The authors have investigated the antibacterial activity of several plant compounds and their effect cytotoxic effect. The compounds have a strong effect on various clarithromycin susceptible and resistant strains of M. abscessus. The study is well designed. However, the introduction needs to be elaborated to put forth a clear and detailed literature review and its development in the field. The role of these plants in folk or cultural medicine and the previous research developments about them should be written with a conclusion of the significance of this study at the end of the introduction. There are technical mistakes in presenting the data. The figures need editing and error bars to be depicted with clarity. The literature references are lacking appropriateness with methodology and findings. Other than that the work is divided into appropriate sub-sections of coherent linkages. The discussion part needs to be more coherent with results, the discussion of the findings with respect to already findings in the literature would broaden its standing and scope.

The experimental design is OK, methods need to be presented in detail to clearly understand the steps performed in a particular method. For statistical analysis, biological or technical replicates are a must, which needs to be incorporated in the methodology section. There is a discrepancy between methods and results.

The findings have been validated with sufficient experimental methods, though technical or biological replicates need attention. Raw data needs to be shared in supplemental. The manuscript is written in good English with few typo errors. Discussion needs to be more coherent and elaborate to increase its significance.

There are certain main concerns, which need to be addressed. Other minor comments are listed in the attached file.

Major comments:

Major Comments:
1. Why RBC and WBCs were chosen for toxicity. It should have been tested with the appropriate cell lines where the M. *abscessus* causes infection in human body, like skin and lung
2. Authors should check the stability of the clarithromycin in culture? It might degrade with time, which leads to the growth at lower concentration with time.

Minor Comments;
Line 69: Authors must introduce these plants, fungi and their antibacterial activity
79: is it plant extracts that were purified or compounds were purified
94: Was this consent required while collecting specimens from patients that they used to generate culture collection at Srinagarind Hospital? it is not clear. Needs more clarification.
99: It would be more clear if authors can mention the media used.
115: why cation adjustment was done, is it to adjust pH? Needs clarification

117: Is this the final concentration or it would be half than this? This needs to be addressed in several places in the document.
128: What does authors mean by 5%? How much was the suspension after wash? it is not clear. Did they made 5% by counting using hemocytometer or what?
130: Is it final concentration after adding the RBC suspension?
131: Is tritron-X100 completely lysing the cells completely (needs reference)?
132: How many RBCs will be in 1ml would it be sufficient to be seen under microscope?
134: Can you read the absorbance at 540 nm, or you used some reagent like drabkin without detergent for absorbance measurement? Does compounds have any color which can affect the absorbance readings? Was proper blank included.
146: How much blood was taken?
154: how many cells were counted under microscope. Number of cells must be given to make it statistically significant.
184: This reference doesn't describe the methodology in detail. This method must be described in detail, or else provide a proper reference.
186: Is it in one experiment the concentration of clarithromycin is its MIC (128ug/ml) which is constant in all 5 wells, where each of 5 wells have one of the 5 concentrations (4MIC, 2MIC, MIC,MIC/2 and MIC/4) of the pure compound. this will determine which of the five concentration will be MIC of the pure compound in combination with drug. Then you repeat in vice versa to determine the MIC of clarithromycin in combination with the drug, whose concentration will be its MIC and is constant in all 5 wells. It must be clearly mentioned. Then how they got 4 here (4/16 for CLA/RL008) in table 3, it (4) is 1/16MIC of CLA (in methods the five concentrations were only tested?????). There seem serious mistakes either in methodology or presentation of the data. Needs details description in methods.
188-189: How the concentration of each compound will be 128 ug/ml in all combinations (the most compounds have MIC of 128ug/ml, at this concentration the growth will be inhibited any how), if 5 concentrations of each compound and clarithromycin are mixed with culture. so authors must describe this method in detail.
205: The MIC ranged from 0.12 to >16. There is no limit of the numbers after 16, it is infinite.
208: In case of inducible it is usually on 14th day, while in case of resistant it is since from 3rd day. Is this resistance phenotypic or genetic?

212: Data must be provided as a supplementary file

213: The concentration should be half when you add equal volume of culture and compound in a well?? There is discrepancy in methods and results.

222, 227: is it final concentration?

228: If it is already mentioned in methods that compounds are pure, then no need to repeat the word pure.

