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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32   Abstract

33   **Background:** Antiplasmodial drug discovery is significant especially from natural sources such

34   as plant bacteria. This research aimed to determine antiplasmodial metabolites of *Streptomyces*

35   spp. against *Plasmodium falciparum* 3D7 by using a metabolomics approach. **Methods:**

36   *Streptomyces* strains' growth curves, namely SUK 12 and SUK 48, were measured and *P.*

37   *falciparum* 3D7 IC_{50} values were calculated. Metabolomics analysis was conducted on both

38   strains' mid-exponential and stationary phase extracts. **Results:** The most successful

39   antiplasmodial activity of SUK 12 and SUK 48 extracts shown to be at the stationary phase with

40   IC_{50} values of 0.8168 ng/mL and 0.1963 ng/mL, respectively. In contrast, the IC_{50} value of

41   chloroquine diphosphate (CQ) for antiplasmodial activity was 0.2812 ng/mL. The univariate

42   analysis revealed that 854 metabolites and 14, 44, and 3 metabolites showed significant differences

43   in terms of strain, fermentation phase, and their interactions. Orthogonal partial least square-

44   discriminant analysis (OPLS-DA), and S-loading plot putatively identified pavettine,

45   aurantioclavine, and 4-butyldiphenylmethane as significant outliers from stationary phase of SUK

46   48. For potential isolation, metabolomics approach may be used as a preliminary approach to

47   rapidly track and identify the presence of antimalarial metabolites before any isolation and

48   purification can be done.

49

50   **Introduction**

51   A large number of imported malaria cases from Europe and the Mediterranean have recently been

52   reported. The increasing number of foreign travelers linked to the hefty inflow of malaria-endemic

53   immigrants. For instance, anopheline vectors, which act as the parasite reservoir and are present

54   in Mediterranean regions, can infect the travelers returning from tropical countries (Dominguez

55   Garcia et al. 2019; Silvia Odolini 2012). In 2017, World Health Organization (WHO) documented

56   that the most malaria cases during that year were reported in Africa (with 92% or 200 million),

57   and followed by Southeast Asia (with 5% or 10.8 million) (WHO 2018).

58   Endophytic *Streptomycetes* are bacteria within ethnomedicinal plants that have symbiotic

59   relationships with the host plants. *Streptomyces* is the biggest genus of actinomycetes, consisting

60   of aerobic Gram-positive bacteria that are capable of generating filamentous branches called

61   mycelia (Gottlieb 1966). The mycelia secrete antibiotics, that may be anti-fungal, antibacterial,

62   and anti-viral, when they sporulate in the dormant phase (Alam et al. 2010; Gramajo et al. 1993;

63   Roszak & Colwell 1987).

64   Previous studies reported that trioxacarcin A and D, produced by marine *Streptomyces* sp.,

65   were more effective in producing antiplasmodial activity than artemisinin (Maskey et al. 2004;

66   Tomita et al. 1981). Furthermore, coronamycin, a novel antiplasmodial agent from *Streptomyces*

67   sp., reportedly possessed antiplasmodial activity against *Plasmodium falciparum* (Ezra et al.

68   2004). Strobel and co-researchers discovered that munumbicin D also had antiplasmodial activity

69   (Castillo et al. 2002). Kakadumycin A, also identified by the same team, isolated from

70   *Streptomyces* sp. NRRL 30566 compound demonstrated promising antiplasmodial activity on

71   *Plasmodium falciparum* (Castillo et al. 2003). Furthermore, the isolated gancidin W of

72   *Streptomyces* sp. SUK 10 inhibited the growth of *Plasmodium berghei (Zin et al. 2017)*.

73   This study aims to determine the antiplasmodial activity on metabolites produced from

74   *Streptomyces* spp. against *Plasmodium falciparum* 3D7 using a metabolomics approach.

75   Metabolomics is a high throughput method utilized to screen the metabolites in organisms or

76   tissues by chromatography techniques coupled with mass spectrometry (Rochfort 2005). Then the

77   metabolites are depicted into a two-dimensional distribution using multivariate analysis, including

78   Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures

79   Discriminant Analysis (OPLS-DA), and further putatively identified using Dictionary of Natural

80   Products (DNP). The metabolites include amino acids, carbohydrates, organic acids, vitamins,

81   antibiotics, and phytochemicals (Wishart 2008). The metabolomics approach uses high analytical

82   techniques to determine metabolites in the biological samples. In this study, a hybrid chemical

83   profile using liquid chromatography and mass spectrometry (LC-MS) with multivariate data

84   analysis (MVA) was used to determine the metabolites present in *Streptomyces kebangsaanensis*

85   SUK 12 and SUK 48. Moreover, mass spectrometry (MS)-based metabolomics helped to fast-track

86   the identification of targeted and untargeted metabolites present in complex extracts during

87   screening.

88

89   **Materials & Methods**

90   **Culture**

91   *Plasmodium falciparum* 3D7 was obtained from the culture collection of the Parasitological

92   Department, Faculty of Medicine, Universiti Malaya (UM) while *Streptomyces kebangsaanensis*

93   SUK 12 and SUK 48 were acquired from the Novel Antibiotics Laboratory, Programme of

94   Biomedical Science, Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM).

95   *Streptomyces kebangsaanensis* SUK 12 was isolated from an ethnomedicinal plant, *Portulaca*

96   *oleracea* L., which was collected from the Nenasi Reserve Forest, Pahang, Malaysia (Sarmin et al.

97   2013). *Streptomyces* sp. SUK 48 was isolated from the fruit of *Brasilia* sp. (Zin 2015).

