# Metabolite Profiling of Endophytic *Streptomyces* spp. and its Antiplasmodial Potential

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**Background:** Antiplasmodial drug discovery is significant especially from natural sources such as plant bacteria. This research aimed to determine antiplasmodial metabolites of *Streptomyces* spp. against *Plasmodium falciparum* 3D7 by using a metabolomics approach. **Methods:** *Streptomyces* strains' growth curves, namely SUK 12 and SUK 48, were measured and *P. falciparum* 3D7 $IC_{50}$ values were calculated. Metabolomics analysis was conducted on both strains' mid-exponential and stationary phase extracts. **Results:** The most successful antiplasmodial activity of SUK 12 and SUK 48 extracts shown to be at the stationary phase with $IC_{50}$ values of 0.8168 ng/mL and 0.1963 ng/mL, respectively. In contrast, the $IC_{50}$ value of chloroquine diphosphate (CQ) for antiplasmodial activity was 0.2812 ng/mL. The univariate analysis revealed that 854 metabolites and 14, 44, and 3 metabolites showed significant differences in terms of strain, fermentation phase, and their interactions. Orthogonal partial least square-discriminant analysis (OPLS-DA), and S-loading plot putatively identified pavettine, aurantioclavine, and 4-butyldiphenylmethane as significant outliers from stationary phase of SUK 48. For potential isolation, metabolomics approach may be used as a preliminary approach to rapidly track and identify the presence of antimalarial metabolites before any isolation and purification can be done.

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**Abstract**

**Background:** Antiplasmodial drug discovery is significant especially from natural sources such as plant bacteria. This research aimed to determine antiplasmodial metabolites of *Streptomyces* spp. against *Plasmodium falciparum* 3D7 by using a metabolomics approach. **Methods:** *Streptomyces* strains' growth curves, namely SUK 12 and SUK 48, were measured and *P. falciparum* 3D7 $IC_{50}$ values were calculated. Metabolomics analysis was conducted on both strains' mid-exponential and stationary phase extracts. **Results:** The most successful antiplasmodial activity of SUK 12 and SUK 48 extracts shown to be at the stationary phase with $IC_{50}$ values of 0.8168 ng/mL and 0.1963 ng/mL, respectively. In contrast, the $IC_{50}$ value of chloroquine diphosphate (CQ) for antiplasmodial activity was 0.2812 ng/mL. The univariate analysis revealed that 854 metabolites and 14, 44, and 3 metabolites showed significant differences in terms of strain, fermentation phase, and their interactions. Orthogonal partial least square-discriminant analysis (OPLS-DA), and S-loading plot putatively identified pavettine, aurantioclavine, and 4-butyldiphenylmethane as significant outliers from stationary phase of SUK 48. For potential isolation, metabolomics approach may be used as a preliminary approach to

rapidly track and identify the presence of antimalarial metabolites before any isolation and purification can be done.

## Introduction

A large number of imported malaria cases from Europe and the Mediterranean have recently been reported. The increasing number of foreign travelers linked to the hefty inflow of malaria-endemic immigrants. For instance, anopheline vectors, which act as the parasite reservoir and are present in Mediterranean regions, can infect the travelers returning from tropical countries (Dominguez Garcia et al., 2019; Silvia Odolini, 2012). In 2017, World Health Organization (WHO) documented that the most malaria cases during that year were reported in Africa (with 92% or 200 million), and followed by Southeast Asia (with 5% or 10.8 million) (WHO 2018).

Endophytic *Streptomycetes* are bacteria within ethnomedicinal plants that have symbiotic relationships with the host plants. *Streptomyces* is the biggest genus of actinomycetes, consisting of aerobic Gram-positive bacteria that are capable of generating filamentous branches called mycelia (Gottlieb 1966). The mycelia secrete antibiotics, that may be anti-fungal, antibacterial, and anti-viral, when they sporulate in the dormant phase (Alam et al., 2010; Gramajo et al., 1993; Roszak & Colwell, 1987).

Previous studies reported that trioxacarcin A and D, produced by marine *Streptomyces* sp., were more effective in producing antiplasmodial activity than artemisinin (Maskey et al., 2004; Tomita et al., 1981). Furthermore, coronamycin, a novel antiplasmodial agent from *Streptomyces* sp., reportedly possessed antiplasmodial activity against *Plasmodium falciparum* (Ezra et al., 2004). Strobel and co-researchers discovered that munumbicin D also had antiplasmodial activity (Castillo et al., 2002). Kakadumycin A, also identified by the same team, isolated from

*Streptomyces* sp. NRRL 30566 compound demonstrated promising antiplasmodial activity on *Plasmodium falciparum* (Castillo et al. 2003). Furthermore, the isolated gancidin W of *Streptomyces* sp. SUK 10 inhibited the growth of *Plasmodium berghei* (Zin et al., 2017*)*.

This study aims to determine the antiplasmodial activity on metabolites produced from *Streptomyces* spp. against *Plasmodium falciparum* 3D7 using a metabolomics approach. Metabolomics is a high throughput method utilized to screen the metabolites in organisms or tissues by chromatography techniques coupled with mass spectrometry (Rochfort, 2005). Then the metabolites are depicted into a two-dimensional distribution using multivariate analysis, including Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA), and further putatively identified using Dictionary of Natural Products (DNP). The metabolites include amino acids, carbohydrates, organic acids, vitamins, antibiotics, and phytochemicals (Wishart 2008). The metabolomics approach uses high analytical techniques to determine metabolites in the biological samples. In this study, a hybrid chemical profile using liquid chromatography and mass spectrometry (LC-MS) with multivariate data analysis (MVA) was used to determine the metabolites present in *Streptomyces kebangsaanensis* SUK 12 and SUK 48. Moreover, mass spectrometry (MS)-based metabolomics helped to fast-track the identification of targeted and untargeted metabolites present in complex extracts during screening.

## Materials & Methods

### Culture

*Plasmodium falciparum* 3D7 was obtained from the culture collection of the Parasitological Department, Faculty of Medicine, Universiti Malaya (UM) while *Streptomyces kebangsaanensis*

SUK 12 and SUK 48 were acquired from the Novel Antibiotics Laboratory, Programme of Biomedical Science, Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM). *Streptomyces kebangsaanensis* SUK 12 was isolated from an ethnomedicinal plant, *Portulaca oleracea* L., which was collected from the Nenasi Reserve Forest, Pahang, Malaysia (Sarmin et al., 2013). *Streptomyces* sp. SUK 48 was isolated from the fruit of *Brasilia* sp. (Zin, 2015).

