

Metabolomics-Guided Isolation of Anti-trypanosomal Metabolites from the Endophytic Fungus *Lasiodiplodia theobromae*

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ABSTRACT

Fungal endophytes offer diverse and unique secondary metabolites, making these organisms potential sources of promising drug leads. The application of high-resolution-liquid chromatography mass spectrometry and nuclear magnetic resonance-based metabolomics to fungal endophytes is practical in terms of dereplication studies and the mining of bioactive compounds. In this paper, we report the application of metabolomics in parallel with anti-trypanosomal assays to determine the ideal conditions for the medium-scale fermentation of the endophyte *Lasiodiplodia theobromae*. The ¹H NMR comparison between the active versus inactive fractions identified several unique chemical fingerprints belonging to the active fractions. Furthermore, by integrating high-resolution-liquid chromatography mass spectrometry data with multivariate data analysis, such as orthogonal partial least squares-discriminant analysis (OPLS-DA) and the bioactivity results of the fractions of *L. theobromae*, the anti-trypanosomal agents were easily discerned. With available databases such as Antibase and Dictionary of Natural Products coupled to MZmine through in-house algorithms optimized in our laboratory, the predicted metabolites were readily identified prior to isolation. Fractionation was performed on the active fractions and three known compounds were isolated, namely, cladospirone B, desmethyl-lasiodiplodin, and *R*-(–)-mellein. Cladospirone B and desmethyl-lasiodiplodin were among the predicted compounds generated by the OPLS-DA S-plot, and these compounds exhibited good activity against *Trypanosoma brucei brucei* with minimum inhibitory concentrations of 17.8 μM and 22.5 μM, respectively.

Introduction

Despite the increasing disinclination of the pharmaceutical industry to pursue natural products in their pipelines, statistical findings show that natural products still play a major role in drug discovery with more than 50% of FDA-approved drugs were derived from natural products [1]. However, natural products research has been found to be too laborious, time-consuming, and uneconomical, which may have led to the declining trend. Nevertheless, with the emergence of new and more advanced technologies such as genomics, transcriptomics, proteomics, metabolomics, and bioinformatics, natural products research has become more competent in finding promising novel drugs for the pipeline [1–4].

The utilization of metabolomics in natural products research is increasingly powerful in several perspectives. Metabolomics is defined as a global study of all or a subset of chemical entities including either or both primary and secondary metabolites that are present in living organisms (cells or tissues) under certain processes [5–7]. Metabolomics, or metabolome mining, in natural products research has been used for dereplication studies of both known and new compounds in crude plant, marine, or microbial extracts [8–18], in differentiating biologically active natural products (NPs) from non-active fractions [19–23], optimizing the production of bioactive secondary metabolites, as well as in developing cultivation processes for large-scale fermentation and understanding their biosynthetic pathways [7, 24].

Endophytic fungi are microorganisms that mutually live inside plant tissues without causing any immediate negative effects towards the host plant for at least a part of the fungal life cycle [25]. The total population of endophytic fungi species has been estimated to be up to 1.3 million [26]. Another study places this estimate at approximately 1.5 million [27]. However, as of the year 2000, only 75 000 fungal species have been identified; the remainder are still untapped and unexplored [28]. Bioactive NPs derived from endophytic fungi display wide ranges of activities, such as (–)-oxysporidinone, (2,6-dihydroxyphenyl)pentan-1-one, and (Z)-1-(2-(2-butyl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one, which exhibited antimicrobial activity [29, 30], pullularin A and hinnulliquinone displayed antiviral activity [31, 32], and spiropreussione A and 9-deacetoxyfumigaclavine C showed anticancer activity [33, 34]. Compounds like cochlioquinone A, isocochlioquinone A, and cercosporin exhibited activity against neglected tropical diseases [35, 36].

Human African trypanosomiasis, or sleeping sickness, is a fatal vector-borne parasitic disease caused by *Trypanosoma brucei brucei* transmitted by the tsetse fly (*Glossina* spp.). This neglected tropical disease occurs only in rural areas of sub-Saharan Africa [37]. To date, only a few drugs have been approved for the treatment of human African trypanosomiasis. These include suramin, pentamidine, melarsoprol, eflornithine, and the combination of nifurtomox/eflornithine. Most of the drugs are old, having been discovered in the 1940s and 1950s, and have adverse effects such as nausea, vomiting, fatigue, seizures, fever, diarrhea, hypoglycemia, abdominal cramping, peripheral neuropathy, hypertension, heart damage, and neutropenia on the patients. For this reason,

► **Table 1** Anti-trypanosomal activity of *L. theobromae* extracts derived from *V. pinnata* in different types of media and incubation periods. MIC was only determined for the bioactive extracts.

| Sample | <i>T. b. brucei</i> 20 µg/mL % of viability | <i>T. b. brucei</i> MIC average ± SD (n = 4) |
|----------|---|--|
| LT-LC-7 | 112.3 | Not tested |
| LT-LC-15 | 106.3 | Not tested |
| LT-LC-30 | 126.0 | Not tested |
| LT-RC-7 | 103.9 | Not tested |
| LT-RC-15 | 94.3 | Not tested |
| LT-RC-30 | 1.4 | 25 ± 1.3 µg/mL |
| Suramin | Not tested | 0.1 ± 0 µM |

