

Assessment of antimycobacterial activities of pure compounds extracted from Thai medicinal plants against clarithromycin-resistant *Mycobacterium abscessus* (#63417)

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Assessment of antimycobacterial activities of pure compounds extracted from Thai medicinal plants against clarithromycin-resistant *Mycobacterium abscessus*

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Background: Infection with *Mycobacterium abscessus* is usually chronic and is associated with clarithromycin resistance. Increasing drug resistance is a major public-health problem and has led to the search for new antimycobacterial agents. We evaluated the antimycobacterial activity, toxicity, and synergistic effects of several plant secondary metabolites against *M. abscessus*.

Methods: Twenty-three compounds were evaluated for antimycobacterial activity against thirty *M. abscessus* clinical isolates by broth microdilution to determine their minimum inhibitory concentration (MIC) values. Toxicity was evaluated using red and white blood cells (RBCs and WBCs). The compounds were used in combination with clarithromycin to investigate the possibility of synergistic activity.

Results: Five out of twenty-three compounds (RL008, RL009, RL011, RL012 and RL013) exhibited interesting antimycobacterial activity against *M. abscessus*, with MIC values ranging from <1 to >128 µg/mL. The extracts did not induce hemolytic effect on RBCs and displayed low toxicity against WBCs. The five least-toxic compounds were tested for synergism with clarithromycin against 7 isolates with inducible clarithromycin resistance and 7 with acquired clarithromycin resistance. The best synergistic results against these isolates were observed for RL008 and RL009 (8/14 isolates; 57%).

Conclusions: This study demonstrates antimycobacterial and synergistic activities of pure compounds extracted from medicinal plants against clarithromycin-resistant *M. abscessus*. The synergistic action of the extracts may be effective for treating infections and should be further studied for the development of novel antimicrobial agents.

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Short title: Plant secondary metabolites against clarithromycin-resistant *M. abscessus*

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45 **Keywords:** antimycobacterial activity, medicinal plants, plant secondary metabolites,
46 *Mycobacterium abscessus*, clarithromycin resistance

Introduction

Nontuberculous mycobacteria (NTM) are found in the environment (soil and water) (Velayati et al. 2014). Some species of NTM can cause life-threatening human diseases with a high mortality rate (Cassidy et al. 2009; Iroh Tam et al. 2015). *Mycobacterium abscessus* is a common NTM species causing chronic infection and is highly associated with drug resistance (Tung et al. 2015). In Thailand, drug-resistant *M. abscessus* infection has become a serious health issue, requiring a prolonged course of treatment, and is associated with treatment failure in up to one-third of cases (Kham-Ngam et al. 2018). Although clarithromycin is a drug of choice, half of the strains present in Thailand are clarithromycin-resistant (Ananta et al. 2018). Therefore, new treatment alternatives are needed to overcome drug-resistant *M. abscessus* infection.

Plants are a source of bioactive compounds and can treat various diseases. Northeastern Thailand has high plant diversity, which remains locally important as a source of traditional medicine. Several research teams have reported anti-*Mycobacterium tuberculosis* activity of plant extracts from *Tetradenia riparia* (see Baldin et al. 2018), *Persea americana* (Jimenez-Arellanes et al. 2013), *Lophira lanceolata* (Nkot et al. 2018) and *Flourensia cernua* (Molina-Salinas et al. 2006). In Thailand, extracts from *Neonothopanus nambi* (see Kanokmedhakul et al. 2012) and *Rothmannia wittii* (Chaipukdee et al. 2016) showed antimycobacterial activity against *M. tuberculosis*. However, the effect of these plant extracts against *M. abscessus* has not been reported.

We aimed to evaluate the antimycobacterial activities of pure compounds extracted from four medicinal plants (*Atalantia monophylla*, *Prismatomeris filamentosa*, *Ageratum conyzoides* and *R. wittii*) and from the cultured mycelium of the luminescent mushroom *N. nambi*. These

compounds were tested against clinical isolates of clarithromycin-susceptible and non-susceptible *M. abscessus*. The toxicity for mammalian cells and synergistic effect with clarithromycin of the selected compounds were also analyzed.

Materials and methods

Pure compounds extracted from local medicinal plants

A total of 23 pure compounds, consisting of 22 compounds isolated from four medicinal plants (*A. monophylla*, *P. filamentosa*, *A. conyzoides* and *R. wittii*) and one compound isolated from the cultured mycelium of a luminescent mushroom (*L. nambi*), were evaluated (Table 1 and Fig. S1). The plant extracts were purified using column chromatography techniques to isolate the pure secondary metabolites, as described previously (Sombatsri et al. 2018). The pure compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions for analysis.

Bacterial isolates

Mycobacterium abscessus clinical isolates were retrieved from the culture collection of Srinagarind Hospital, Khon Kaen University, Thailand. All specimens were fully anonymized before they were accessed. The colony morphology of each isolate was noted. The species identification of *M. abscessus* was performed using INNO-LiPA Mycobacteria v2 (Innogenetics GmbH, Heiden, Germany), Genotype Mycobacterium CM/AS assay (Hain Lifescience GmbH, Nehren, Germany) or Molecutech REBA Myco-ID (YD Diagnostics CORP, Gyeonggi-do, Korea). All *M. abscessus* isolates were sub-cultured on Lowenstein-Jensen (LJ) solid medium and then incubated at 37°C for 7 days before further analysis. Informed consent is not required

for this study. All specimens including isolates and blood samples were obtained from routine practice in which patient's information were deidentified. This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE611496).

Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) for clarithromycin was determined according to published protocols (Kham-Ngam et al. 2019). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2018). Clarithromycin susceptibility was read at 3, 5, and 14 days. A reading at day 3 was used to test for inducible resistance according to previously described protocols (Ananta et al. 2018). Inducible resistance was inferred by changes in MIC values from “susceptible” at day 3 to “resistant” at day 14. Isolates that were resistant on day 3 and thereafter were regarded as demonstrating acquired resistance. All clarithromycin-susceptible and clarithromycin-resistant (both inducible and acquired resistance) *M. abscessus* clinical isolates were used for further analysis.