98

99   ***Streptomyces* spp. growth condition**

100   The SUK 12 and SUK 48 strains were grown in nutrient broth (pH 7.0) on an orbital shaker at 160

101   rpm, 28°C for 21 days. The dry weight was collected daily by centrifugation at 4000 rpm for 15

102   mins at 25°C and dried at 70°C. The growth curves of both strains were plotted using Microsoft

103   Excel. The growth rate and generation time were estimated by the following calculation:

104

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

106   Generation time $= \dfrac{1}{k}$

107

108   ***Streptomyces* spp. extracts preparation**

109   The crude extracts prepared in the 14-day culture of both strains' blocks (1 cm^2) were used to

110   inoculate in nutrient broth for 5, 12, and 14 days for SUK 12, and 7, 14, and 21 days for SUK 48.

111   The broth cultures were also incubated on an orbital shaker (Protech, Malaysia) at 160 rpm, 28°C.

112   Both strains' cultures were extracted using 3-fold ½ volume of ethyl acetate. The organic layer

113   (ethyl acetate) was discarded and dried under vacuum using a rotary evaporator (Buchii,

114   Swirtzerland).

115

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

139   ranging from 10 µg/mL to 10^{-6} µg/mL. Next, 100 µL of the diluted extracts and CQ were

140   transferred into a sterile 96-wells plate. For negative and positive control wells, 100 µL of CM

141   was transferred into this 96-well plate.

142

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

144   Parasite culture (iRBC) with 10% parasitaemia was selected and centrifuged at 1800 rpm for 5

145   mins to obtain the cell pellets. The cell pellets were diluted to 2% parasitaemia with fresh RBC.

146   Approximately 2 µL iRBC was transferred into every well of diluted extracts, control drug, CQ,

147   and positive control. Then, 2 µL of fresh RBC was added into negative control, and the 96-well

148   was incubated at 37°C, 5% carbon dioxide (CO_2) for 48 hs. The plate was then frozen overnight

149   at -20°C prior the pLDH assay started (Makler et al. 1993).

150

151   **Parasite lactate dehydrogenase (pLDH assay)**

152   Upon overnight freezing at -20°C, the 96-well plate was defrosted at 37°C for 20 mins and re-

153   frozen at -20°C for 30 mins. This step was repeated 3 times to break the parasite cells. At the same

154   time, Malstat reagent and NBT-PES (nitroblue tetrazolium-phenazine ethosulfate) were also

155   prepared in the dark. About 100 µL of Malstat reagent and 25 µL of NBT-PES were added to a

156   new 96-well plate. After the freezing and defrosting processes finished, 15 µL of each defrost

157   culture well plate was transferred into the wells of a new plate that contained Malstat reagent and

158   NBT-PES. The 96-well plate was then incubated for 1 h at room temperature in the dark.

159   Absorbance readings at the wavelength of 650 nm (A_{650}) were measured for the 96-well plate using

160   a spectrophotometer (Tecan M200, Switzerland). The positive control was assumed to be the

161 maximum level of lactate dehydrogenase enzyme production. The negative control was the blank

162 (Makler et al. 1993; Trager & Jensen 1976). Inhibition of parasite (%) was calculated as follows:

163 $\qquad \dfrac{(A650\ average\ for\ diluted\ sample\ -\ A650\ average\ negative\ control)\ \ X100}{(A650\ average\ positive\ control\ -\ A650\ average\ negative\ control)}$

164

165 A sigmoid graph was plotted using Graphpad PRISM version 7. The value of parasite inhibition

166 50% (IC_{50}) was determined from the graph.

167

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

169 Approximately 3 mg/mL extracts of S12D5 (SUK 12 day to harvest fermented broth is 5), S12D12,

170 S48D7, and S48D14 were sent for LC-MS analysis (Rosli et al. 2017). Scientific C-18 column

171 Thermo was chromatographically separated by an UltiMate 3000 UHPLC (Dionex) system

172 (AcclaimTM Polar Advantage II, 3 mm to 150 mm, 3 μm particle size). A flow rate of 0.4 mL/min

173 at 40°C was maintained with water that contained 0.1% formic acid and 100% acetonitrile with a

174 total operating time of 22 mins. The gradient started for 3 mins at 5% of solvent B, then grew to

175 80% of solvent B for 7 mins and stayed at 80% of solvent B for 5, or 10-15 mins. At last, the

176 gradient turned to 5% of solvent B in 7 mins (15-22 mins). The ESI-positive ionization was

177 performed with the use of MicrOTOF-Q III (Bruker Deltonic) with the settings: capillary voltage

178 4500 V; pressure of 1.2 bar, and dry gas flow 8 L/min at 200°C; 50-1000 Da m/z, respectively.

179

180 **Mass spectrometry data handling**

181 Raw material in "d format" was supplied to Bruker Compass Data Analysis Viewer version 4.2

182 (Bruker Daltonics, Germany) and imported into the Profile Analysis 2.0 data bucketing software

183 (Bruker Daltonics, Germany) (Mamat et al. 2018; Veeramohan et al. 2018). The parameters for

184   compound detecting were: signal/noise threshold: 5; correlation coefficient: 0.7; minimum

185   compound length: 8; smoothing width: 2. Compound detection was done using Find Molecular

186   Features (FMF). The composite bucket table was calculated using advanced bucketing features as

187   time alignment parameters. The time interval was between 0.00 mins and 22.04 mins, and the mass

188   range was between 49 m/z and 1001 m/z. For standardized settings, the data was uploaded to the

189   MetaboAnalyst 3.0 server (www.metaboanalyst.ca). The normalizing features used were: internal

190   standard caffeine: 195.088 m/z, RT: 7.98 mins; transformation: log; scaling: pareto. (Xia et al.