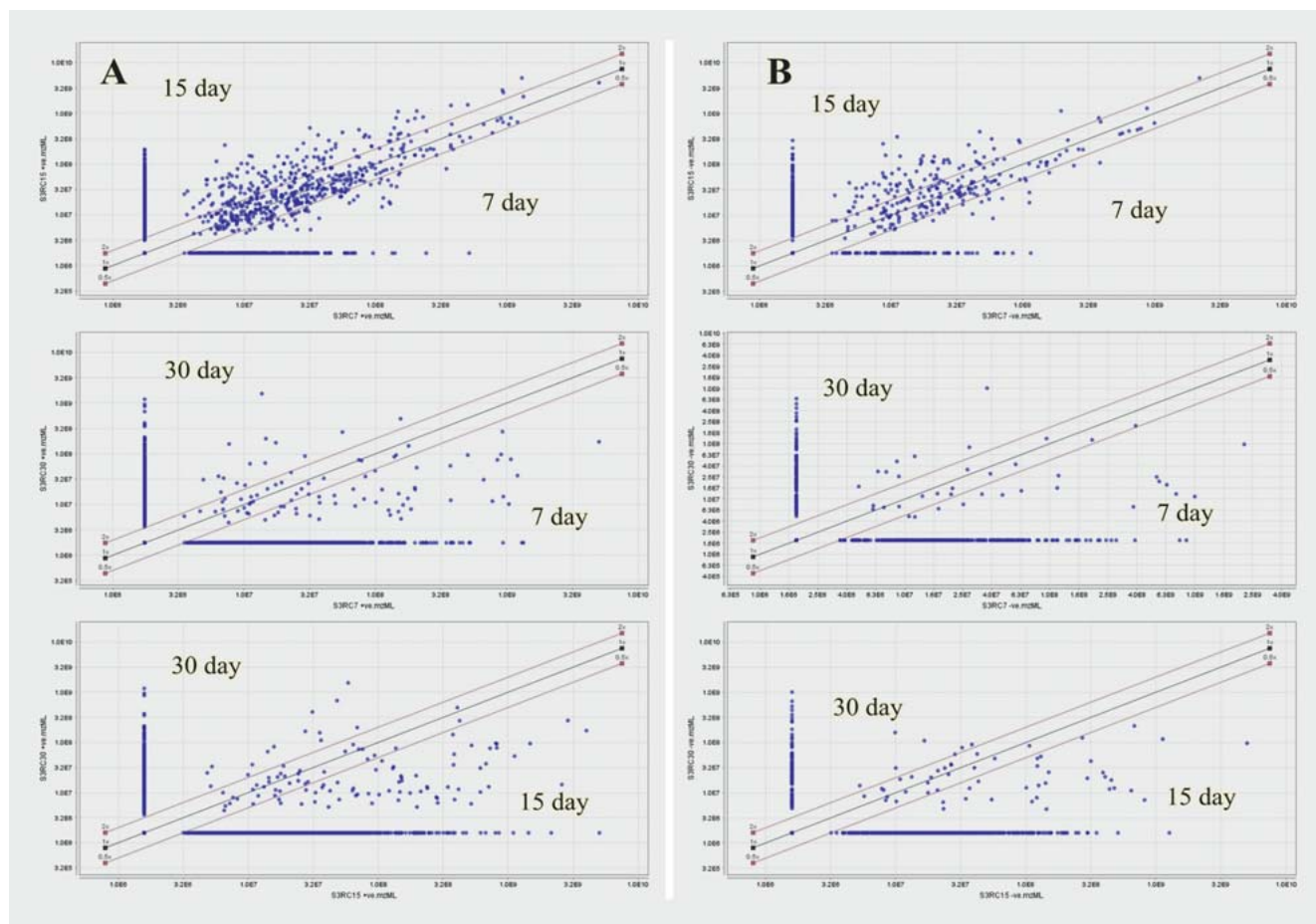
LT = *L. theobromae*.

mining and developing new human African trypanosomiasis drugs from natural products is crucial and essential because various natural sources including plants, microorganisms, animals, and marine organisms offer a high number of NPs with diverse chemical structures and novel pharmacological mechanism of action [38].

The aim of this study is to adopt an untargeted HR-LCMS and NMR-based metabolomics approach to determine the optimal fermentation conditions of *Lasiodiplodia theobromae* for medium scale-up, and also to capture and trace the production of active anti-trypanosomal metabolites by using statistical multivariate data analysis of generated HR-LCMS data, such as principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA). To verify the dereplication results obtained from HR-LCMS data, 1D and 2D ¹H NMR data were utilized. Finally, the isolation of active metabolites was performed based on the outcome of HR-LCMS and NMR-based metabolomics profile data.