**_Streptomyces_ spp. growth condition**

The SUK 12 and SUK 48 strains were grown in nutrient broth (pH 7.0) on an orbital shaker at 160 rpm, 28°C for 21 days. The dry weight was collected daily by centrifugation at 4000 rpm for 15 mins at 25ºC and dried at 70ºC. The growth curves of both strains were plotted using Microsoft Excel. The growth rate and generation time were estimated by the following calculation:

$$\text{Growth rate, k} = \frac{\log(\text{Higher dry weight, } X1) - \log(\text{lower dry weight, } X0)}{0.301\text{t (time between two intervals)}}$$

$$\text{Generation time} = \frac{1}{k}$$

**_Streptomyces_ spp. extracts preparation**

The crude extracts prepared in the 14-day culture of both strains' blocks (1 $cm^2$) were used to inoculate in nutrient broth for 5, 12, and 14 days for SUK 12, and 7, 14, and 21 days for SUK 48. The broth cultures were also incubated on an orbital shaker (Protech, Malaysia) at 160 rpm, 28ºC. Both strains' cultures were extracted using 3-fold ½ volume of ethyl acetate. The organic layer (ethyl acetate) was discarded and dried under vacuum using a rotary evaporator (Buchii, Swirtzerland).

116 ***In vitro* antimalarial assay**

117 The 96-well plate *in vitro* antimalarial activity includes a series of extracts dilution (complete
118 media or CM and extracts), positive drug control (CM and CQ), positive control (CM plus iRBC,
119 infected red blood cells), and negative control (CM plus fresh RBC). The positive control was the
120 maximum parasite lactate dehydrogenase (pLDH) enzyme absorbed in cells, and the negative
121 control was a blank (Lambros & Vanderberg, 1979).

122

123 ***Plasmodium falciparum* 3D7 culture**

124 The volume of iRBC was calculated then a complete media (containing RPMI 1640, 2.3 g/L
125 sodium bicarbonate, 4 g/L dextrose, 5.957 g/L HEPES, 0.05 g/L hypoxanthine, 5 g/L Albumax II,
126 0.025 g/L gentamycin sulfate, 0.292 g/L L-glutamine) and fresh red blood cells (RBC) were added
127 into the culture to make the final values of 1% parasitaemia and 2% hematocrit. The parasitaemia
128 level was measured using a thin blood film (TBS) that was stained with 10% Giemsa and observed
129 under a light microscope with 1000x magnification. Next, when the parasitaemia level reached
130 within 5-7%, iRBC was synchronized with 5% sorbitol to obtain the ring stage of the parasite
131 (Amir et al., 2016; Lambros & Vanderberg, 1979).

132

133 **Dilution of *Streptomyces spp.* extracts**

134 *Streptomyces* spp. extracts stock solution was prepared using 10 mg/mL dimethyl sulfoxide
135 (DMSO) and complete medium (CM). The final concentration of the prepared DMSO was less
136 than 1% to prevent its toxic effect on the parasite. Next, the stock solution of *Streptomyces* spp.
137 extracts was serially tenfold-diluted eight times (starting at 1000 µg/mL and ending at 0.0001
138 µg/mL). For the control, the drug chloroquine diphosphate (CQ) was used in various dilutions

ranging from 10 µg/mL to $10^{-6}$ µg/mL. Next, 100 µL of the diluted extracts and CQ were transferred into a sterile 96-wells plate. For negative and positive control wells, 100 µL of CM was transferred into this 96-well plate.

**Incubation of *Streptomyces spp.* extracts with parasite**

Parasite culture (iRBC) with 10% parasitaemia was selected and centrifuged at 1800 rpm for 5 mins to obtain the cell pellets. The cell pellets were diluted to 2% parasitaemia with fresh RBC. Approximately 2 µL iRBC was transferred into every well of diluted extracts, control drug, CQ, and positive control. Then, 2 µL of fresh RBC was added into negative control, and the 96-well was incubated at 37°C, 5% carbon dioxide ($CO_2$) for 48 hs. The plate was then frozen overnight at -20°C prior the pLDH assay started (Makler et al., 1993).

**Parasite lactate dehydrogenase (pLDH assay)**

Upon overnight freezing at -20°C, the 96-well plate was defrosted at 37°C for 20 mins and re-frozen at -20°C for 30 mins. This step was repeated 3 times to break the parasite cells. At the same time, Malstat reagent and NBT-PES (nitroblue tetrazolium-phenazine ethosulfate) were also prepared in the dark. About 100 µL of Malstat reagent and 25 µL of NBT-PES were added to a new 96-well plate. After the freezing and defrosting processes finished, 15 µL of each defrost culture well plate was transferred into the wells of a new plate that contained Malstat reagent and NBT-PES. The 96-well plate was then incubated for 1 h at room temperature in the dark. Absorbance readings at the wavelength of 650 nm ($A_{650}$) were measured for the 96-well plate using a spectrophotometer (Tecan M200, Switzerland). The positive control was assumed to be the

maximum level of lactate dehydrogenase enzyme production. The negative control was the blank (Makler et al. 1993; Trager & Jensen 1976). Inhibition of parasite (%) was calculated as follows:

$$(A650\ average\ for\ diluted\ sample\ -\ A650\ average\ negative\ control)\ \ X100$$
$$(A650\ average\ positive\ control\ -\ A650\ average\ negative\ control)$$

A sigmoid graph was plotted using Graphpad PRISM version 7. The value of parasite inhibition 50% ($IC_{50}$) was determined from the graph.

**Liquid Chromatography-Mass Spectrometry (LC-MS) analysis**

Approximately 3 mg/mL extracts of S12D5 (SUK 12 day to harvest fermented broth is 5), S12D12, S48D7, and S48D14 were sent for LC-MS analysis (Rosli et al., 2017). Scientific C-18 column Thermo was chromatographically separated by an UltiMate 3000 UHPLC (Dionex) system (AcclaimTM Polar Advantage II, 3 mm to 150 mm, 3 μm particle size). A flow rate of 0.4 mL/min at 40°C was maintained with water that contained 0.1% formic acid and 100% acetonitrile with a total operating time of 22 mins. The gradient started for 3 mins at 5% of solvent B, then grew to 80% of solvent B for 7 mins and stayed at 80% of solvent B for 5, or 10-15 mins. At last, the gradient turned to 5% of solvent B in 7 mins (15-22 mins). The ESI-positive ionization was performed with the use of MicrOTOF-Q III (Bruker Deltonic) with the settings: capillary voltage 4500 V; pressure of 1.2 bar, and dry gas flow 8 L/min at 200°C; 50-1000 Da m/z, respectively.