Antimycobacterial assay

The antimycobacterial assay was carried out using a broth microdilution method to determine the MIC values according to the CLSI guideline. Two-fold serial dilutions of pure compounds were prepared directly in a 96-well microtiter plate. Individual colonies of *M. abscessus* were suspended in demineralized water to obtain a density corresponding to McFarland Standard 0.5. Then, 50 µL of cell suspension were transferred into a tube of cation-adjusted Mueller-Hinton broth (TREK Diagnostic Systems, Ohio, USA) with TES buffer to

achieve a final cell concentration of approximately 5×10^5 CFU/mL. One-hundred microliters of this inoculum were mixed with 100 μ L of pure compound (1, 2, 4, 8, 16, 32, 64 and 128 μ g/mL) and were then added to each well of the 96-well plate. Following incubation for 3-5 days at 37°C, MICs were visually determined as the lowest concentration of the compound that completely inhibited the mycobacterial growth.

Hemolytic assay

Hemolytic activity was determined according to protocols published previously (Lima Viana et al. 2018). Briefly, six milliliters of blood from a single healthy volunteer were collected and transferred into a heparin collection tube. Whole blood was centrifuged at 5,000 rpm for 5 min and the plasma was then discarded. Human red blood cells (RBCs) were isolated and washed three times with 1% sterile phosphate buffer saline (PBS) solution (pH 7.4). The final cell suspension was adjusted to 5% of RBCs in 1% PBS. Two-hundred microliters of RBC suspension were transferred into the tubes containing different concentrations of pure compounds (1, 2, 4, 8, 16, 32, 64 and 128 μ g/mL in 1% PBS). Positive and negative controls were used, these being 1% Triton X-100 solution and 1% PBS, respectively. The final volume of each experiment was 1.0 mL. The solutions were incubated at 37°C for 1 h. After incubation, the suspensions were then centrifuged at $3,000 \times g$ for 2 min. Then, 100 μ L of supernatant from each tube were transferred into a 96-well plate for measurement of the absorbance at 540 nm using a microplate reader (each absorbance was measured twice). In addition, RBC morphology was observed under a light microscope and recorded. All tests were performed in duplicate for each test compounds. Hemolytic activity was calculated by the following equation:

$$\text{Hemolysis (\%)} = [(A_s - A_n) / (A_c - A_n)] \times 100$$

Where A_s refers to the absorbance of the sample, A_n refers to the absorbance of the negative control (RBCs with PBS) and A_c refers to the absorbance of the positive control (RBCs with Triton X-100).

Cell viability assay

To assay the toxicity of each tested compound for human white blood cells, the trypan-blue exclusion test was used (Strober 2015). Briefly, white blood cells (WBCs) were isolated from the blood of a healthy volunteer using the Ficoll density gradient technique. Whole blood was carefully transferred into a tube containing Ficoll solution (ratio 1:1). Then, the cells were centrifuged at 1,500 rpm for 10 min at 20°C and then the WBC layer was transferred into a new tube. WBCs were washed three times with 1% PBS at 1,500 rpm for 5 min at 20°C and re-suspended with 1 mL of RPMI-1640 media (Gibco™, New York, USA). Fifty microliters of WBC suspension were transferred into individual wells of a 96-well plate and then 50 µL of pure compounds at different concentrations (ranging from 1 to 128 µg/mL) were added. The 96-well plate was incubated at 37°C for 1 h. Then, 20 µL of the suspension was mixed with 20 µL of 0.4% trypan blue solution in buffered isotonic salt solution (0.81% NaCl and 0.06% K₂HPO₄) and incubated for 3 min at room temperature. The viable and dead cells were counted under a light microscope using a hemocytometer. As a negative control, WBC suspension was treated with PBS. The test was performed in duplicate for each test compounds. Dead cells were calculated using the following equation:

$$\text{Dead cells (\%)} = (\text{number of dead cells} / \text{total number of cells}) \times 100$$

Genome sequencing and analysis

Genomic DNA of the 30 *M. abscessus* clinical isolates was extracted using the cetyltrimethylammonium bromide-sodium chloride (CTAB) method (De Almeida et al. 2013) and was sent for genome sequencing (NovogeneAIT, Hong Kong) using an Illumina HiSeq platform generating 150-bp paired-end reads.

The quality of raw sequences was checked using FastQC version 0.11.7 (Andrews 2010). Trimmomatic (v0.36) software (Bolger et al. 2014) was used to remove low-quality reads. High-quality paired-end reads were then mapped to the *M. abscessus* ATCC 19977 reference genome (GenBank accession number CU458896.1) using BWA-mem (v.0.7.17) (Li 2013). For converting SAM to BAM format, sorting and indexing the bam files, SAMtools v0.1.19 algorithm was used (Li et al. 2009). GATK version 4.0.5. (McKenna et al. 2010) was used for realignment, generating coverage statistics and mapping details. Both GATK and SAMtools were used for variant calling and filtering, including single nucleotide polymorphisms (SNPs) and small indels.

For phylogenetic analysis, a WGS-based phylogeny was analyzed using mpileup, VCF and coverage files. Maximum likelihood analysis was performed using MEGA-7 (Qasim et al. 2018) with the general time-reversible (GTR) and gamma model. Support for individual nodes was assessed using 1000 bootstrap replicates. The phylogenetic tree was visualized using iTol software (<https://itol.embl.de/>). The raw sequence data have been deposited into NCBI GenBank under Bioproject PRJNA523980.

