturing, also the authers would like to thank Dr. Rothwelle Tate for using his lab facilities in sequencing study.

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