**Mass spectrometry data handling**

Raw material in "d format" was supplied to Bruker Compass Data Analysis Viewer version 4.2 (Bruker Daltonics, Germany) and imported into the Profile Analysis 2.0 data bucketing software (Bruker Daltonics, Germany) (Mamat et al. 2018; Veeramohan et al. 2018). The parameters for

compound detecting were: signal/noise threshold: 5; correlation coefficient: 0.7; minimum compound length: 8; smoothing width: 2. Compound detection was done using Find Molecular Features (FMF). The composite bucket table was calculated using advanced bucketing features as time alignment parameters. The time interval was between 0.00 mins and 22.04 mins, and the mass range was between 49 m/z and 1001 m/z. For standardized settings, the data was uploaded to the MetaboAnalyst 3.0 server (www.metaboanalyst.ca). The normalizing features used were: internal standard caffeine: 195.088 m/z, RT: 7.98 mins; transformation: log; scaling: pareto. (Xia et al. 2015). Normalized and validated data tables were exported to SIMCA P+ version 15 from Umetrics AB, Umea, Sweden. The PCA, the examination of fundamental variations in samples, and the presentation of outliers were conducted prior to the sample classification of Model OPLS-DA. With 100 random permutations using cross-validations and responsive permutation tests, the robustness of OPLS-DA was validated. The two-way ANOVA type was "ANOVA" in subjects. The significance threshold was set as the *p*-value of correction lower than 0.05. The false discovery rate was less than 0.05.

**Metabolite identification**

The search and manual verification of secondary metabolites in the LC–MS analyses were done. Online databases, namely METLIN and Dictionary of Natural Products (DNP), were examined for the value of a molecular ion of interest (Mazlan et al., 2019; Smith et al., 2005). The databases were used to identify molecular weight metabolites within a specified m/z value query tolerance range.

**DNA extraction**

First, neutral lysis of genome DNA was done for isolation, followed by extraction, then precipitation of phenol chloroforms and isopropanol. The protocol was used to extract the DNA (Kieser 1984). For several changes, *Streptomyces* mycelium, in a 1.5 mL of Eppendorf tube was interrupted by vortexing with 500 µL of lysozyme. The samples were then added with 25 µL of 50 mg/mL of lysozyme and 3 µL of 10 mg/mL of RNase. Finally, the purity and concentration of the DNA were determined on a Thermo Scientific nano-drop 2000C machine. SUK 12 DNA sequence was obtained from the previous study (Sarmin et al., 2013).

**16S rRNA molecular identification and phylogenetic tree analysis**

Polymerase chain reaction (PCR) amplified the 16S rRNA gene sequence using universal 16SrRNA bacterial gene primers as described earlier (Coombs & Franco, 2003). For SUK 12 and SUK 48, 1416 and 1463 nucleotides respectively, were near full-length 16S rRNA gene sequences. The sequences were compared using BLAST and EzTaxon e-databases with the GenBank database (Altschul et al., 1997). The comprehensive sequence similarity calculation was based on the EzTaxon server's global alignment algorithm (Kim et al., 2012). The sequence was also matched several times with 16S rRNA gene sequences, which are available in GenBank/EMBL with the CLUSTAL W programme, for closely related species of *Streptomyces* (Thompson et al., 1994). The neighbour-joining (NJ) approach (Saitou & Nei, 1987), available in MEGA software package 7 version, used the reconstructions of the phylogenetic trees (Tamura et al., 2013). In accordance with the two-parameter Kimura model, the matrices of distance were calculated (Kimura, 1980). Moreover, the tree was made with the maximum likelihood (ML) (Kimura, 1980). By conducting a bootstrap analysis-based of 1000 replicates, the topology of the trees was evaluated (Felsenstein, 1985).

230

231 **Results**

232

233 **Growth curve and effectiveness of antiplasmodial activity of *Streptomyces* spp.**

234 The growth curve showed that the generation time of *Streptomyces* sp. SUK 12 was faster than
235 SUK 48's with 1.16 h/generation and 4.56 h/generation, respectively (Figure 1 A and B).
236 Meanwhile, the antiplasmodial activity revealed that the crude extract of *Streptomyces* sp. SUK
237 48 on day 14 was more potent (with an $IC_{50}$ value of 0.1963 ng/mL) than that of SUK 48 on day
238 7 and SUK 12 on day 5 and day 10 (Table 1).

239

240 **Metabolomics approach**

241 The metabolite profiles in extracts using two-way ANOVA (univariate analysis) were compared
242 to those using PCA, OPLS-DA, and S-Plot (multivariate analysis). Two-way ANOVA summarizes
243 and simplifies each sample metabolites (Figure 2). Approximately, 96 metabolites were common
244 in the type of strain, fermentation time, and interaction between both (time and strain type). While
245 44 metabolites were significant in strain type, 14 metabolites were significant in fermentation time,
246 and three metabolites were significant in the interaction between both (Supplementary Table 1).
247 The variation and diversity of the extracts were examined in a PCA model (Figure 3A). The PCA
248 model, however, gave a low Q2 = 0.271 preview. *Streptomyces* sp. extracts cannot be
249 distinguished by any significant variation. The OPLS-DA scores scatter plot of the crude extracts
250 SUK 48 showed distinct separation between day 7 and day 14 (Figure 3B). Nevertheless, the crude
251 extract of SUK 12 on day 5 and day 12 was not well distributed. Meanwhile, the OPLS-DA scores
252 scatter plot (Figure 3C) and S-loadings plot (Figure 3D) of SUK 48 crude extracts between day 7

253 and day 14 gave a good distribution. Overall, the metabolites separation from SUK 48 day 14
254 extracts could be seen as in Figure 4. The secondary metabolite at m/z 195.092 was putatively
255 identified as 1-Vinyl-β-carboline (pavettine) whereas metabolites at m/z 225.163 and 225.153
256 were putatively identified as 4-butyldiphenylmethane. Metabolites presence at m/z 227.135,
257 227.145, and 227.153 were putatively identified as aurantioclavine; (-)-form. All the outliers in
258 the crude extracts of SUK 48 day 14 mentioned as above are known metabolites in Table 2. Then,
259 metabolites at m/z 211.135 and 211.123 were putatively identified as 2,5-Dimethyl-3-(2-
260 phenylethenyl)pyrazine, while metabolites at m/z 245.117 and 245.137 were 3,3-Bis(4-
261 hydroxyphenyl)-1-propanol. The secondary metabolite at m/z 195.149 was represented as 6-(1-
262 Methylethyl)-3-(2-methylpropyl)-2(1H)-pyrazinone, while at m/z 155.078 was trifluoromethyl
263 piperazine. These metabolites were the outliers in SUK 48 day 7 (Table 3).
264
265 ***Streptomyces* spp. identification using molecular analysis.**
266 Phylogenetic tree analysis of both SUKs showed that SUK 48 and SUK 12, using the NJ tree,
267 belonged to different clades (Figure 5) where the bootstrap value was 98%. Moreover, the ML tree
268 (supplement Figure 1) supported this report with a bootstrap value of 95%.
269
270
271 **Discussion**
272 There are four stages of bacteria growth: lag, log, stationary, and death. The crucial phase for
273 producing antibiotics is the stationary phase in *Streptomyces* spp. (Chandrakar & Gupta, 2019;
274 Chen et al., 2020). *Streptomyces lactamdurans* that produces the antibiotic cephamycin C has a
275 generation time of 7.5 hs (Ginther, 1979). Furthermore, faster growth is potentially essential in the