Synergism

Combinations of plant secondary metabolites and clarithromycin were evaluated using a microdilution checkerboard method (Doern 2014). Five concentrations of each test compound

and of clarithromycin (Sigma-Aldrich, Missouri, USA) were prepared (4MIC, 2MIC, MIC, MIC/2 and MIC/4), and *M. abscessus* cell suspensions prepared as for previous experiments were used. Fifty microliters of each pure compound and clarithromycin were mixed in a 96-well plate, and 100 μ L of inoculum were then added (final concentration of each pure compound was 128 μ g/mL and of clarithromycin ranged from 4 to 512 μ g/mL). The plate was incubated at 37°C for 7 days (for isolates with acquired resistance) or 14 days (for isolates with inducible resistance). The results were recorded and interpreted as fractional inhibitory concentration index (FICI). The FICI value was calculated using the following equation:

$$FICI = [A] / (A) + [B] / (B)$$

Where [A] refers to MIC (A) in combination with (B), (A) refers to MIC (A) alone, [B] refers to MIC (B) in combination with (A), and (B) refers to MIC (B) alone.

FICI values of ≤ 0.5 , $>0.5-1.0$, $>1.0-4.0$ and >4.0 were interpreted as indicating “synergy”, “additive”, “indifference” and “antagonism”, respectively.

Results

Identification and characteristics of *M. abscessus* isolates used

Thirty clinical isolates of *M. abscessus* on LJ solid medium were recovered. Characteristics of these isolates are described in **Fig. 1**. Thirteen isolates were identified as belonging to *M. abscessus* subsp. *abscessus* and 17 isolates to *M. abscessus* subsp. *massiliense* (**Fig. 1**). Based on their phylogenetic relationships, these isolates were not clonal strains (**Fig. 1**). The MIC values for clarithromycin among the *M. abscessus* isolates ranged from 0.12 to ≥ 16 μ g/mL. Ten isolates were phenotypically susceptible to clarithromycin (ranging from 0.12 to 2

μg/mL), while eleven and nine isolates exhibited inducible and acquired resistance to clarithromycin (both ≥ 16 μg/mL), respectively (Fig. 1 and Table 2).

Antimycobacterial activity of plant secondary metabolites

The MIC values of 23 pure compounds were determined by broth microdilution against 30 clinical isolates of *M. abscessus*, including clarithromycin-susceptible and -resistant isolates. The MIC cut-off value of 128 μg/mL for at least one isolate was chosen for the selection of potentially useful test compounds. Five compounds (RL008, RL009, RL011, RL012 and RL013) were particularly effective in suppressing *M. abscessus*. The MIC values for these compounds ranged from <1 to >128 μg/mL (Table 2). The five effective pure compounds were selected for further analysis.

Toxicity testing on human RBCs and WBCs

The hemolytic activity of the five selected compounds (RL008, RL009, RL011, RL012 and RL013) on human RBCs was evaluated. None showed any hemolytic effect on RBCs ($<1\%$) at various concentrations (1, 2, 4, 8, 16, 32, 64, and 128 μg/mL) (Fig. 2). RBC morphologies under the light microscope were displayed as the cell shrinks (Fig. 3). These findings indicate that the compounds were not harmful to human RBCs.

For WBCs, the percentage of dead cells following exposure to each tested compound is presented in Fig. 4. The toxic effects were concentration-dependent. All five compounds caused death of 15-20% of cells at concentrations of 64-128 μg/mL, except for compound RL013, for which the mortality rate was significantly lower (3-8%). These results suggest that the pure compounds have a low toxicity towards human WBCs.

Synergistic antimycobacterial activity of pure compounds and clarithromycin

Based on phenotypic results, fourteen *M. abscessus* isolates (7 isolates with inducible and 7 isolates with acquired resistance to clarithromycin) were randomly selected to study the synergistic effect against these of five pure compounds in combination with clarithromycin. Results (Table 3) showed that the highest degree of synergism was observed for the RL008/CLA and RL009/CLA combinations (FICI ranging from 0.13 to 0.50), which inhibited eight *M. abscessus* isolates (57%) (Fig. 1). The second strongest synergistic activity was observed for the RL012/CLA combination, followed by the RL013/CLA combination, which showed synergistic effects against seven (50%) and six isolates (42.9%), respectively. The RL011/CLA combination showed the lowest synergistic effect, inhibiting only five isolates (35.7%).

Discussion

Mycobacterium abscessus mostly occurs as a chronic infection and is an important cause of morbidity and mortality (Cassidy et al. 2009). The emergence, evolution and spread of *M. abscessus* infection is highly associated with drug resistance and treatment failure (Tung et al. 2015). Antibiotic resistance to *M. abscessus* is a major public health concern worldwide, including in Thailand (Imwidthaya et al. 1990; Phowthongkum et al. 2005). Clarithromycin, a macrolide antibiotic, has a broad spectrum of antimicrobial activity that inhibits a range of Gram-positive and Gram-negative microorganisms (van der Paardt et al. 2017). It is often a drug of choice for the treatment of serious infections caused by *M. abscessus*. However, *M. abscessus* clinical isolates with reduced susceptibility to clarithromycin have emerged, resulting in a prolonged treatment course and poor clinical outcomes (Li et al. 2017). Clarithromycin

monotherapy is associated with treatment failure. A combination of antimicrobial agents may be of therapeutic benefit and efficacious in the treatment of infections caused by clarithromycin-resistant *M. abscessus*.  here is therefore a need to search for new sources of antimycobacterial substances. Plants produce a variety of bioactive compounds, sometimes with known therapeutic properties.  They are good sources of powerful antibiotic metabolites and can treat various diseases (Hernandez-Garcia et al. 2019). This study was conducted to evaluate the antimycobacterial activity of different secondary metabolites of plant origin against clarithromycin-susceptible and -resistant *M. abscessus* isolates.