191   2015). Normalized and validated data tables were exported to SIMCA P+ version 15 from

192   Umetrics AB, Umea, Sweden. The PCA, the examination of fundamental variations in samples,

193   and the presentation of outliers were conducted prior to the sample classification of Model OPLS-

194   DA. With 100 random permutations using cross-validations and responsive permutation tests, the

195   robustness of OPLS-DA was validated. The two-way ANOVA type was "ANOVA" in subjects.

196   The significance threshold was set as the *p*-value of correction lower than 0.05. The false discovery

197   rate was less than 0.05.

198

199   **Metabolite identification**

200   The search and manual verification of secondary metabolites in the LC–MS analyses were done.

201   Online databases, namely METLIN and Dictionary of Natural Products (DNP), were examined for

202   the value of a molecular ion of interest (Mazlan et al. 2019; Smith et al. 2005). The databases were

203   used to identify molecular weight metabolites within a specified m/z value query tolerance range.

204

205   **DNA extraction**

206 First, neutral lysis of genome DNA was done for isolation, followed by extraction, then

207 precipitation of phenol chloroforms and isopropanol. The protocol was used to extract the DNA

208 (Kieser 1984). For several changes, *Streptomyces* mycelium, in a 1.5 mL of Eppendorf tube was

209 interrupted by vortexing with 500 μL of lysozyme. The samples were then added with 25 μL of 50

210 mg/mL of lysozyme and 3 μL of 10 mg/mL of RNase. Finally, the purity and concentration of the

211 DNA were determined on a Thermo Scientific nano-drop 2000C machine. SUK 12 DNA sequence

212 was obtained from the previous study (Sarmin et al. 2013).

213

214 **16S rRNA molecular identification and phylogenetic tree analysis**

215 Polymerase chain reaction (PCR) amplified the 16S rRNA gene sequence using universal

216 16SrRNA bacterial gene primers as described earlier (Coombs & Franco, 2003). For SUK 12 and

217 SUK 48, 1416 and 1463 nucleotides respectively, were near full-length 16S rRNA gene sequences.

218 The sequences were compared using BLAST and EzTaxon e-databases with the GenBank database

219 (Altschul et al. 1997). The comprehensive sequence similarity calculation was based on the

220 EzTaxon server's global alignment algorithm (Kim et al. 2012). The sequence was also matched

221 several times with 16S rRNA gene sequences, which are available in GenBank/EMBL with the

222 CLUSTAL W programme, for closely related species of *Streptomyces* (Thompson et al. 1994).

223 The neighbour-joining (NJ) approach (Saitou & Nei 1987), available in MEGA software package

224 7 version, used the reconstructions of the phylogenetic trees (Tamura et al. 2013). In accordance

225 with the two-parameter Kimura model, the matrices of distance were calculated (Kimura 1980).

226 Moreover, the tree was made with the maximum likelihood (ML) (Kimura 1980). By conducting

227 a bootstrap analysis-based of 1000 replicates, the topology of the trees was evaluated (Felsenstein

228 1985).

229

Results

231

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

233 The growth curve showed that the generation time of *Streptomyces* sp. SUK 12 was faster than

234 SUK 48's with 1.16 h/generation and 4.56 h/generation, respectively (Figure 1 A and B).

235 Meanwhile, the antiplasmodial activity revealed that the crude extract of *Streptomyces* sp. SUK

236 48 on day 14 was more potent (with an IC_{50} value of 0.1963 ng/mL) than that of SUK 48 on day

237 7 and SUK 12 on day 5 and day 10 (Table 1).

238

Metabolomics approach

240 The metabolite profiles in extracts using two-way ANOVA (univariate analysis) were compared

241 to those using PCA, OPLS-DA, and S-Plot (multivariate analysis). Two-way ANOVA summarizes

242 and simplifies each sample metabolites (Figure 2). Approximately, 96 metabolites were common

243 in the type of strain, fermentation time, and interaction between both (time and strain type). While

244 44 metabolites were significant in strain type, 14 metabolites were significant in fermentation time,

245 and three metabolites were significant in the interaction between both (Supplementary Table 1).

246 The variation and diversity of the extracts were examined in a PCA model (Figure 3A). The PCA

247 model, however, gave a low Q2 = 0.271 preview. *Streptomyces* sp. extracts cannot be

248 distinguished by any significant variation. The OPLS-DA scores scatter plot of the crude extracts

249 SUK 48 showed distinct separation between day 7 and day 14 (Figure 3B). Nevertheless, the crude

250 extract of SUK 12 on day 5 and day 12 was not well distributed. Meanwhile, the OPLS-DA scores

251 scatter plot (Figure 3C) and S-loadings plot (Figure 3D) of SUK 48 crude extracts between day 7

252 and day 14 gave a good distribution. Overall, the metabolites separation from SUK 48 day 14

253 extracts could be seen as in Figure 4. The secondary metabolite at m/z 195.092 was putatively

254 identified as 1-Vinyl-β-carboline (pavettine) whereas metabolites at m/z 225.163 and 225.153

255 were putatively identified as 4-butyldiphenylmethane. Metabolites presence at m/z 227.135,

256 227.145, and 227.153 were putatively identified as aurantioclavine; (-)-form. All the outliers in

257 the crude extracts of SUK 48 day 14 mentioned as above are known metabolites in Table 2. Then,

258 metabolites at m/z 211.135 and 211.123 were putatively identified as 2,5-Dimethyl-3-(2-

259 phenylethenyl)pyrazine, while metabolites at m/z 245.117 and 245.137 were 3,3-Bis(4-

260 hydroxyphenyl)-1-propanol. The secondary metabolite at m/z 195.149 was represented as 6-(1-

261 Methylethyl)-3-(2-methylpropyl)-2(1H)-pyrazinone, while at m/z 155.078 was trifluoromethyl

262 piperazine. These metabolites were the outliers in SUK 48 day 7 (Table 3).