Results and Discussion

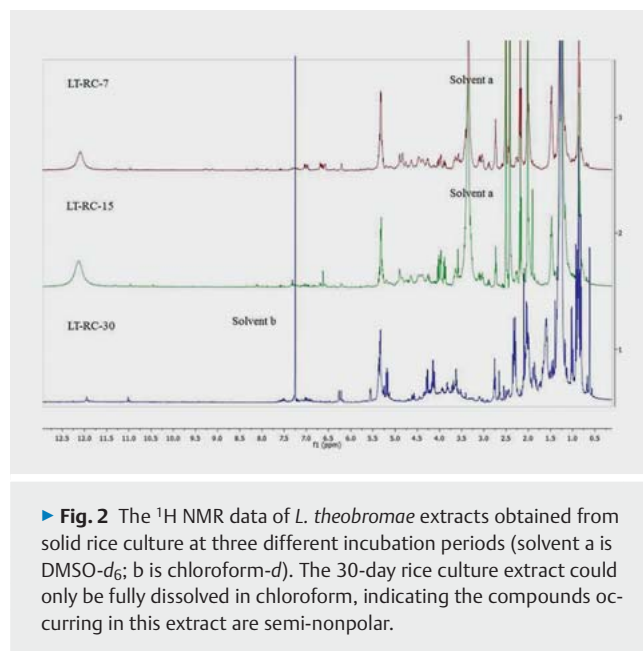
In search of the best condition for scaling up the culture of the endophyte *L. theobromae* obtained from the leaves of *Vitex pinnata*, HR-LCMS and NMR-based metabolomics along with the bioassay data were utilized. The fungus was grown in solid rice cultures and liquid Wickerham cultures for 7, 15, and 30 days, after which the metabolites were extracted. Three different incubation times were chosen based on the fungal life cycle [39]. In this case, the first 7 days represent the germination phase, while the 15-day and 30-day cultures cover the hyphal growth stage and sporing phase, respectively, of *L. theobromae*. The production of secondary metabolites was monitored using HR-LCMS and NMR at each of the growth phases parallel to the bioassay results. Based on the bioassay results (► **Table 1**), the 30-day rice culture extract exhibited the strongest activity against *T. b. brucei* with a minimum inhibitory concentration (MIC) of less than 25 µg/mL. The HR-LCMS raw data was processed using MZMine 2.10 [40]. The results of the assay were reproducible between the scale-up batches.



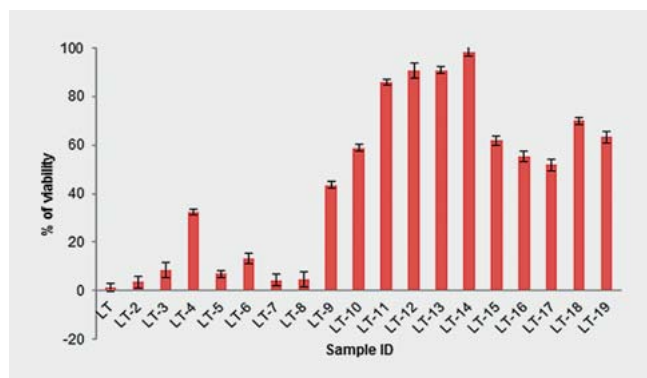
► **Fig. 1** Scatter plot of the ion peaks of the *L. theobromae* extracts from different days (A) in positive ionization and (B) in negative ionization.

Metabolite production and distribution between cultures were analyzed through ion peak scatter plots (► **Fig. 1**). Based on the MS data, the occurrences of the metabolites on the 7th and 15th days were similar, while a decrease in metabolite production was observed on the 30th day. However, the ion chromatogram both in positive and negative modes revealed a different set of metabolites on the 30th day to those of the 7th and 15th day extracts (► **Fig. 1**). Moreover, the ^1H NMR data revealed findings complementary to the MS data (► **Fig. 2**). Therefore, the bioactive 30-day rice culture condition was chosen for scale-up and further isolation work.

The medium-scale 30-day rice culture extract of *L. theobromae* was fractionated yielding 19 fractions (LT-1 until LT-19). These were submitted for the anti-trypanosomal activity and subjected to MS- ^1H NMR-based metabolomics data profiling. The nonpolar fractions LT-2 to LT-8 exhibited strong bioactivity, except for LT-4, which showed only moderate activity against *T. b. brucei* (► **Fig. 3**). LT-1 was excluded from the bioassay screening because it contained only fatty acids, as indicated by its ^1H NMR data (► **Fig. 4**). The ^1H NMR data of the 19 fractions (► **Fig. 4**) were analyzed, and unique chemical fingerprints of the active fractions were detected. Among these active fractions, two distinctive subgroups, LT-2 to LT-4 and LT-6 to LT-8, clustered together, as they



► **Fig. 2** The ^1H NMR data of *L. theobromae* extracts obtained from solid rice culture at three different incubation periods (solvent a is $\text{DMSO}-d_6$; b is $\text{chloroform}-d$). The 30-day rice culture extract could only be fully dissolved in chloroform, indicating the compounds occurring in this extract are semi-nonpolar.