Researchers have isolated several such compounds and demonstrated their activities against mycobacteria, including *M. tuberculosis* (Baldin et al. 2018; Chaipukdee et al. 2016; Jimenez-Arellanes et al. 2013; Jyoti et al. 2016; Kanokmedhakul et al. 2012; Molina-Salinas et al. 2006; Naik et al. 2014; Nkot et al. 2018). However, antimycobacterial activities of secondary metabolites against *M. abscessus* have rarely been reported. We used 23 secondary metabolites isolated from *A. monophylla*, *P. filamentosa*, *A. conyzoides*, *R. wittii* and *N. nambi* against *M. abscessus* with different clarithromycin-resistance levels. The most effective compounds were RL008, RL009, RL011, RL012, and RL013, which exhibited MIC values ranging from <1 to >128 µg/mL. Previous reports also showed that colony morphology was not associated with susceptibility to first-line antibiotics (Ruger et al. 2014). However, Clary et al. (2018) reported that the colony morphotypes of *M. abscessus* were associated with biofilm formation and prolonged intracellular survival.

Checking the toxicity of secondary metabolites on both human RBCs and WBCs is of importance when selecting candidates for antimycobacterial drugs. Our results demonstrate that the selected secondary metabolites are not harmful towards RBCs. The RBC shrinkage observed



at high compound concentrations was an osmotic effect due to the extra-cellular concentration. These results agree with those from a previous study (Lima Viana et al. 2018), which evaluated the antimicrobial activity of *Bixa orellana* secondary metabolites to treat *Mycobacterium* infections. In this study, the secondary metabolites tested did not induce significant toxicity in human RBCs. The plant secondary metabolites that we tested killed 3% to 20% of WBCs at their MIC levels (128 µg/mL). The RL0013 compound caused the fewest WBC deaths (≈3%). Although toxicity for WBCs was quite high, these compounds had potential to inhibit clarithromycin-resistant *M. abscessus* isolates. Another study reported that human WBCs were similarly affected by plant secondary metabolites (Maiti et al. 2016).

In this study, a combination of the tested compounds and clarithromycin had synergistic effects on some *M. abscessus* isolates with acquired or inducible clarithromycin resistance. No antagonistic effect of combining these substances was found. Among the five effective compounds tested, RL008 and RL009 proved to be the best ones in a combined treatment with clarithromycin, frequently showing a synergistic effect. They were particularly efficient against *M. abscessus* isolates, with the FICI values ranging from 0.13 to 0.5. With the synergistic effects of RL008 and RL009, the average MICs of clarithromycin alone were reduced up to 4-fold (i.e., reduced from 512 to 32 µg/mL). Both RL008 and RL009 had low toxicity against RBCs and WBCs at the MIC levels. These results are consistent with those of Rahgozar *et al.* (2018), who found that the best synergistic results against *Mycobacterium bovis* were obtained for extracts of *Lavandula stoechas* and *Datura stramonium* in combination with ethambutol. In addition, Lopes *et al.* (2014) reported that a synergism was observed against *M. tuberculosis* with eupomatenoid-5 (EUP-5), extracted from *Piper solmsianum* C. DC. var. *solmsianum* plus rifampicin, and EUP-5 plus ethambutol combinations. Similar observations of synergism between plant secondary

metabolites and various drugs against mycobacteria have been reported in other studies (Aro et al. 2016; Mossa et al. 2004; Naik et al. 2014). We observed that synergistic effects of combining the test compounds with clarithromycin occurred against *M. abscessus* isolates with inducible as well as acquired clarithromycin resistance. Therefore, plant secondary metabolites could be used for treatment of both forms of resistance. However, the association between antimycobacterial susceptibility and clarithromycin-resistance type remains unclear.

Nowadays, the frequent treatment failure of *M. abscessus* infection is a major public health concern. Although our combinations of pure compounds and clarithromycin were not synergistic against all isolates, almost 60% of clarithromycin-resistant *M. abscessus* isolates (showing either inducible or acquired resistance) were inhibited. This might be applied in development of alternative treatments for *M. abscessus* infection.

The core structure of both RL008 and RL009 is an anthraquinone, an aromatic organic compound. This study supports previous findings in the literature that anthraquinone compounds possess antimicrobial properties against Gram-positive bacteria, Gram-negative bacteria, and fungi. (Comini et al. 2011; Kemege et al. 2017; Lu et al. 2011; Xu et al. 2017).

Limitation of this study should be acknowledged. These combinations of secondary metabolites and clarithromycin that we tested *in vitro* should be investigated *in vivo* for more conclusive results. Further work is necessary using structural variants of the plant secondary metabolites identified here to improve their antimycobacterial efficacy. Additional studies might also evaluate antifungal, antiviral and antiparasitic activities of these compounds.

In conclusion, we report that five compounds isolated from medicinal plants have potent antimycobacterial effects, which are enhanced synergistically when combined with clarithromycin against clarithromycin-resistant *M. abscessus* clinical isolates. They also showed

acceptable results in toxicity test towards RBCs and WBCs. These compounds might be used as an alternative treatment and should be further studied to develop anti-tuberculous drugs.

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Table 1 (on next page)

Compounds used in this study.

Table 1 Compounds used in this study.