263

264 ***Streptomyces* spp. identification using molecular analysis.**

265 Phylogenetic tree analysis of both SUKs showed that SUK 48 and SUK 12, using the NJ tree,

266 belonged to different clades (Figure 5) where the bootstrap value was 98%. Moreover, the ML tree

267 (supplement Figure 1) supported this report with a bootstrap value of 95%.

268

269

270 **Discussion**

271 There are four stages of bacteria growth: lag, log, stationary, and death. The crucial phase for

272 producing antibiotics is the stationary phase in *Streptomyces* spp. (Chandrakar & Gupta 2019;

273 Chen et al. 2020). *Streptomyces lactamdurans* that produces the antibiotic cephamycin C has a

274 generation time of 7.5 hs (Ginther 1979). Furthermore, faster growth is potentially essential in the

275   enzyme production of actinomycetes. The effective mycelial fragmentation by the enhanced

276   expression SsgA has significant consequences for antibiotic production, with increasing

277   undecylprodigiosin, but a complete block in the production of actinorhodin (van Wezel et al.

278   2009). SUK 48 could therefore be a potential antibiotic producer as its ability as slow-growing

279   capacity. The stationary phase of bacterial growth is the survival phase in which they actively

280   secrete secondary metabolites to combat oxidative stress and malnutrition for survival purposes

281   (Banchio & Gramajo 2002; Undabarrena et al. 2017).

282   Chemometrics is a chemical discipline that uses mathematical and statistical logic-based

283   methods to design optimal measurement procedures and tests, and provide maximum chemical

284   information through the analysis of chemical data (Massart & Buydens 1988). From a

285   metabolomics perspective, the univariate analysis is a statistical test used to independently measure

286   metabolites that have significantly increased or decreased between different groups. This test is

287   important for significance testing of the tens to hundreds of metabolites to reduce the probability

288   of false positives caused by multiple tests. Of the 854 metabolites analyzed in two-way ANOVA,

289   14 were significant in the fermentation time (mid-exponential and stationary phase), whereas, 44

290   and 14 metabolites were significant in strain and time respectively, and interaction between the

291   both were three metabolites were significant. In addition, there were 96 metabolites significant in

292   strain, time, and interaction of both. There were two antimalarials identified in the list of 96

293   metabolites, gancidin W, antimycin and hydroquinine. Gancidin W has been reported isolated

294   from *Streptomyces gancidicus* and poses antimalarial agent against *Plasmodium berghei* (Aiso K

295   1956; Wakaki et al. 1958; Zin et al. 2017). Besides, antimycin had been reported was isolated from

296   *Streptomyces blastmyceticus* and antiplasmodial activity against *Plasmodium falciparum* K1

297   (Gomez-Lorenzo et al. 2018; Nakayama et al. 1956). While hydroquinine was first isolated from

298   the reduction of quinine and was determined as an antimalarial agent (Buttle et al. 1938; Griffin et

299   al. 2012). Pavettine is an alkaloid compound which found in SUK 48 on day 14, was reported to

300   have anti-cancer where IC_{50} was 100 ng/mL against leukemic cell lines (Figuerola & Avila 2019).

301   Furthermore, the antimicrobial activity of pavettine against *Bacillus subtilis* and *Candida albicans*

302   reported minimum inhibitory concentration (MIC) values from 1.9 µg/disc to 3.8 µg/disc (Sudha

303   & Masilamani 2012). Pavettine was previously mainly found in *Pavetta lanceolata, Cribricellina*

304   *cribraria, Costaticella hastata*, and *Soulamea raxinifolia* (Blackman & Walls 1995; Jordaan

305   1968). Further isolation and purification of putative metabolites and identification and elucidation

306   of molecule structure is required by using MS/MS, 1D- and 2D-NMR.

307   Phylogenetic tree analysis of both SUKs showed that SUK 48 and SUK 12 belonged to

308   different clades. The reliability test was over 70%, which was 98% at nodes between SUK 12 and

309   SUK 48. This result suggested that both SUKs were different subspecies. Distance matrices of the

310   neighbouring tree construction were calculated using Kimura, the two-parameter correction model

311   (Kimura 1980), and a bootstrap analysis based on 1000 replications was performed to evaluate the

312   topology of the neighbouring joining trees (Felsenstein 1985). SUK 12 was a novel species

313   (Sarmin et al. 2013) with the antiplasmodial agent (Remali 2016). We believe SUK 48 is

314   presumably a novel species (data not published). The selected outgroup, *Kitasatospora setae* KM-

315   6054[T] was used based on the previous study (Sarmin et al. 2013).