enzyme production of actinomycetes. The effective mycelial fragmentation by the enhanced expression SsgA has significant consequences for antibiotic production, with increasing undecylprodigiosin, but a complete block in the production of actinorhodin (van Wezel et al., 2009). SUK 48 could therefore be a potential antibiotic producer as its ability as slow-growing capacity. The stationary phase of bacterial growth is the survival phase in which they actively secrete secondary metabolites to combat oxidative stress and malnutrition for survival purposes (Banchio & Gramajo, 2002; Undabarrena et al., 2017).

Chemometrics is a chemical discipline that uses mathematical and statistical logic-based methods to design optimal measurement procedures and tests, and provide maximum chemical information through the analysis of chemical data (Massart & Buydens, 1988). From a metabolomics perspective, the univariate analysis is a statistical test used to independently measure metabolites that have significantly increased or decreased between different groups. This test is important for significance testing of the tens to hundreds of metabolites to reduce the probability of false positives caused by multiple tests. Of the 854 metabolites analyzed in two-way ANOVA, 14 were significant in the fermentation time (mid-exponential and stationary phase), whereas, 44 and 14 metabolites were significant in strain and time respectively, and interaction between the both were three metabolites were significant. In addition, there were 96 metabolites significant in strain, time, and interaction of both. There were two antimalarials identified in the list of 96 metabolites, anisomycin, and hydroquinine. Anisomycin was first isolated from *Streptomyces griseolus* and *Streptomyces roseochromogenes* by Sobin and Tanner (Sobin, 1954) in 1954 and it was reported to possess antimalarial activity against *Plasmodium falciparum* (Ekong et al., 1990*),* while hydroquinine was first isolated from the reduction of quinine and was determined as an antimalarial agent (Buttle et al., 1938; Griffin et al., 2012). Pavettine is an alkaloid compound

299 which found in SUK 48 on day 14, was reported to have anti-cancer where $IC_{50}$ was 100 ng/mL
300 against leukemic cell lines (Figuerola & Avila, 2019).

301 Furthermore, the antimicrobial activity of pavettine against *Bacillus subtilis* and *Candida*
302 *albicans* reported minimum inhibitory concentration (MIC) values from 1.9 µg/disc to 3.8 µg/disc
303 (Sudha & Masilamani, 2012). Pavettine was previously mainly found in *Pavetta lanceolata,*
304 *Cribricellina cribraria, Costaticella hastata*, and *Soulamea raxinifolia* (Blackman & Walls, 1995;
305 Jordaan, 1968). Aurantioclavine (-)-form was reported to produce similar functions as
306 benzodiazepines drugs that can treat anxiety, sleep deprivation, and panic disorder (Griffin, et al.
307 2013; Selala et al., 1989). In addition, it was used as a precursor of communesin B (coumarins,
308 which had anticancer properties) (Trost & Osipov, 2015). Moreover, it was reportedly found in
309 *Penicillium aurantiovirens* as well (Selala et al., 1989). No biological activity was documented by
310 the compounds listed in SUK 48 on day 7 and 4-butyldiphenylmethane in SUK 48 on day 14. The
311 2,5-Dimethyl-3-(2-phenylethenyl)pyrazine metabolites of secretion of ant *Iridomyrmex humilis*
312 (John Buckingham et al., 2010). While metabolites at m/z 245.117 and 245.137 were putatively
313 identified as 3,3-Bis(4-hydroxyphenyl)-1-propanol which has been isolated from *Streptomyces*
314 *albospinus* strain 15-4-2 (Chapman & Hall, 2007). Meanwhile, the metabolite at m/z 195.149 was
315 represented as 6-(1-Methylethyl)-3-(2-methylpropyl)-2(1H)-pyrazinone, has been mainly found in
316 *Aspergillus flavus* (Chapman & Hall, 2007). Further isolation and purification of putative
317 metabolites and identification and elucidation of molecule structure is required by using MS/MS,
318 1D- and 2D-NMR.

319 Phylogenetic tree analysis of both SUKs showed that SUK 48 and SUK 12 belonged to
320 different clades. The reliability test was over 70%, which was 98% at nodes between SUK 12 and
321 SUK 48. This result suggested that both SUKs were different subspecies. Distance matrices of the

neighbouring tree construction were calculated using Kimura, the two-parameter correction model (Kimura, 1980), and a bootstrap analysis based on 1000 replications was performed to evaluate the topology of the neighbouring joining trees (Felsenstein, 1985). SUK 12 was a novel species (Sarmin et al., 2013) with the antiplasmodial agent (Remali, 2016). We believe SUK 48 is presumably a novel species (data not published). The selected outgroup, *Kitasatospora setae* KM-6054$^T$ was used based on the previous study (Sarmin et al., 2013).

## Conclusion

SUK 48 at the stationary phase produced more metabolites with antimicrobial and anticancer properties than SUK 48 at the mid-exponential phase. Therefore, SUK 48 at the stationary phase was the potential antimalarial extract candidate for the further purification process. The metabolomics approach is a rapid tool that dereplicated known antimalarial metabolites and determined those at specific fermentation time. Further study will focus on the fractionation and purification of possible antimalarial compounds from *Streptomyces* spp. SUK 48. Meanwhile, an *in silico* study using molecular docking (MD) will be conducted to verify the potential interaction between complex protein-ligands in developing a potential new antiplasmodial agent.

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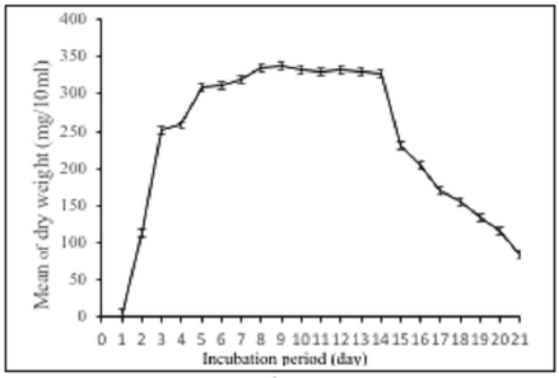
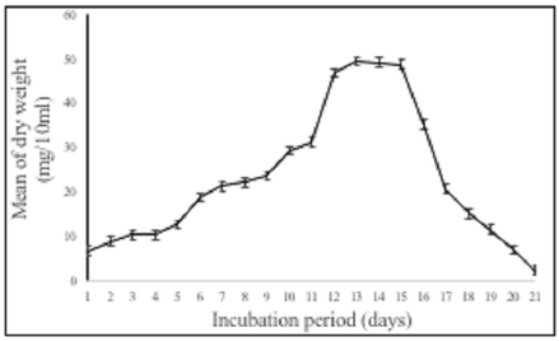
# Figure 1

Growth curve of Streptomyces spp.