► **Fig. 3** Anti-trypanosomal activity of *L. theobromae* fractions against *T. b. brucei*. LT: *L. theobromae* extract as the positive control; LT2-LT19: *L. theobromae* fractions.

shared similar spectral data. Fraction LT-5 was a mixture of both groups. The ^1H NMR spectra of fractions LT-2 to LT-5 displayed a pair of *meta*-coupled aromatic protons at δ_{H} 6.23 and 6.28 ($J = 2.7$ Hz) and a multiplet peak at δ_{H} 5.15, which may be an olefinic or oxygenated methine. The upfield shift of the *meta*-coupled aromatic protons at the 6 ppm region suggested the presence of an electron withdrawing group such as a hydroxyl or halogen substituent. In fractions LT-5 to LT-8, two *meta*-coupled doublets at δ_{H} 6.18 and 6.25 ($J = 2.6$ Hz) were observed (designated by red arrows on ► **Fig. 4**). The proton signals between 4.2–4.8 ppm revealed the presence of oxygenated methines while proton signals between 6.6 to 7.7 ppm indicated the presence of aromatic compounds in fractions LT-6 to LT-8.

Supervised methods of multivariate data analysis were used to analyze the similarity and differences of the data sets between 19 samples. PCA was used in an earlier step to observe an overview of variance between the fractions and metabolites generated from MS data and also to identify any outliers. The distribution difference of the type of metabolites between active vs. inactive fractions of *L. theobromae* against *T. b. brucei* was analyzed by subjecting the data to OPLS-DA. The results of the analysis led to the prediction of compounds that contribute towards the anti-trypanosomal activity of the fractions. For the OPLS-DA model (► **Fig. 5A**), the MS-based metabolomics data set was assigned as the X independent variable, while the fractions' anti-trypanosomal response was the Y dependent variable. The quality of the OPLS-DA model was measured by two parameters, R² (goodness of fit) and Q² (predictability), and the model's R²X was 0.261, R²Y was 0.955, and Q² was 0.733. These results were interpreted that 26.1% of the X variables could be used to describe 95.5% of the variation between active fractions and inactive fractions, while this model has 73.3% of the average predictability. The value of R²Y and Q² was greater than 50%, indicating a well-fitted model exhibiting good prediction [41]. The quality and robustness of the OPLS-DA model was validated by a permutation test ($n = 100$). The Q² intercept value was -0.368 (below 0.05), showing that the original model is statistically effective (► **Fig. 5B**) [42]. For the OPLS-DA scores plot (► **Fig. 5A**), the active fractions were grouped together versus the inactive ones. Under the active

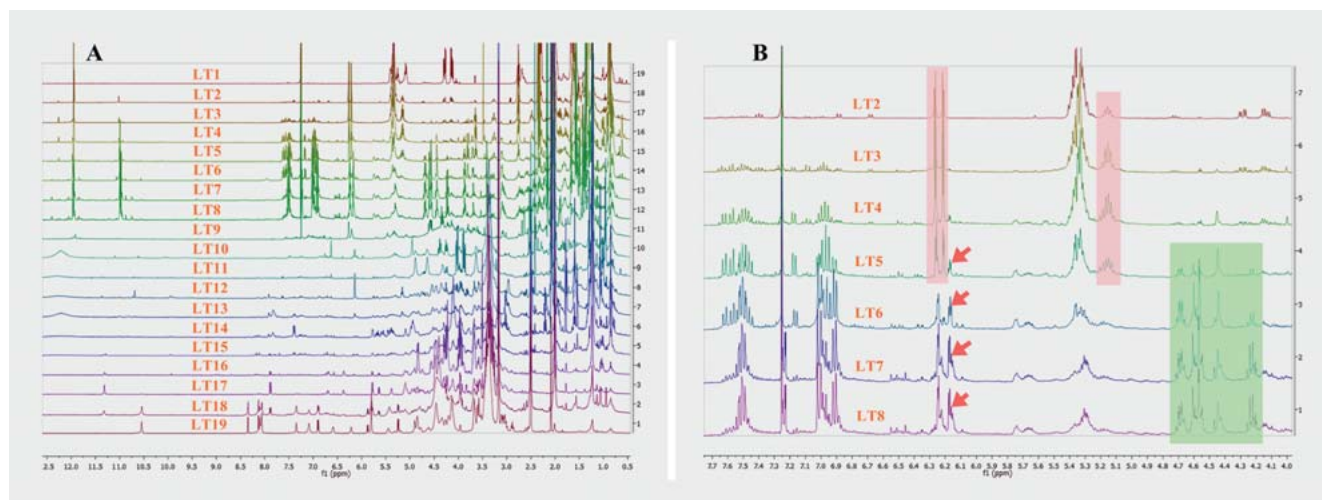
group, fractions LT-2 to LT-8 were clustered together, indicating a shared set of metabolites, while fractions LT-9 to LT-19 were observed as the inactive group. The generated S-plot (► **Fig. 5D**) determined the “end point” or unique compounds for each of the respective groups, indicating the metabolites that are potentially responsible for the bioactivity against *T. b. brucei*, which discriminated the active from the inactive fractions of *L. theobromae*. Eight metabolites were identified from Antibase as shown in ► **Table 2**. The end point compounds were targeted for bioassay-guided isolation work (► **Fig. 6**).