No.*	Code	Compound	Source	References
1	RL001	<i>N</i> -methylcycloatalaphylline A	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
2	RL006	yukocitrine	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
3	RL002	<i>N</i> -methylatalaphylline	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
4	RL007	atalaphylline	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
5	RL004	atalaphylline-3,5-dimethyl ether	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
6	RL003	2,2-dimethylchromenocoumarin	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
7	RL005	auraptene	Roots of <i>A. monophylla</i>	(Pailee <i>et al.</i> , 2020)
8	RL009	rubiadin-1-methyl ether	Roots of <i>P. filamentosa</i>	(Wisetsai <i>et al.</i> , 2021)
9	RL010	rubiadin	Roots of <i>P. filamentosa</i>	(Wisetsai <i>et al.</i> , 2021)
10	RL011	knoxiadin	Roots of <i>P. filamentosa</i>	(Wang <i>et al.</i> , 1985)
11	RL008	nordamnacanthol	Roots of <i>P. filamentosa</i>	(Wisetsai <i>et al.</i> , 2021)
12	RL012	damnacanthol	Roots of <i>P. filamentosa</i>	(Wisetsai <i>et al.</i> , 2021)
13	RL013	damnacanthol	Roots of <i>P. filamentosa</i>	(Wisetsai <i>et al.</i> , 2021)
14	RL014	3',4',7-tri- <i>O</i> -methyluteolin	Flowers of <i>A. conyzoides</i>	(Ahond <i>et al.</i> , 1990)
15	RL015	4',7-di- <i>O</i> -methylapigenin	Flowers of <i>A. conyzoides</i>	(Ahond <i>et al.</i> , 1990)
16	RL016	4'- <i>O</i> -methylapigenin	Flowers of <i>A. conyzoides</i>	(Yim <i>et al.</i> , 2003)
17	RL017	2'-hydroxy-4,4',6'-trimethoxychalcone	Flowers of <i>A. conyzoides</i>	(Sukari M. A, 2004)
18	RL020	3,5-dihydroxycinnamate	Roots of <i>R. wittii</i>	(Wisetsai <i>et al.</i> , 2020)
19	RL021	lippianoside B	Roots of <i>R. wittii</i>	(Wisetsai <i>et al.</i> , 2020)
20	RL022	rothmannioside C	Roots of <i>R. wittii</i>	(Wisetsai <i>et al.</i> , 2020)
21	RL023	rothmannioside A	Roots of <i>R. wittii</i>	(Wisetsai <i>et al.</i> , 2020)
22	RL024	rothmannioside B	Roots of <i>R. wittii</i>	(Wisetsai <i>et al.</i> , 2020)
23	RL019	aurisin A	Cultured mycelium of <i>N. nambi</i>	(Kanokmedhakul <i>et al.</i> , 2012)

* Compounds **1–22** were isolated from four medicinal plants (*Atalantia monophylla*, *Prismatomeris filamentosa*, *Crinum asiaticum*, and *Rothmannia wittii*). Compound **23** was isolated from the cultured mycelium of the luminescent mushroom *Neonothopanus nambi*.

Table 2(on next page)

Antimycobacterial activity screening of five plant secondary metabolites against 30 *M. abscessus* isolates.

1 **Table 2** Antimycobacterial activity screening of five plant secondary metabolites against 30 *M. abscessus* isolates

Isolates	Organism	Subspecies	Colony morphology	MIC value of clarithromycin (µg/mL)						DST interpretation	Antimycobacterial screening against <i>M. abscessus</i> isolates				
				Day 3		Day 5		Day 14			MIC value of pure compounds (µg/mL)				
				MIC	Phenotype	MIC	Phenotype	MIC	Phenotype		RL008	RL009	RL011	RL012	RL013
80097	<i>M. abscessus</i>	<i>abscessus</i>	Mixed	0.25	Susceptible	8	Resistant	16	Resistant	Inducible	128	16	64	64	128
80167	<i>M. abscessus</i>	<i>massiliense</i>	Rough	≥16	Resistant	≥16	Resistant	≥16	Resistant	Acquired	128	8	64	64	128
80225	<i>M. abscessus</i>	<i>abscessus</i>	Rough	0.25	Susceptible	4	Intermediate	≥16	Resistant	Inducible	<1	<1	<1	<1	<1
80448	<i>M. abscessus</i>	<i>massiliense</i>	Rough	0.12	Susceptible	0.25	Susceptible	2	Susceptible	Susceptible	128	128	128	128	128
80524	<i>M. abscessus</i>	<i>massiliense</i>	Mixed	0.5	Susceptible	2	Susceptible	2	Susceptible	Susceptible	128	128	128	128	128
80700	<i>M. abscessus</i>	<i>massiliense</i>	Rough	>16	Resistant	>16	Resistant	>16	Resistant	Acquired	>128	128	64	>128	128
80824	<i>M. abscessus</i>	<i>massiliense</i>	Mixed	0.25	Susceptible	0.5	Susceptible	1	Susceptible	Susceptible	128	16	32	128	128
80838	<i>M. abscessus</i>	<i>abscessus</i>	Mixed	1	Susceptible	16	Resistant	16	Resistant	Inducible	128	128	128	128	>128
80866	<i>M. abscessus</i>	<i>massiliense</i>	Smooth	0.12	Susceptible	0.12	Susceptible	>16	Resistant	Inducible	128	128	128	128	128
80901	<i>M. abscessus</i>	<i>abscessus</i>	Rough	1	Susceptible	8	Resistant	16	Resistant	Inducible	>128	128	128	128	>128
80988	<i>M. abscessus</i>	<i>abscessus</i>	Rough	0.12	Susceptible	0.25	Susceptible	16	Resistant	Inducible	128	>128	64	128	>128
81103	<i>M. abscessus</i>	<i>abscessus</i>	Smooth	2	Susceptible	2	Susceptible	16	Resistant	Inducible	128	128	64	128	128
81350	<i>M. abscessus</i>	<i>massiliense</i>	Rough	≤0.06	Susceptible	0.12	Susceptible	0.12	Susceptible	Susceptible	>128	128	128	128	128
81422	<i>M. abscessus</i>	<i>abscessus</i>	Rough	0.5	Susceptible	1	Susceptible	≥16	Resistant	Inducible	>128	128	128	128	>128
81463	<i>M. abscessus</i>	<i>massiliense</i>	Smooth	≤0.06	Susceptible	0.12	Susceptible	0.25	Susceptible	Susceptible	128	128	64	128	128
81499	<i>M. abscessus</i>	<i>massiliense</i>	Rough	16	Resistant	16	Resistant	16	Resistant	Acquired	128	128	64	128	128
81618	<i>M. abscessus</i>	<i>abscessus</i>	Mixed	16	Resistant	16	Resistant	16	Resistant	Acquired	128	16	32	128	128
81652	<i>M. abscessus</i>	<i>massiliense</i>	Mixed	0.12	Susceptible	0.25	Susceptible	1	Susceptible	Susceptible	128	8	128	64	128
81702	<i>M. abscessus</i>	<i>massiliense</i>	Rough	>16	Resistant	>16	Resistant	>16	Resistant	Acquired	>128	128	64	>128	128
81838	<i>M. abscessus</i>	<i>massiliense</i>	Rough	16	Resistant	16	Resistant	≥16	Resistant	Acquired	128	128	128	128	128
82119	<i>M. abscessus</i>	<i>abscessus</i>	Smooth	2	Susceptible	16	Resistant	16	Resistant	Inducible	128	16	64	64	128
82154	<i>M. abscessus</i>	<i>abscessus</i>	Smooth	0.5	Susceptible	8	Resistant	16	Resistant	Inducible	128	32	64	64	128
82593	<i>M. abscessus</i>	<i>massiliense</i>	Rough	≤0.06	Susceptible	0.12	Susceptible	0.5	Susceptible	Susceptible	>128	128	128	>128	>128