**Figure 1**. (A) The growth curve of *Streptomyces* sp. SUK 12 and (B) SUK 48.




A

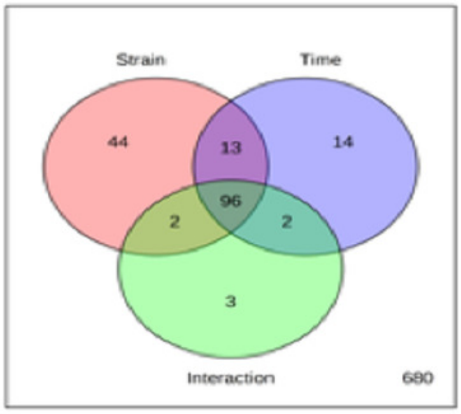



B

# Figure 2

Two-way ANOVA (Analysis of Variance)

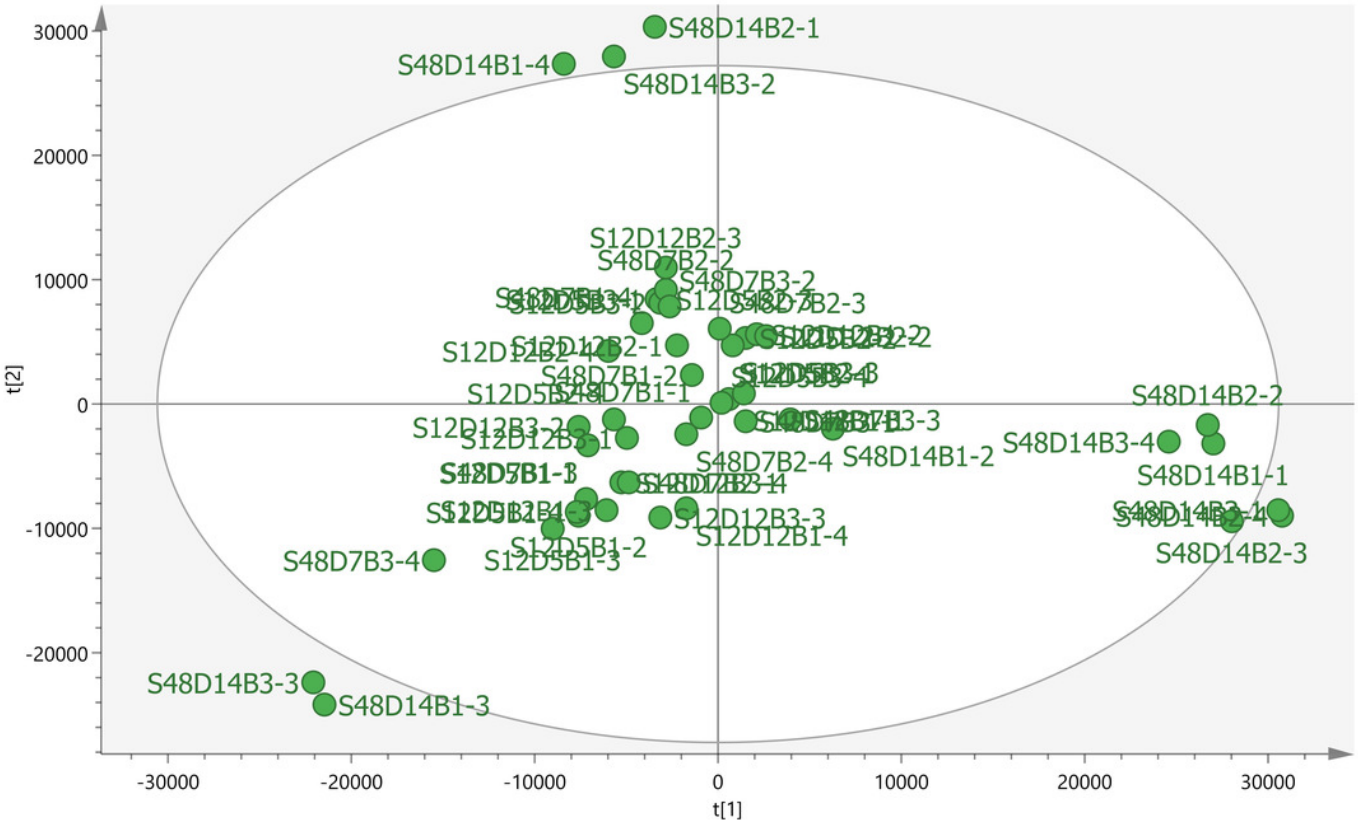
Two-way ANOVA (Analysis of Variance). The red circle with 155 metabolites represents type of strain, SUK 12 and SUK 48, whereas blue circle with 125 metabolites represents fermentation time and green circle with 103 metabolites represents interaction between both (time and strain type).