316

317   **Conclusion**

318   SUK 48 at the stationary phase produced more metabolites with antimicrobial and

319   anticancer properties than SUK 48 at the mid-exponential phase. Therefore, SUK 48 at the

320   stationary phase was the potential antimalarial extract candidate for the further purification

321 process. The metabolomics approach is a rapid tool that dereplicated known antimalarial

322 metabolites and determined those at specific fermentation time. Further study will focus on the

323 fractionation and purification of possible antimalarial compounds from *Streptomyces* spp. SUK

324 48. Meanwhile, an *in silico* study using molecular docking (MD) will be conducted to verify the

325 potential interaction between complex protein-ligands in developing a potential new

326 antiplasmodial agent.

327

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336 (DNP) database library.

337

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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.

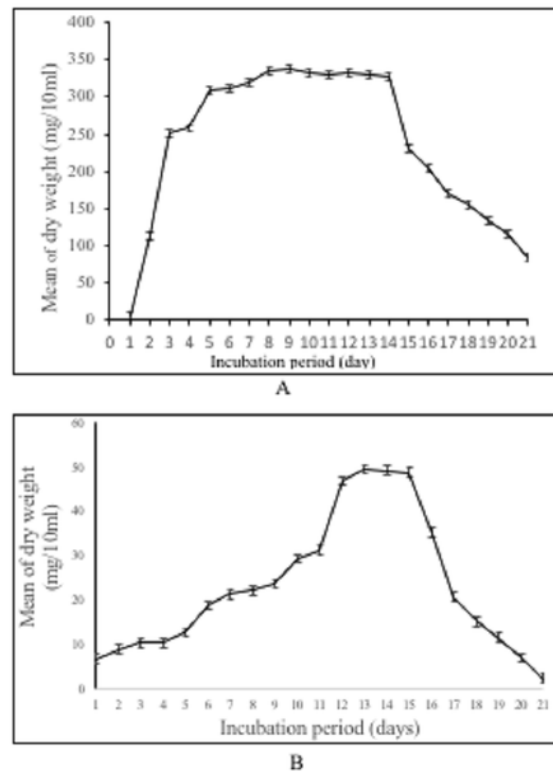



Figure 2

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).

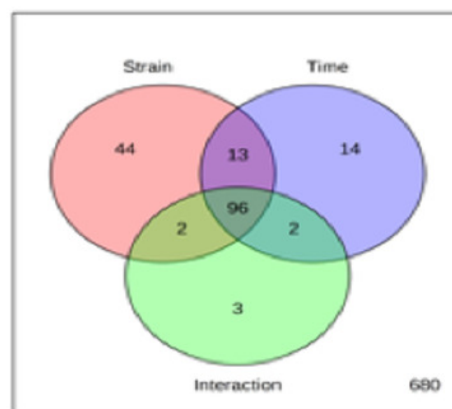



Figure 3

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)