The active metabolites listed from the dereplication step of the HR-LCMS data were identified as palmarumycin CP17 (5), cladospirone B (6), and desmethyl-lasiodiplodin (8). Further analysis of the ^1H - ^1H COSY NMR spectrum of fraction LT-3 revealed substructures belonging to the predicted active metabolites. For example, the correlation of the *meta*-coupled aromatic protons at δ_{H} 6.23 and 6.28 as well as that of the methyl doublet at δ_{H} 1.35 with one oxygenated methine proton at δ_{H} 5.15, which further correlated with the aliphatic chain, was characteristic of the desmethyl-lasiodiplodin structure (8) (**Table S1**, **Fig. S1**, Supporting Information). Substructures of palmarumycin CP17 and cladospirone B were elucidated in the same manner.

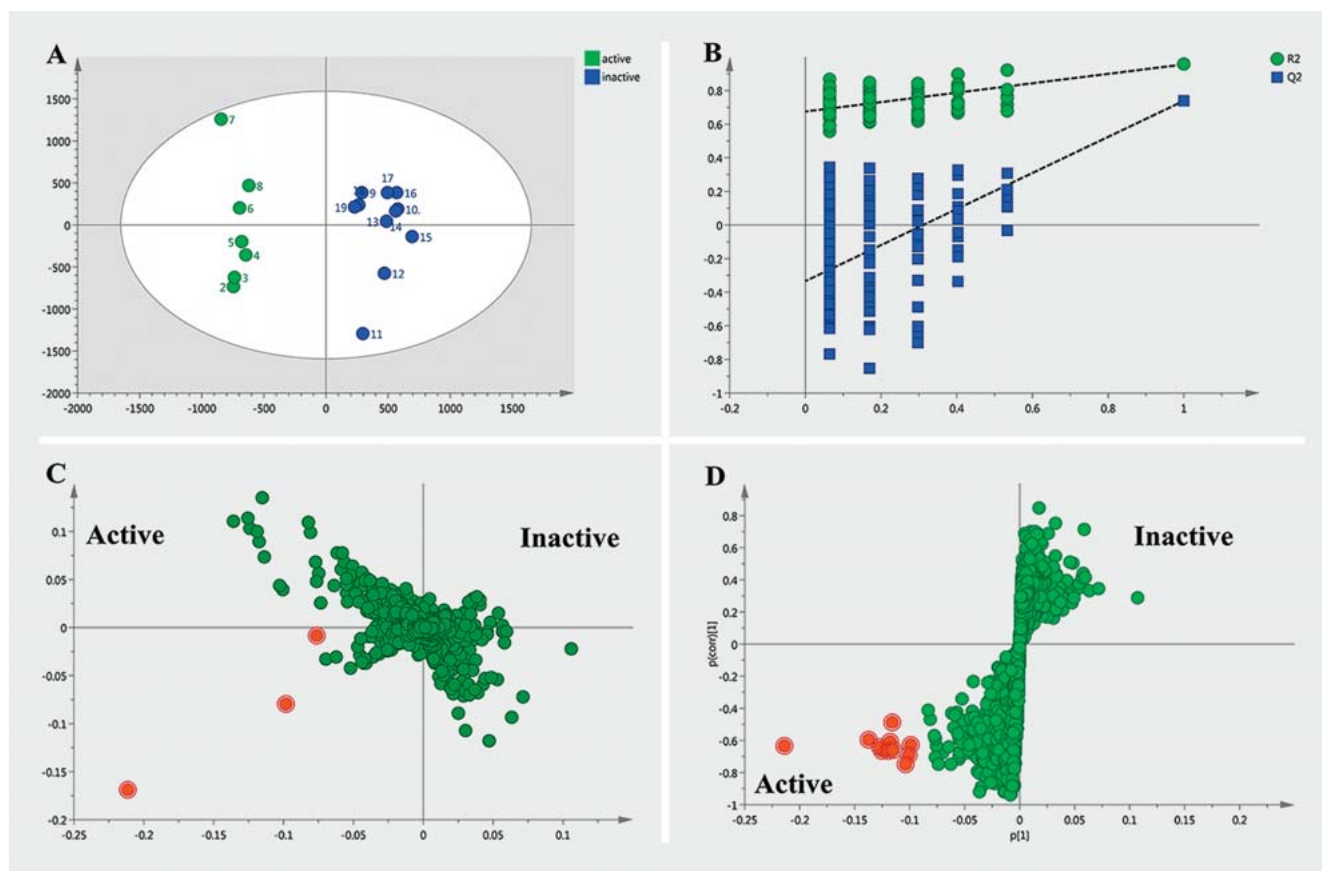
Fraction LT-7, an outlier in the active group, was also selected based on the OPLS-DA results, which indicated the bioactive metabolite. Among these metabolites, the structure of the compound 6-oxo-de-O-methylasiodiplodin (2) was confirmed by its COSY spectral data, which exhibited correlations similar to desmethyl-lasiodiplodin (8), as shown in **Fig. S2A**, Supporting Information. In the aromatic region, the COSY spectrum revealed correlations as in preussomerin-C (**Fig. S2B**, Supporting Information).