# Figure 3

Principal component analysis

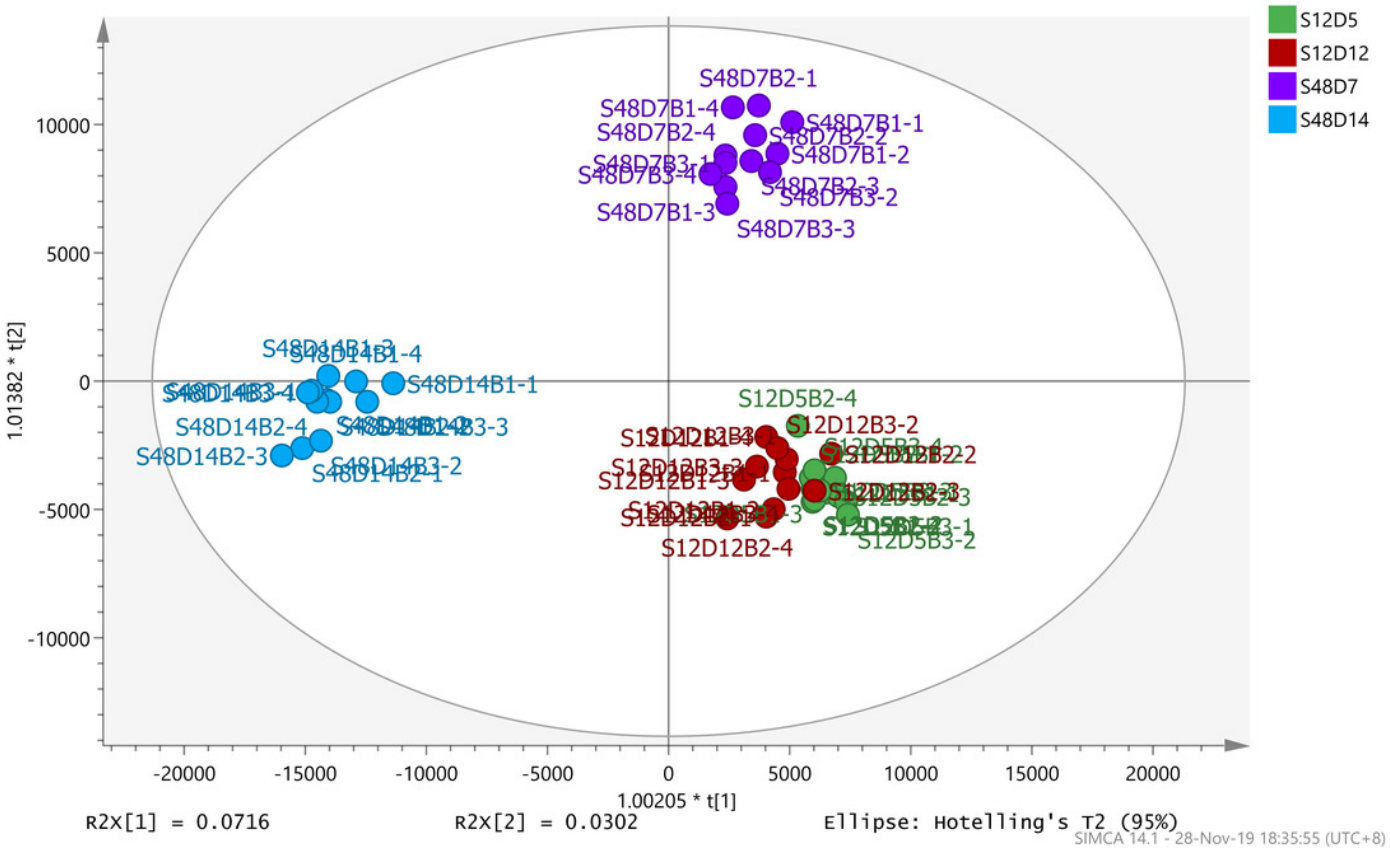
(A) PCA scores scatter plot of SUK 12 (day 5 and 12) and SUK 48 (day 7 and 14) crude extracts (R2 = 0.47; and Q2 = 0.271)



# Figure 4

OPLS-DA scores scatter plot

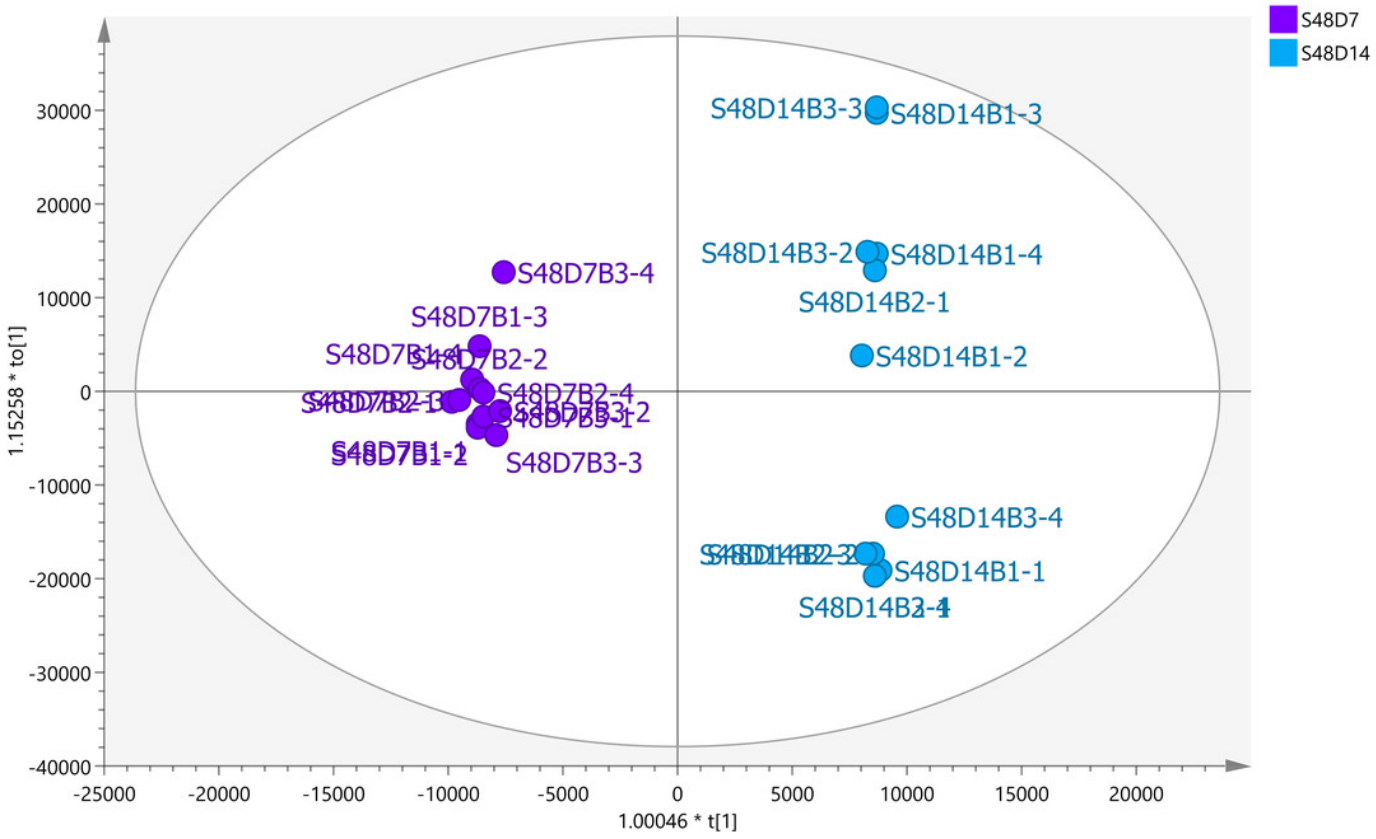
(B) OPLS-DA scores scatter plot between SUK 12 and SUK 48 crude extracts (R2(Y) = 0.651; Q2 = 0.395