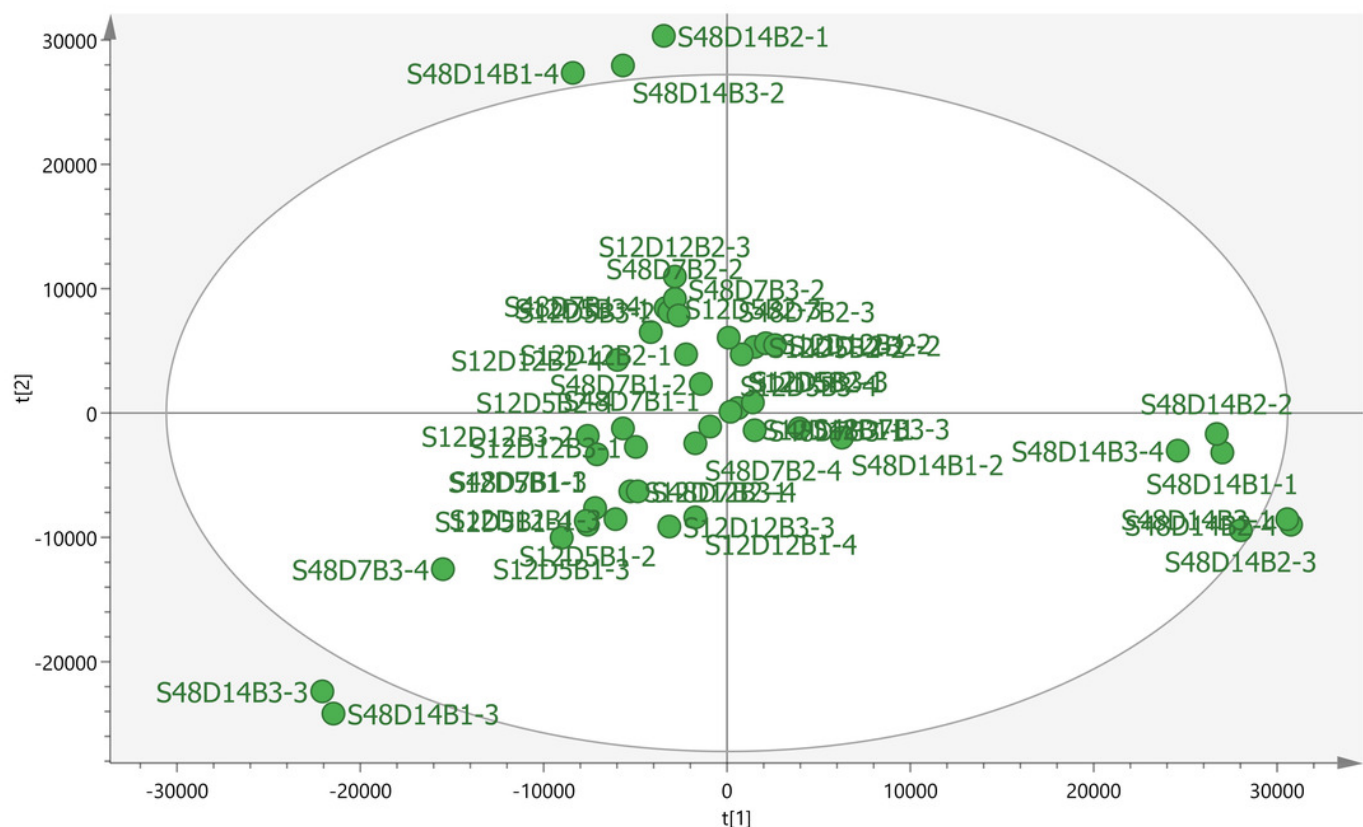



Figure 4

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

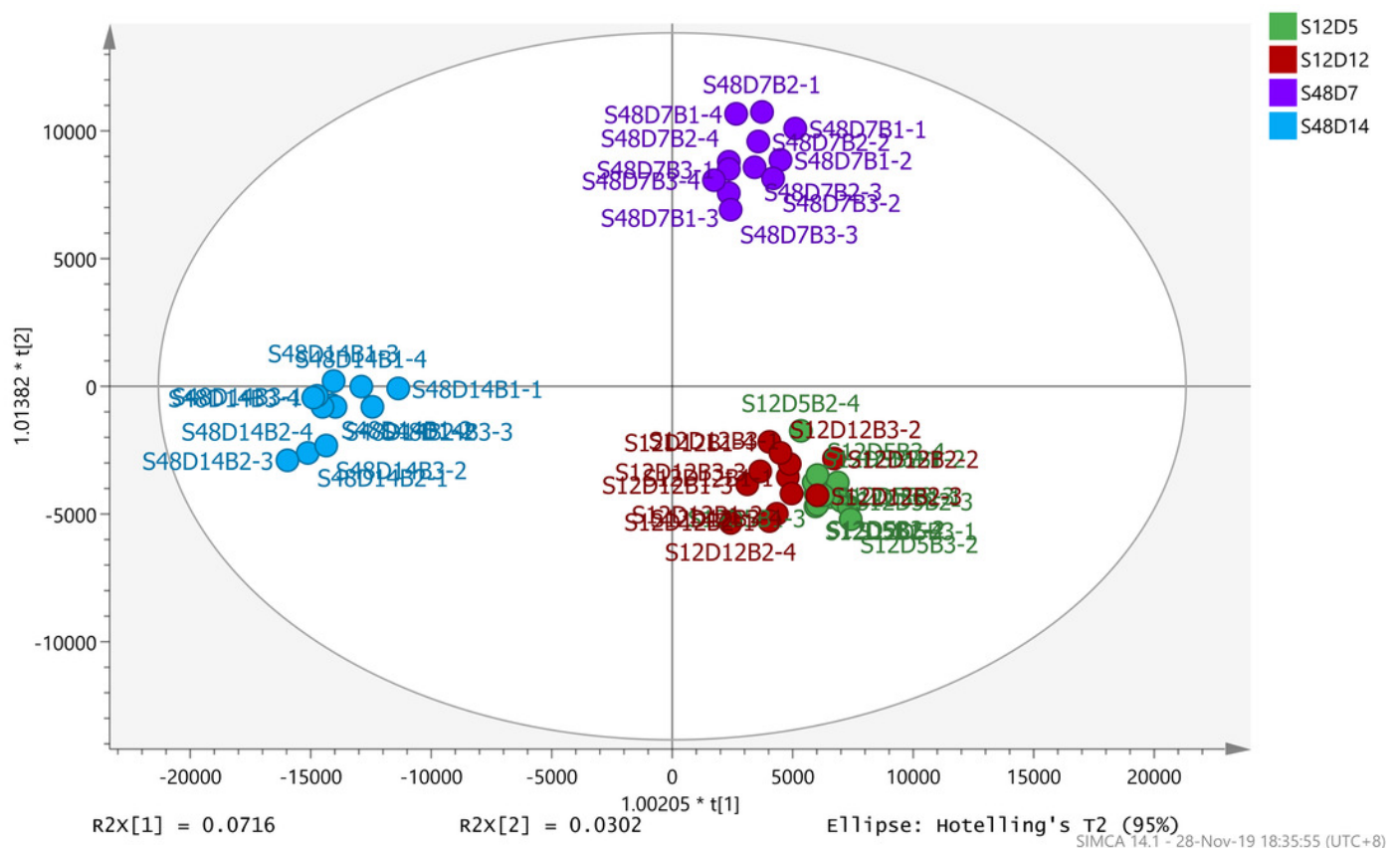



Figure 5

OPLS-DA scores scatter plot

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

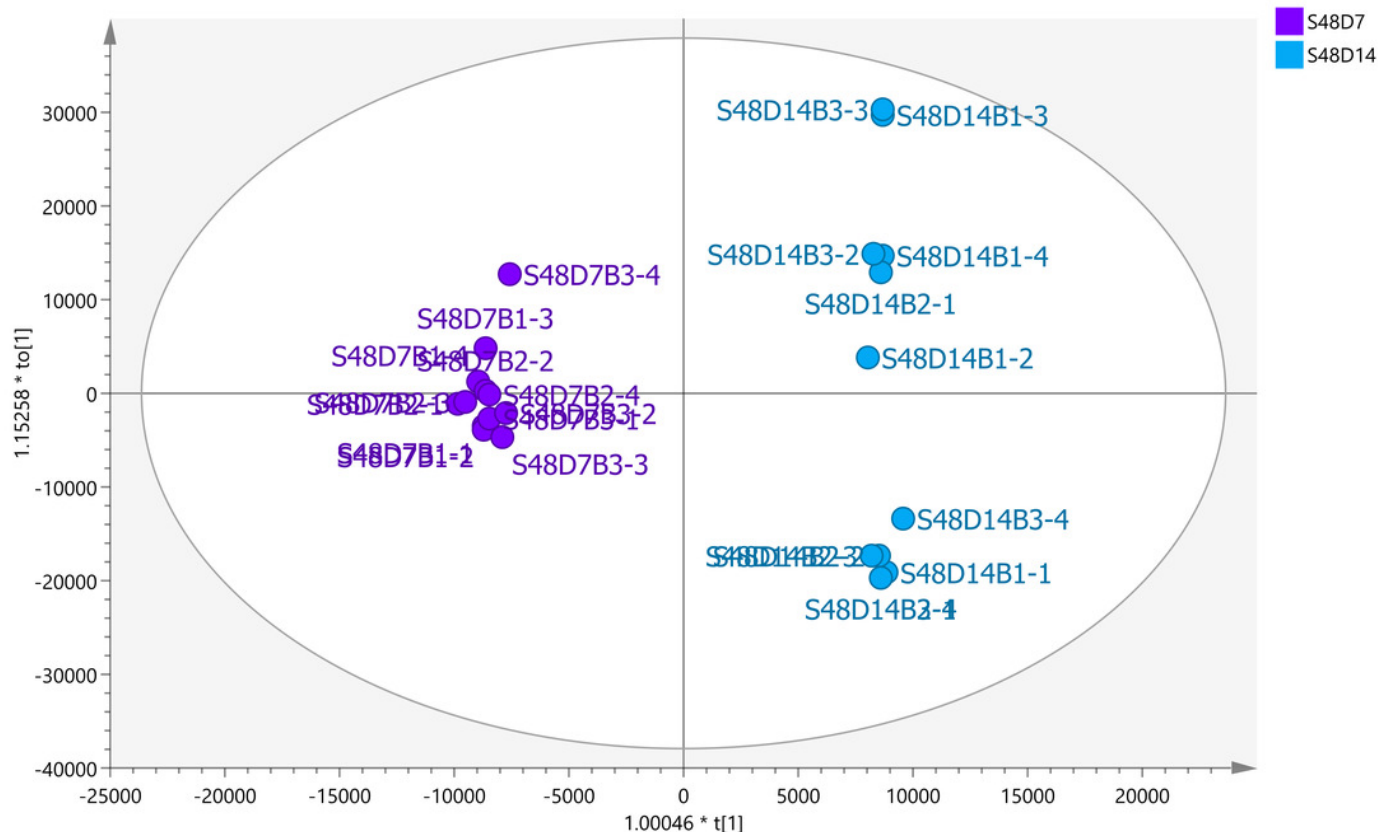



Figure 6

S-Plot

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