Isolation and purification of the compounds in the active group was performed by high-throughput medium pressure liquid chromatography (MPLC). Three known compounds were isolated and elucidated based on their NMR and MS data as cladospirone B (6) [43], desmethyl-lasiodiplodin (8) [44], and *R*-(–)-mellein (9) [45] (chemical structures shown in ► **Fig. 6**). The isolation of cladospirone B (6) and desmethyl-lasiodiplodin (8) confirmed the putative identification of the bioactive metabolites predicted from the S-plot of the OPLS-DA model. *R*-(–)-mellein (9) is a structurally close analogue of 6,8-dihydroxy-3-methylisocoumarin (1). All isolated compounds were tested against *T. b. brucei*, cladospirone B (6) and desmethyl-lasiodiplodin (8) had MICs of 17.8 and 22.5 μM , respectively. All three metabolites were checked again in the S-plot and *R*-(–)-mellein was located in the middle of the plot, suggesting that the anti-trypanosomal activity would be less (► **Fig. 7**); this is indeed the case as confirmed by the bioassay results (► **Table 3**). Due to low concentration, palmarumycin CP17 was not isolated. Desmethyl-lasiodiplodin (8) has been known to exhibit anticancer activity against MCF-7 via apoptosis with an IC₅₀ sevenfold more potent than its toxicity on normal cells [46]. On the other hand, cladospirone B (6) has been reported to be inactive in antibacterial and antifungal assays [45].

In our screening program, we observed that the crude extract of *L. theobromae* from agar plates showed anti-trypanosomal activity against *T. b. brucei*. In order to find the optimal conditions to grow *L. theobromae*, HR-LCMS-based metabolomics was ap-



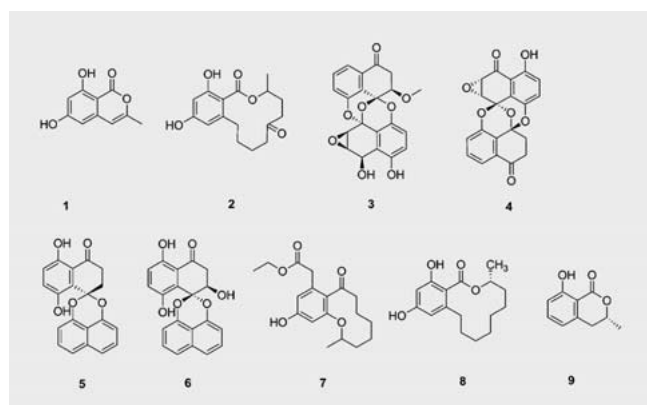
► **Fig. 4** A The ^1H NMR spectra of the 19 fractions. B The expansion of the ^1H NMR data of active anti-trypanosome fractions highlighting several unique chemical fingerprints found only in these fractions.



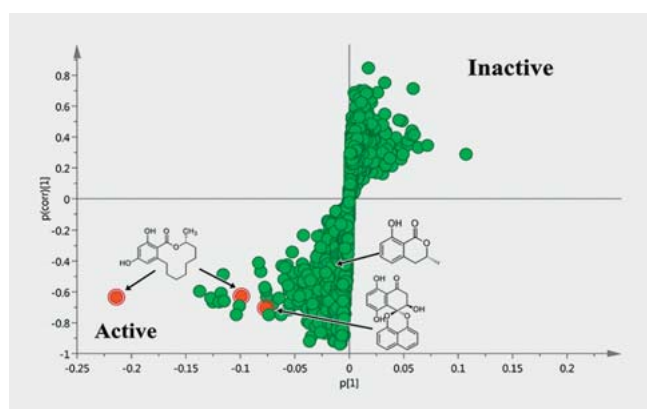
► **Fig. 5** Multivariate analysis of *L. theobromae* fractions and anti-trypanosomal activity data correlation. A The score scatter plot of OPLS-DA shows the samples were grouped based on their bioactivity. B The permutation test result of the OPLS-DA model. (C) The loading scatter plot of OPLS-DA shows the m/z values of active metabolites. (D) The S-plot generated from the OPLS-DA model shows the end point compounds that are the predicted metabolites responsible for the bioactivity (highlighted in red).

► **Table 2** Putatively identified unique metabolites of *L. theobromae* active fractions obtained from S-plot “end point” data as shown on ► **Fig. 5D**. (P = positive mode; N = negative mode).

| Ionization mode | MS <i>m/z</i> | Rt (min) | Chemical formula | Name |
|-----------------|---------------|----------|--|--------------------------------------|
| N | 191.035 | 11.32 | C ₁₀ H ₈ O ₄ | 6,8-Dihydroxy-3-methylisocoumarin(1) |
| N | 291.124 | 13.17 | C ₁₆ H ₂₀ O ₅ | 6-Oxo-de-O-methylasiodiplodin (2) |
| P | 293.139 | 13.71 | C ₁₆ H ₂₀ O ₅ | 6-Oxo-de-O-methylasiodiplodin (2) |
| N | 395.077 | 13.60 | C ₂₁ H ₁₆ O ₈ | Preussomerin-C (3) |
| N | 363.051 | 14.61 | C ₂₀ H ₁₂ O ₇ | Preussomerin-H (4) |
| N | 333.077 | 17.61 | C ₂₀ H ₁₄ O ₅ | Palmarumycin CP17 (5) |
| N | 349.072 | 13.71 | C ₂₀ H ₁₄ O ₆ | Cladospirone B (6) |
| P | 321.170 | 19.14 | C ₁₈ H ₂₄ O ₅ | Phomopsin B (7) |
| N | 319.155 | 19.16 | C ₁₈ H ₂₄ O ₅ | Phomopsin B (7) |
| N | 277.144 | 21.79 | C ₁₆ H ₂₂ O ₄ | Desmethyl-lasiodiplodin (8) |
| P | 279.159 | 21.80 | C ₁₆ H ₂₂ O ₄ | Desmethyl-lasiodiplodin (8) |
| N | 555.296 | 21.79 | | Complex of 277.144 |



► **Fig. 6** Chemical structures of unique metabolites (1–8) predicted from the S-plot and further secondary metabolites (6, 8, 9) isolated from an active fraction of *L. theobromae* (Group 1).