82895	<i>M. abscessus</i>	<i>massiliense</i>	Rough	0.25	Susceptible	0.5	Susceptible	2	Susceptible	Susceptible	128	>128	32	128	>128
82998	<i>M. abscessus</i>	<i>abscessus</i>	Smooth	16	Resistant	16	Resistant	16	Resistant	Acquired	128	128	128	128	128
83305	<i>M. abscessus</i>	<i>abscessus</i>	Smooth	16	Resistant	16	Resistant	16	Resistant	Acquired	>128	128	128	128	128
83310	<i>M. abscessus</i>	<i>abscessus</i>	Rough	8	Resistant	8	Resistant	≥16	Resistant	Acquired	128	>128	128	>128	>128
83347	<i>M. abscessus</i>	<i>massiliense</i>	Mixed	0.25	Susceptible	0.5	Susceptible	0.5	Susceptible	Susceptible	128	128	128	128	>128
90919	<i>M. abscessus</i>	<i>massiliense</i>	Mixed	0.12	Susceptible	0.12	Susceptible	0.5	Susceptible	Susceptible	128	128	128	128	>128
826492	<i>M. abscessus</i>	<i>massiliense</i>	Mixed	0.12	Susceptible	0.25	Susceptible	16	Resistant	Inducible	128	16	64	64	128

2 **Note:** MIC, minimum inhibitory concentration; DST, drug susceptibility testing; Acquired, acquired resistance; Inducible, inducible

3 resistance

4

Table 3(on next page)

Synergistic activity of five plant secondary metabolites combined with clarithromycin against 14 clarithromycin-resistant *M. abscessus* isolates.

Table 3 Synergistic activity of five plant secondary metabolites combined with clarithromycin against 14 clarithromycin-resistant *M. abscessus* isolates.

Isolates	Susceptibility profile	Individual MIC (µg/mL)						Combination MIC (µg/mL)					FICI				
		CLA	RL008	RL009	RL011	RL012	RL013	CLA/RL008	CLA/RL009	CLA/RL011	CLA/RL012	CLA/RL013	RL008	RL009	RL011	RL012	RL013
81499	Acquired	64	128	128	64	128	128	4/16	32/128	64/128	4/8	16/64	0.19	1.50	2.00	0.13	0.75
82998	Acquired	16	128	128	128	128	128	8/32	2/32	8/64	8/128	8/32	0.75	0.38	1.00	1.50	0.75
83305	Acquired	16	>128	128	128	128	128	16/128	2/128	16/128	4/128	4/64	2.00	1.13	2.00	1.25	0.75
83310	Acquired	4	128	>128	128	>128	>128	1/8	2/8	1/8	4/8	1/8	0.31	0.56	0.31	1.06	0.31
81618	Acquired	64	128	16	32	128	128	64/128	32/64	64/128	64/128	64/128	2.00	1.00	2.00	2.00	2.00
80700	Acquired	512	>128	128	64	>128	128	512/128	512/128	512/128	512/128	512/128	2.00	2.00	2.00	2.00	2.00
81838	Acquired	16	128	128	128	128	128	1/8	1/8	1/8	1/8	1/8	0.13	0.13	0.13	0.13	0.13
82119	Inducible	512	128	16	64	64	128	32/8	512/128	512/64	64/128	512/64	0.50	2.00	1.50	1.13	1.5
80097	Inducible	512	128	16	64	64	128	32/8	64/32	32/64	64/32	128/64	0.13	0.38	0.56	0.38	0.75
80838	Inducible	128	128	128	128	128	>128	32/8	32/16	64/8	16/16	16/32	0.31	0.38	0.56	0.25	0.38
80988	Inducible	4	128	>128	64	128	>128	2/8	1/32	1/8	2/8	2/8	0.56	0.50	0.31	0.56	0.56
80901	Inducible	16	>128	128	128	128	>128	4/8	4/8	4/8	4/8	4/16	0.31	0.31	0.31	0.31	0.38
82154	Inducible	32	128	32	64	64	128	4/16	8/16	8/16	8/32	8/16	0.25	0.38	0.38	0.50	0.38
81103	Inducible	64	128	128	64	128	128	32/8	16/32	16/128	8/32	16/32	0.56	0.50	1.25	0.38	0.50

Note: MIC, minimum inhibitory concentration; CLA, clarithromycin; Acquired, acquired resistance; Inducible, inducible resistance; FICI, fractional inhibitory concentration index

FICI interpretation: ≤0.5: synergy; >0.5-1.0: additive; >1.0-4.0: indifference; >4.0: antagonism (Doern 2014)

6 Grey-shaded boxes shows synergistic effect

Figure 1

Characteristics of 30 *Mycobacterium abscessus* isolates and antimycobacterial activities of five plant secondary metabolites.