# Figure 5

OPLS-DA scores scatter plot

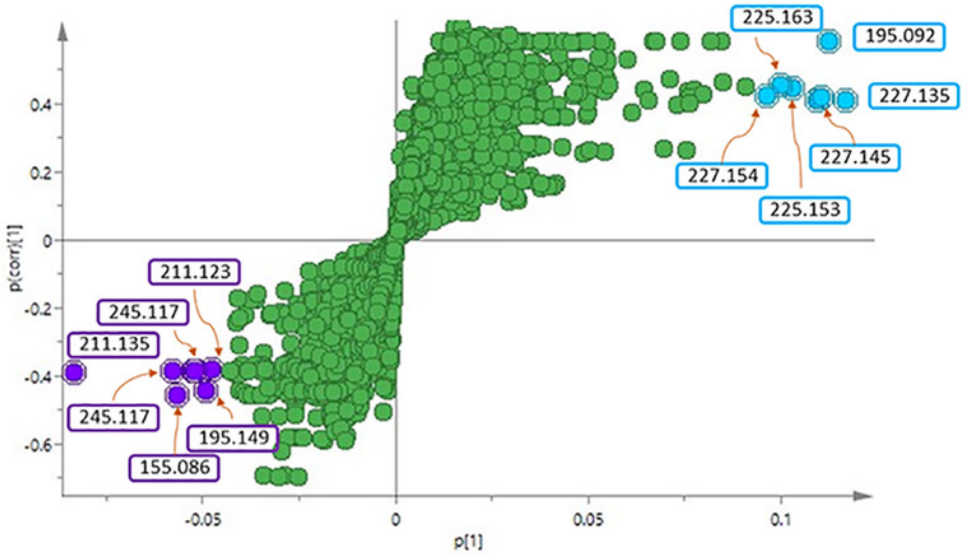
OPLS-DA scores scatter plot between S48D7 and S48D14, (R2(Y) = 0.996; Q2 = 0.61



# Figure 6

S-Plot

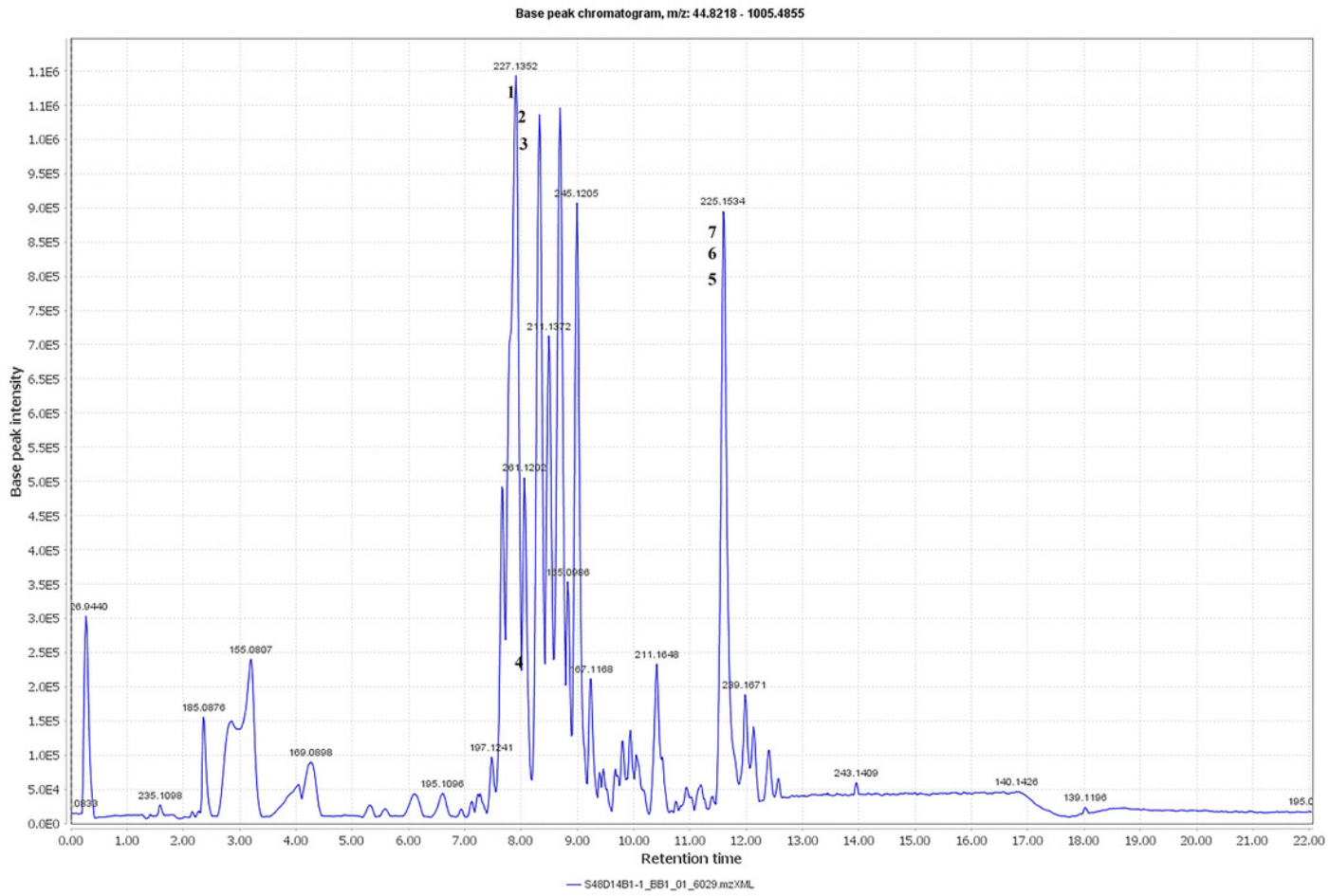
S-plot of SUK48 day 7 vs day 14 metabolites.