► **Fig. 7** Three isolated compounds from *L. theobromae* labelled in the S-plot. *R*-(–)-mellein was in the middle of the plot, suggesting less anti-trypanosomal activity for this compound.

plied, which resulted in the selection of solid rice culture for 30 days as the best conditions for medium-scale fermentation. Fractionation was performed on the crude extract, and based on the 1D ¹H NMR data comparison of 19 fractions, several unique chemical fingerprints in the active fractions were highlighted. Furthermore, by utilizing the HR-LCMS data for multivariate analysis such as OPLS-DA, consequently, a set of the metabolites that were predicted to be active, was generated. All predicted metabolites were easily identified with the aid of AntiBase coupled to MZMine. The application of ¹H NMR and COSY allowed for the detection of the predicted metabolites in the active fractions as well as the confirmation of the dereplication results obtained from the HR-LCMS data. Three known compounds were isolated and identified as cladospirone B (6), desmethyl-lasiodiplodin (8), and *R*-(–)-mellein (9). To the best of our knowledge, this is the first report of isolation of cladospirone B (6) from *L. theobromae*. It is also the first report to indicate the good anti-trypanosomal activity of cladospirone B (6) and desmethyl-lasiodiplodin (8) against *T. b. brucei* in comparison with suramin (MIC value of 0.1 ± 0 μM). Our strategy of emphasizing HR-LCMS and NMR-based metabolomics to search for active anti-trypanosomal compounds has therefore been proven to be effective. In conclusion, this study determined that the combination of HR-LCMS and NMR-based metabolomics is a powerful and advantageous decision-making tool in mining active metabolites of *L. theobromae* against *T. b. brucei* and is also promising for implementation in other drug discovery programs elsewhere.

Materials and Methods

Fungal sampling

The fungus *L. theobromae* was isolated from fresh healthy leaves of *V. pinnata* collected in April 2011 near Kuala Terengganu, Malaysia. The plant was identified by Dr. Nashriyah Mat from the Faculty of Bioresources and Food Industry, Universiti Sultan Zainal

► **Table 3** Anti-trypanosomal activity of isolated compounds obtained from *L. theobromae* fermented in solid rice culture for 30 days.

| Compound | Anti-trypanosomal activity (<i>T. b. brucei</i>) MIC average \pm SD (n = 4) (μ M) |
|------------------------------|---|
| Cladospiron-B (6) | 17.8 \pm 0 |
| Desmethyl-lasiiodiplodin (8) | 22.5 \pm 1.50 |
| R-(–)-mellein (9) | > 100 \pm 2.75 |
| Suramin | 0.1 \pm 0 |

Abidin, and a voucher specimen was deposited (collection number VP 01).

Samples were kept in zip lock bags and stored at 4°C until the isolation of endophytic fungi was performed 4 days later upon arrival at the University of Strathclyde in Glasgow. The surfaces of the leaves and stems were sterilized with 70% iso-propanol for 2 min and subsequently rinsed in sterile water. Small tissue samples from inside the leaves and stems were cut aseptically and pressed onto agar plates (composition of isolation medium: 15 g/L malt extract, 15 g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4–7.8, adjusted with 10% NaOH or 36.5% HCl). Chloramphenicol (\geq 98%; purity Sigma-Aldrich) was added to inhibit bacterial growth. The plates were left for a few days until fungal growth was observed. Reinoculation onto new malt agar plates was repeated several times until pure strains were attained.

Identification of fungal strains

The fungal strain was identified using DNA amplification and sequencing of the internal transcribed spacer (ITS) region as described previously [47]. The sequence data has been submitted to GenBank with accession number KC960898. The fungal strain was identified as *L. theobromae*. A voucher strain was submitted and is kept at the Natural Product Metabolomics Laboratory, SIPBS.

Small- and medium-scale fermentation

The fungal strain was cultivated on malt agar plates for 7 days at 30°C. The colonies and agar were cut into small pieces and were placed in liquid or solid media. The liquid medium used was Wickerham medium, consisting of 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose, and distilled water adding up to 1000 mL in 2 L Erlenmeyer flasks, pH 7.2–7.4, adjusted with 10% NaOH or 36.5% HCl. The solid medium used was rice medium, with 100 g of long grain rice and 100 mL of distilled water autoclaved together in 1 L Erlenmeyer flasks. The strain was grown for 3 different incubation times: 7, 15, and 30 days, under static conditions at room temperature. For medium-scale fermentation, *L. theobromae* was cultivated in 1 L flasks using the optimal conditions as determined by the small-scale cultures. In this case, the growth of the fungus on rice medium for 30 days under static conditions was determined to be the most favorable condition for the production of anti-trypanosomal metabolites.