All 30 isolates were either *M. abscessus* subspecies *abscessus* (red box) or subspecies *massiliense* (red border) based on genome analysis. A bootstrap consensus tree was inferred from 1,000 replicates. Red circles refer to bootstrap values and the size of each circle is proportional to its value (the largest red circle indicates a value of 100%). *Mycobacterium chelonae* was used as the outgroup and three reference strains of *M. abscessus* were included for comparison. Colony morphology was classified as rough (blue box), smooth (light green box) or mixed (purple box). Clarithromycin (CLA) susceptibility profiles showed acquired resistance (red box), inducible resistance (orange box), or susceptibility to CLA (green box). MIC values for the five compounds tested against the *M. abscessus* isolates ranged from 8-128 µg/mL (open circles) or were >128 µg/mL (shaded circles). Light blue, blue, dark blue, dark purple and purple circles refer to compounds labeled as RL008, RL009, RL011, RL012, and RL013, respectively. Synergistic activity of compounds in combination with clarithromycin is represented as no synergism (open box) and synergism (shaded box). Pink, red, dark blue, yellow and light green boxes refer to combinations RL008/CLA, RL009/CLA, RL011/CLA, RL012/CLA, and RL013/CLA, respectively.

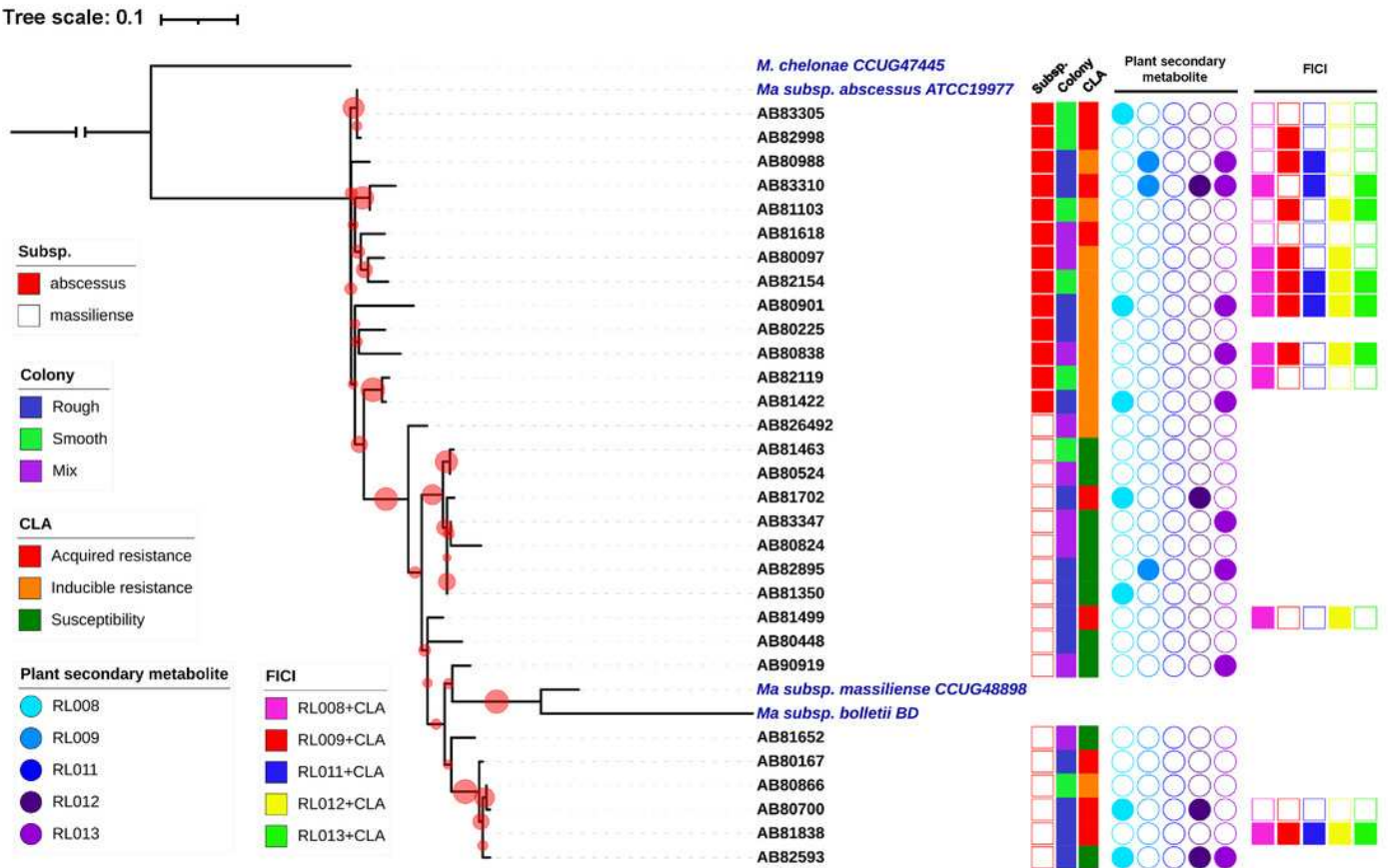


Figure 2

Hemolytic activity of plant secondary metabolites against red blood cells (RBCs).

RBC suspension was incubated for 1 h with different concentrations of (A) RL008, (B) RL009, (C) RL011, (D) RL012 and (E) RL013. The hemolytic activity is presented as the percentage of hemolysis. Data are expressed as mean $\pm$ SD. 