# Figure 7

Total ion chromatogram

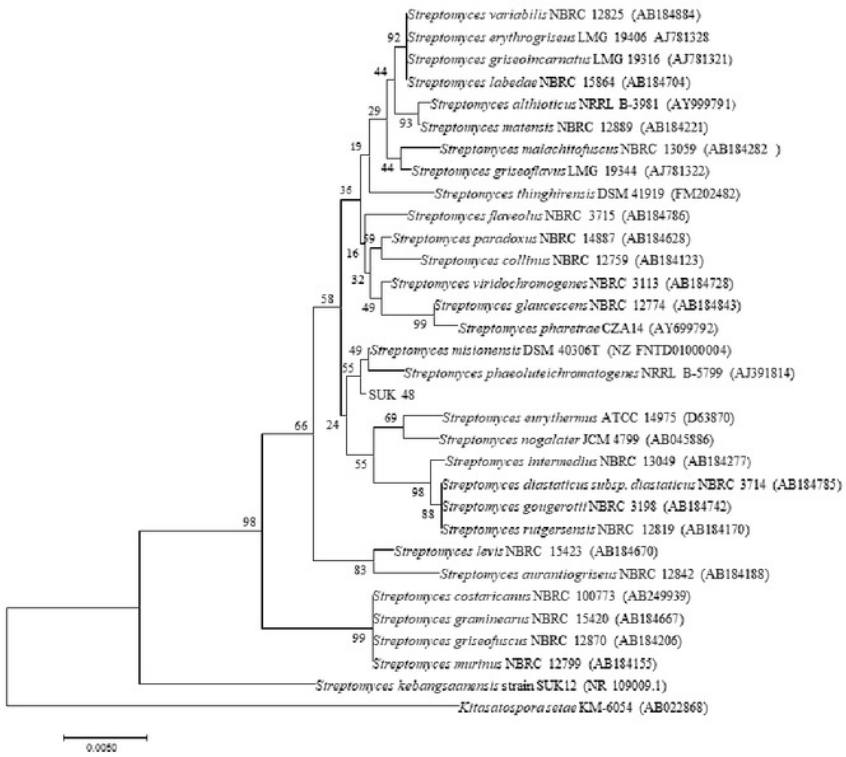
Total ion chromatogram of the crude extract of SUK48 on day 14 with labelled of significant peaks of outliers (1-6).



# Figure 8

Phylogenetic tree

Phylogenetic tree of full length 16S rRNA nucleotide sequences using Neighbour Joining method showing the relationship of strain SUK12 and SUK 48 with closely related members of the genus *Streptomyces* and *Kitasatospora setae* KM-6054$^{T}$ as the outgroup. Numbers at the nodes indicate levels of bootstrap support based on 1000 replication. Bar, 0.005 changes per nucleotide.



## Table 1(on next page)

Anti-plasmodial activity

Anti-plasmodial activity of *Streptomyces* spp. extracts

1 Table 1: Anti-plasmodial activity of *Streptomyces* spp. extracts

| Sample (Growth phase) | Inhibitory concentration ( $IC_{50}$ in ng/mL ± SEM) |
|---|---|
| S12D5 (mid exponential phase) | 18.62 ± 0.00 |
| S12D12 (stationary phase) | 0.8168 ± 0.174 |
| S12D14 (death phase) | 62.29 ± 0.00 |
| S48D7 (mid exponential phase) | 0.1980 ± 0.099 |
| S48D14 (stationary phase) | 0.1963 ± 0.17 |
| S48D21 (death phase) | 527.4 ± 0.00 |
| Chloroquine diphosphate (control drug) | 0.2821 ± 0.00 |

2

## Table 2(on next page)

Identification of significant metabolites of SUK48 day 14 extracts

Putatively identified metabolites using DNP and METLIN that presence in the crude extracts of SUK48 day 14 highlighted S-Plot.

Table 2: Putatively identified metabolites using DNP and METLIN that presence in the crude extracts of SUK48 day 14 highlighted S-Plot.

| No | m/z value | Retention time (minutes) | Molecular Weight | Monoisotopic mass | Compound Name | Tolerance (ppm) | Chemical Formula | Sources |
|---|---|---|---|---|---|---|---|---|
| 1 | 227.154 | 7.91 | 226.1457 | 226.1470 | Aurantioclavine; (-)-form | -0.3401 | $C_{15}H_{18}N_2$ | *Penicillium aurantiovirens* |
| 2 | 227.145 | 7.91 | 226.1377 | 226.1470 | Aurantioclavine; (-)-form | -0.3401 | $C_{15}H_{18}N_2$ | *Penicillium aurantiovirens* |
| 3 | 227.135 | 7.91 | 226.1277 | 226.1470 | Aurantioclavine; (-)-form | -0.3401 | $C_{15}H_{18}N_2$ | *Penicillium aurantiovirens* |
| 4 | 195.092 | 7.94 | 194.0844 | 194.0844 | 1-Vinyl-β-carboline (pavettine) | 0.02 | $C_{13}H_{10}N_2$ | *Pavetta lanceolata* , *Cribricellina cribraria*, *Costaticella hastata* and *Soulamea raxinifolia* |
| 5 | 225.163 | 11.60 | 224.1565 | 224.1565 | 4-Butyldiphenylmethane | 3 | $C_{17}H_{20}$ | Chemically synthesized |
| 6 | 225.153 | 11.60 | 224.1460 | 224.1460 | 4-Butyldiphenylmethane | 3 | $C_{17}H_{20}$ | Chemically synthesized |

## Table 3(on next page)

Identification of significant outliers from SUK48 day 7 extracts

Putatively identified metabolites using DNP and METLIN that presence in the crude extracts of SUK48 day 7 highlighted S-Plot

Table 3: Putatively identified metabolites using DNP and METLIN that presence in the crude extracts of SUK48 day 7 highlighted S-Plot

| No | m/z value | Retention time | Molecular Weight | Monoisotopic mass | Compound Name | Tolerance (ppm) | Chemical Formula | Sources |
|---|---|---|---|---|---|---|---|---|
| 1. | 211.135 | 8.68 | 210.1353 | 210.1157 | 2,5-Dimethyl-3-(2-phenylethenyl)pyrazine | -1.7585 | $C_{14}H_{14}N_2$ | *Iridomyrmex humilis* |
| 2. | 211.123 | 8.68 | 210.1153 | 210.1157 | 2,5-Dimethyl-3-(2-phenylethenyl)pyrazine | -1.7585 | $C_{14}H_{14}N_2$ | *Iridomyrmex humilis* |
| 3. | 245.117 | 8.98 | 244.1099 | 244.1099 | 3,3-Bis(4-hydroxyphenyl)-1-propanol | -0.1998 | $C_{15}H_{16}O_3$ | *Streptomyces albospinus* 15-4-2 |
| 4. | 245.137 | 8.98 | 244.1298 | 244.1099 | 3,3-Bis(4-hydroxyphenyl)-1-propanol | -0.1998 | $C_{15}H_{16}O_3$ | *Streptomyces albospinus* 15-4-2 |
| 5. | 195.149 | 10.47 | 194.1415 | 194.1419 | 6-(1-Methylethyl)-3-(2-methylpropyl)-2(1H)-pyrazinone | -2.1236 | $C_{11}H_{18}N_2O$ | *Aspergillus flavus* |
| 6. | 155.078 | 3.20 | 154.0718 | 154.0718 | 2-(Trifluoromethyl)piperazine | 6 | $C_5H_9F_3N_2$ | Chemically synthesized |