Extraction and isolation of pure compounds

The medium-scale rice cultures were extracted with ethyl acetate and homogenized as finely as possible using a T18 Basic Ultra-Turrax (IKA) at maximum speed. These were then kept overnight. The extract was subsequently dried under *vacuo* using a rotary evaporator (Buchi Labortechnik AG). The crude extract (5.0 g) was then partitioned with 10% *n*-hexane and 90% methanol to remove fatty acids. The extract containing methanol-soluble compounds (2.0 g) was collected for further isolation work. The fractionation of the methanol extract was accomplished using MPLC. Linear gradient elution was employed with hexane (A) and ethyl acetate (B) as the mobile phase at a flow rate of 20 mL/min. A pre-packed silica column (20–45 μ m, 23 \times 110 mm, Silica VersaPak cartridge) was used. It was connected to a Buchi Pump Manager C-615 coupled to binary pumps (Buchi Modules C-601). 100% A was run for 5 min, followed by 100% A to 100% B for 20 min, and finished with 100% B for the last 5 min. The total run time was 30 min. Fractions were collected in collection tubes automatically every 2 mL using a fraction collector Frac 920 (GE Healthcare Bio-Sciences AB). Fractions with similar TLC profiles were pooled together, yielding a total of 19 fractions. Fraction LT-2 (435 mg) was further subjected to MPLC on a prepacked silica column (20–45 μ m, 23 \times 53 mm, Silica VersaPak cartridge) utilizing an isocratic gradient system (80% *n*-hexane: 20% ethyl acetate) for 30 min at a flow rate of 20 mL/min. In total, 300 collection tubes of fractions (2 mL in each tube) were collected automatically using their TLC profiles. Similar fractions were pooled, resulting in 17 fractions and giving 3 known compounds, **6** (3 mg), **8** (73 mg), and **9** (11 mg) with a purity > 95% (determined by HPLC).

In vitro anti-trypanosomal assay

The samples were prepared to a final concentration of 10 mg/mL (stock solution) by being dissolved in the appropriate amount of DMSO. To screen for *in vitro* activity, a concentration of 200 μ g/mL was used. This was achieved by diluting the stock solution 1 in 10 with HMI-9 (drug solution). Four μ L of drug solution were transferred to 96 μ L of HMI-9 in the 96-well plate. One hundred μ L of trypanosome suspension, consisting of *T. b. brucei* S427 blood stream form at 3×10^4 trypanosomes/mL, were then added to the 96-well plate to make the final concentration of the compounds range from 100 μ g/mL to 0.17 μ g/mL. DMSO was used as the negative control (concentration of 1 to 0.002%) and suramin (Calbiochem-Novabiochem Co., purity > 98% by HPLC) was selected as the positive control (concentration of 1 to 0.008 μ M). The plate was incubated for 48 h at 37°C, 5% CO₂ with a humidified atmosphere, after which 20 μ L of Alamar blue were added. The plate was again incubated for another 24 h under the same conditions. The fluorescence was measured using a Wallac Victor microplate reader (PerkinElmer) with excitation at 530 nm and emission at 590 nm. The results were calculated as percentages of control values. All samples that exhibited > 90% inhibition were selected for the MIC assay to determine the MIC value.

Nuclear magnetic resonance instrumentation

One- and two-dimensional ¹H and ¹³C NMR spectra were recorded at 400 MHz on a JEOL-LA400 FT-NMR spectrometer system with a 40TH5AT/FG probe (JEOL LTD.). Compounds **6** and **8** were reconsti-

tuted in deuterated chloroform (CDCl_3) while compound **9** was reconstituted in deuterated DMSO ($\text{DMSO-}d_6$).

High-resolution-liquid chromatography mass spectrometry procedure

HR-LCMS was measured using an Accela 600 HPLC pump with Accela autosampler and UV/Vis detector (Thermo Scientific) and an Orbitrap Exactive mass spectrometer (Thermo Fisher Scientific, Inc.). Analysis of samples was done using similar protocols described previously [23, 47, 48].

High-resolution-liquid chromatography mass spectrometry data processing

Initially the raw HR-LCMS data were sliced into two data sets according to ionization mode using the MassConvert tool from ProteoWizard (<http://proteowizard.sourceforge.net/>). The sliced data were imported to MZMine 2.10 (<http://sourceforge.net/projects/mzmine/>), software developed for the differential analysis of mass spectrometry data. The data processing step was performed in the same manner as explained previously [23], albeit with slightly modified parameters. In this analysis, the data set was crop filtered from 0.1 to 35 min and the retention time normalizer was not applied because only one batch of data was used.

Statistical analysis

MS spectral data were converted to an ASCII text file and imported to MS Excel. The data was sorted to exclude background peaks that belonged to the MeOH blank. The sorted data were then exported to the SIMCA-P software 14.0 version (Umetrics). Pareto scaling was employed on the MS data set. Finally, PCA, OPLS-DA, and S-plot were performed.

Supporting information

The ^1H , ^{13}C NMR and HMBC as well as ESI-MS data of the isolated compounds are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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