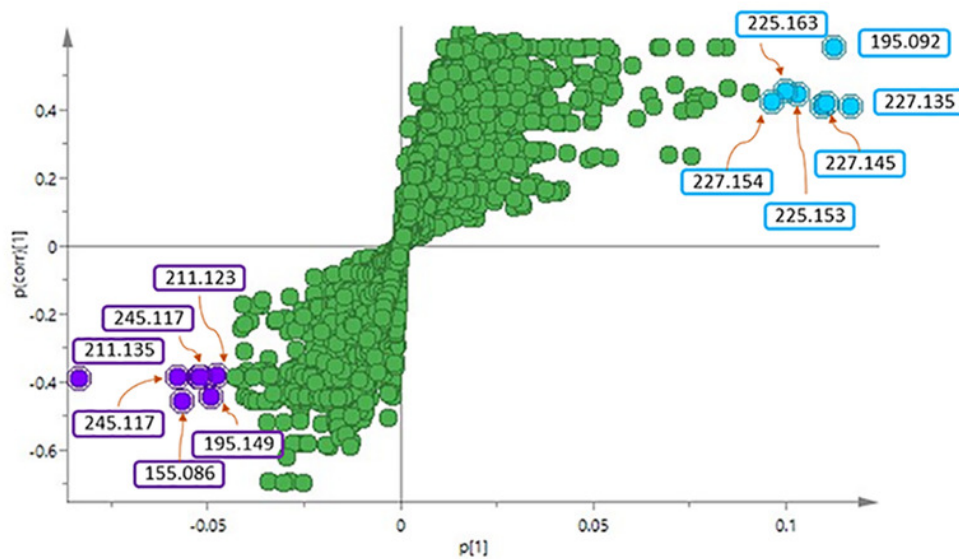



Figure 7

Total ion chromatogram

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

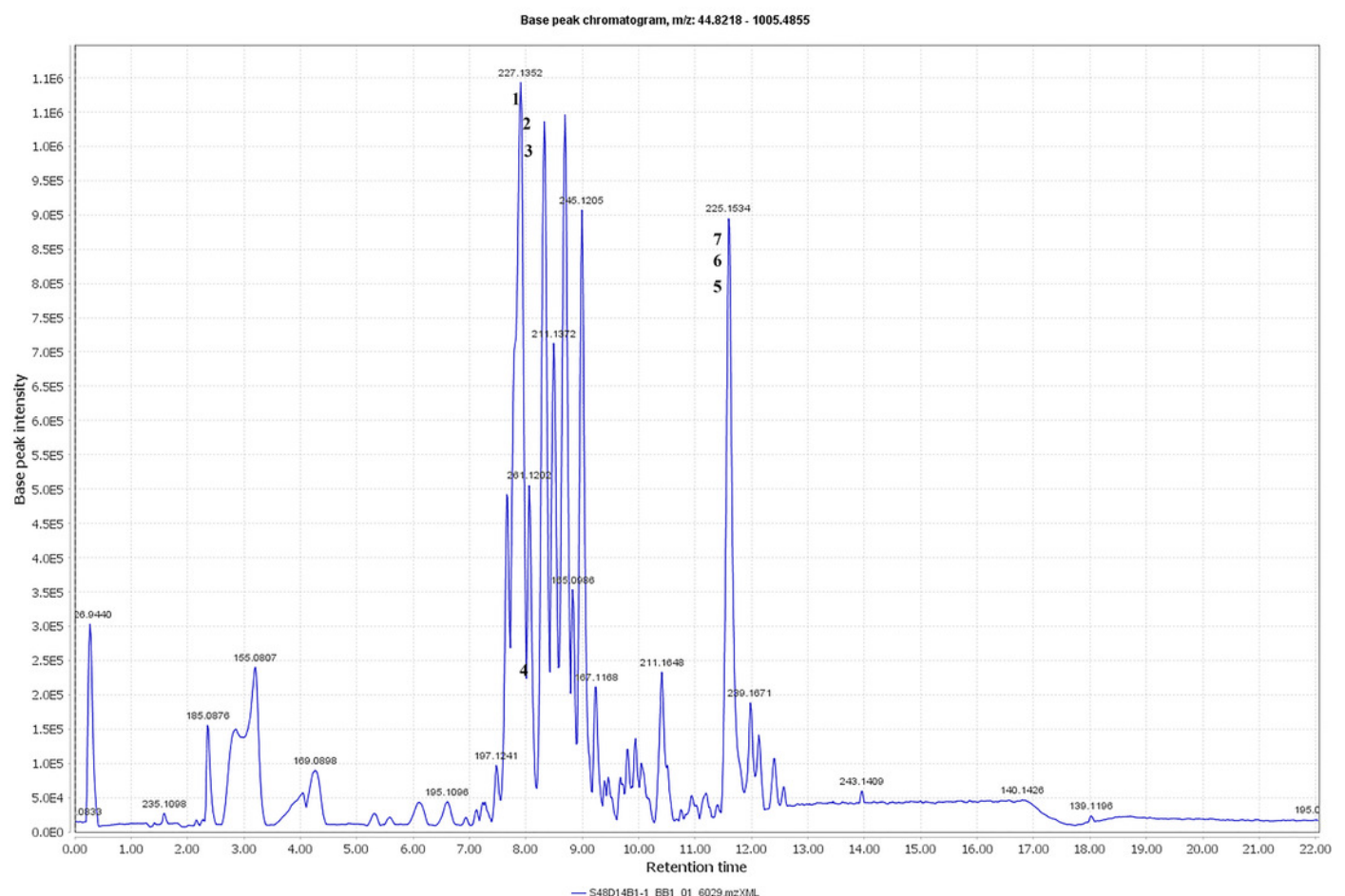
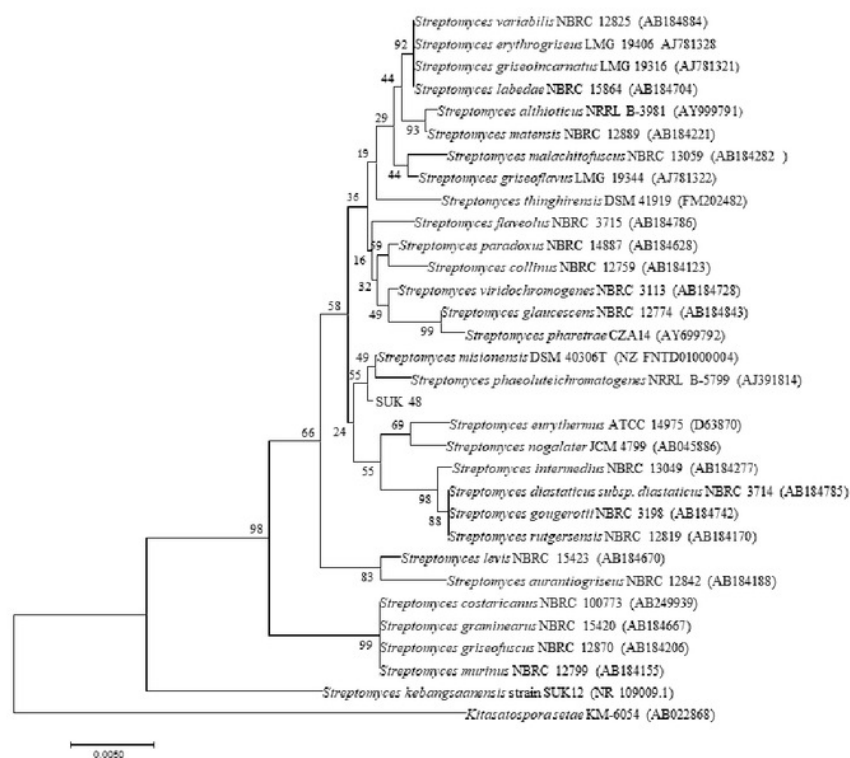



Figure 8

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.

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

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.

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

5

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

1 Table 3: Putatively identified metabolites using DNP and METLIN that presence in the crude extracts of SUK48 day 7 highlighted S-
2 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