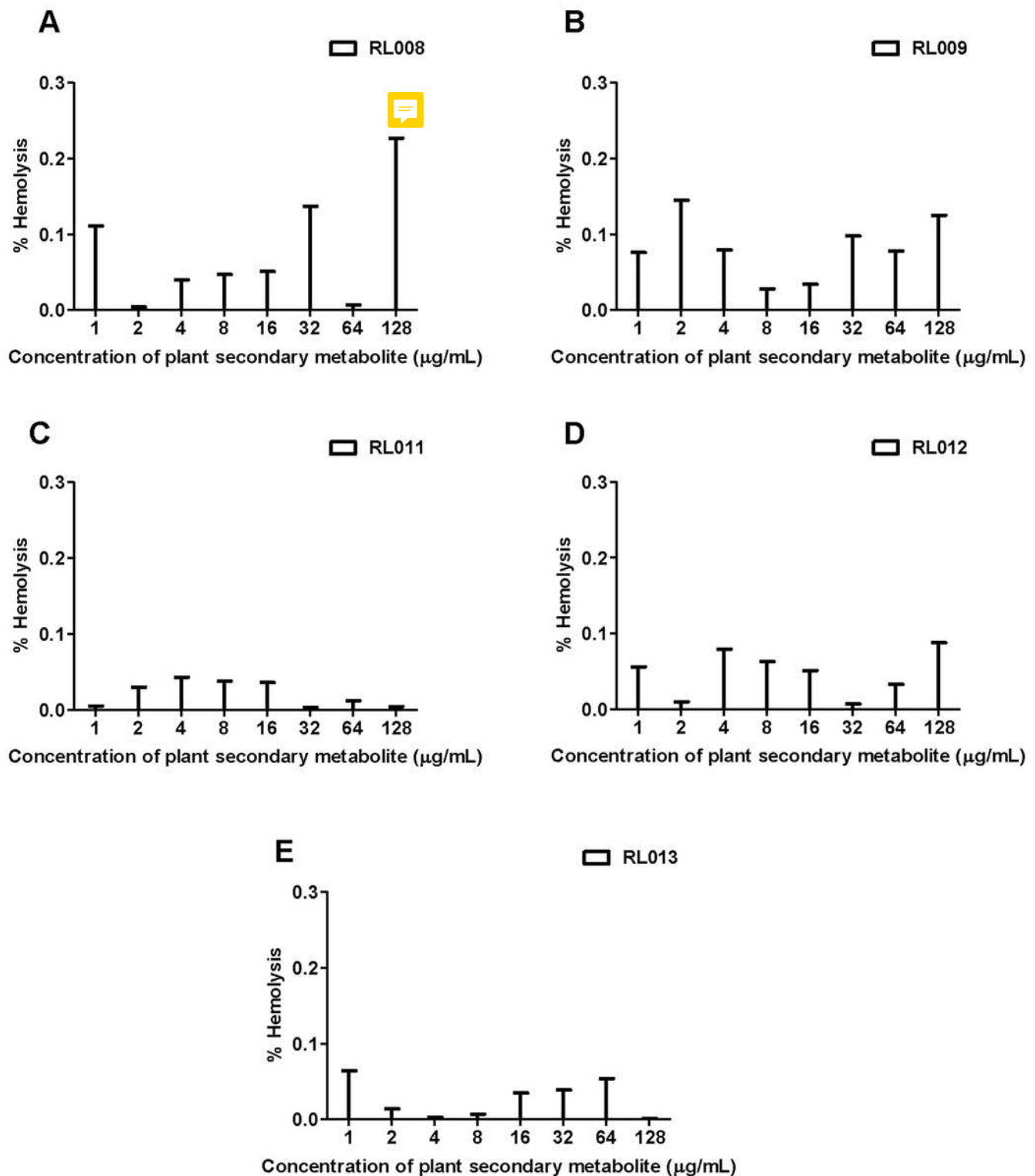


Figure 3

Red blood cell (RBC) morphology under the light microscope.

RBC suspension was incubated for 1 h with (A) 1% Triton-X (positive control, showing complete hemolysis), (B) 1% PBS (negative control, showing no hemolysis) and (C) 128 $\mu\text{g/mL}$ of RL013 (showing some shrinkage due to osmotic effects).

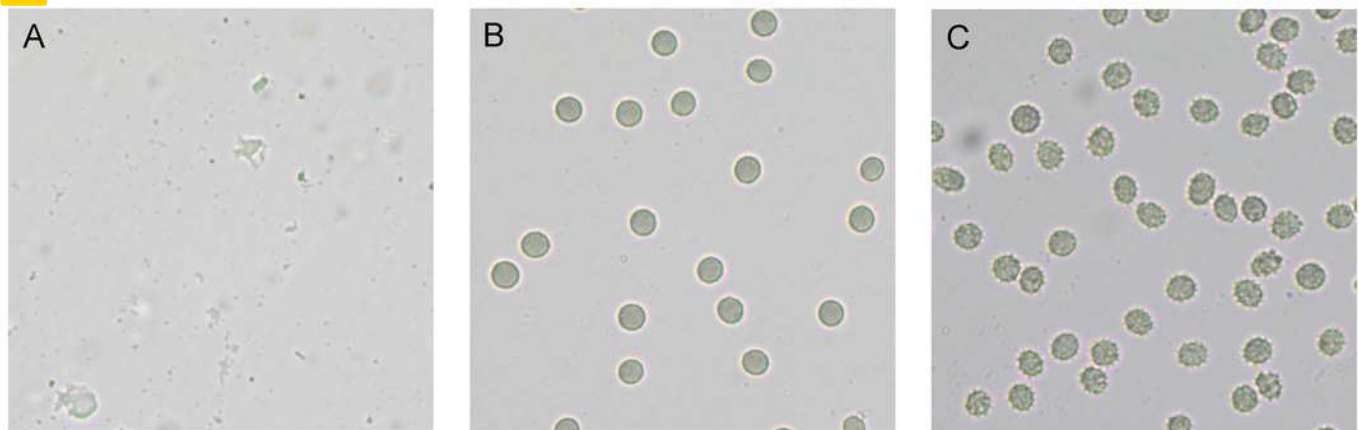


Figure 4

Effect of plant secondary metabolites on white blood cells (WBCs).

WBCs were incubated for 1 h with different concentrations of (A) RL008, (B) RL009, (C) RL011, (D) RL012, and (E) RL013. The toxicity of each secondary metabolite is presented as the percentage of dead cells. Data are expressed as mean $\pm$ SD.