268-269: <1 can be any fraction like 0. 01, 0.0001 and so on. >128 could be 128mg or 128g/ml

281: caused what?

Table 1 Legend: *Crinum asiaticum* not in the table.

Figure 2: SD can not be calculate for duplicate samples. SD Error bar is not visible. The SD bar must be on the top of a bar to clearly distinguish it. Secondary metabolite (ug/ml) is sufficient in XX'. It would appear better if at one concentration (like 2ug/ml) is chosen on which all the five compound are compared in a single bar graph with SD and P values using appropriate statistical analysis.

Figure 3: It is better to show morphology of RBCs for all the five compounds. For SD, you need to have either three biological or experimental replicates.

Figure 4. It would be more appropriate if the effect of all the compound at 128 ug/ml is compared in a single graph with p values using appropriate statistical analysis. For SD, you need to have either three biological or experimental replicates.

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

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- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Conclusions are well stated, linked to original research question & limited to supporting results.

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Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

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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[¶] These authors contributed equally to this work.

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

Abstract

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Keywords: antimycobacterial activity, medicinal plants, plant secondary metabolites, *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 medicines. 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 compunds 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 (*N. 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 guidelines. 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 µL of pure compound (1, 2, 4, 8, 16, 32, 64 and 128 µg/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 µg/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×*g* for 2 min. Then, 100 µL 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:

$$\text{Hemolysis (\%)} = [(As - An) / (Ac - An)] \times 100$$

Where *As* refers to the absorbance of the sample, *An* refers to the absorbance of the negative control (RBCs with PBS) and *Ac* 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 saline solution (0.81% NaCl and 0.06% K_2HPO_4) 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 indel.

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). The 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 µL of inoculum were then added. Final concentration of each pure compound was 128 µg/mL and of clarithromycin ranged from 4 to 512 µg/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 µg/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*. There 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 concentrations. 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 RBCsThe 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; Kemegne 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	*N*-methylcycloatalaphylline A	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
2	RL006	yukocitrine	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
3	RL002	*N*-methylatalaphylline	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
4	RL007	atalaphylline	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
5	RL004	atalaphylline-3,5-dimethyl ether	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
6	RL003	2,2-dimethylchromenocoumarin	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
7	RL005	auraptene	Roots of *A. monophylla*	(Pailee *et al.*, 2020)
8	RL009	rubiadin-1-methyl ether	Roots of *P. filamentosa*	(Wisetsai *et al.*, 2021)
9	RL010	rubiadin	Roots of *P. filamentosa*	(Wisetsai *et al.*, 2021)
10	RL011	knoxiadin	Roots of *P. filamentosa*	(Wang *et al.*, 1985)
11	RL008	nordamnacanthal	Roots of *P. filamentosa*	(Wisetsai *et al.*, 2021)
12	RL012	damnacanthal	Roots of *P. filamentosa*	(Wisetsai *et al.*, 2021)
13	RL013	damnacanthol	Roots of *P. filamentosa*	(Wisetsai *et al.*, 2021)
14	RL014	3',4',7-tri-*O*-methylluteolin	Flowers of *A. conyzoides*	(Ahond *et al.*, 1990)
15	RL015	4',7-di-*O*-methylapigenin	Flowers of *A. conyzoides*	(Ahond *et al.*, 1990)
16	RL016	4'-*O*-methylapigenin	Flowers of *A. conyzoides*	(Yim *et al.*, 2003)
17	RL017	2'-hydroxy-4,4',6'-trimethoxychalcone	Flowers of *A. conyzoides*	(Sukari M. A, 2004)
18	RL020	3,5-dihydroxycinnamate	Roots of *R. wittii*	(Wisetsai *et al.*, 2020)
19	RL021	lippianoside B	Roots of *R. wittii*	(Wisetsai *et al.*, 2020)
20	RL022	rothmannioside C	Roots of *R. wittii*	(Wisetsai *et al.*, 2020)
21	RL023	rothmannioside A	Roots of *R. wittii*	(Wisetsai *et al.*, 2020)
22	RL024	rothmannioside B	Roots of *R. wittii*	(Wisetsai *et al.*, 2020)
23	RL019	aurisin A	Cultured mycelium of *N. nambi*	(Kanokmedhakul *et al.*, 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.

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

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

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

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)

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.

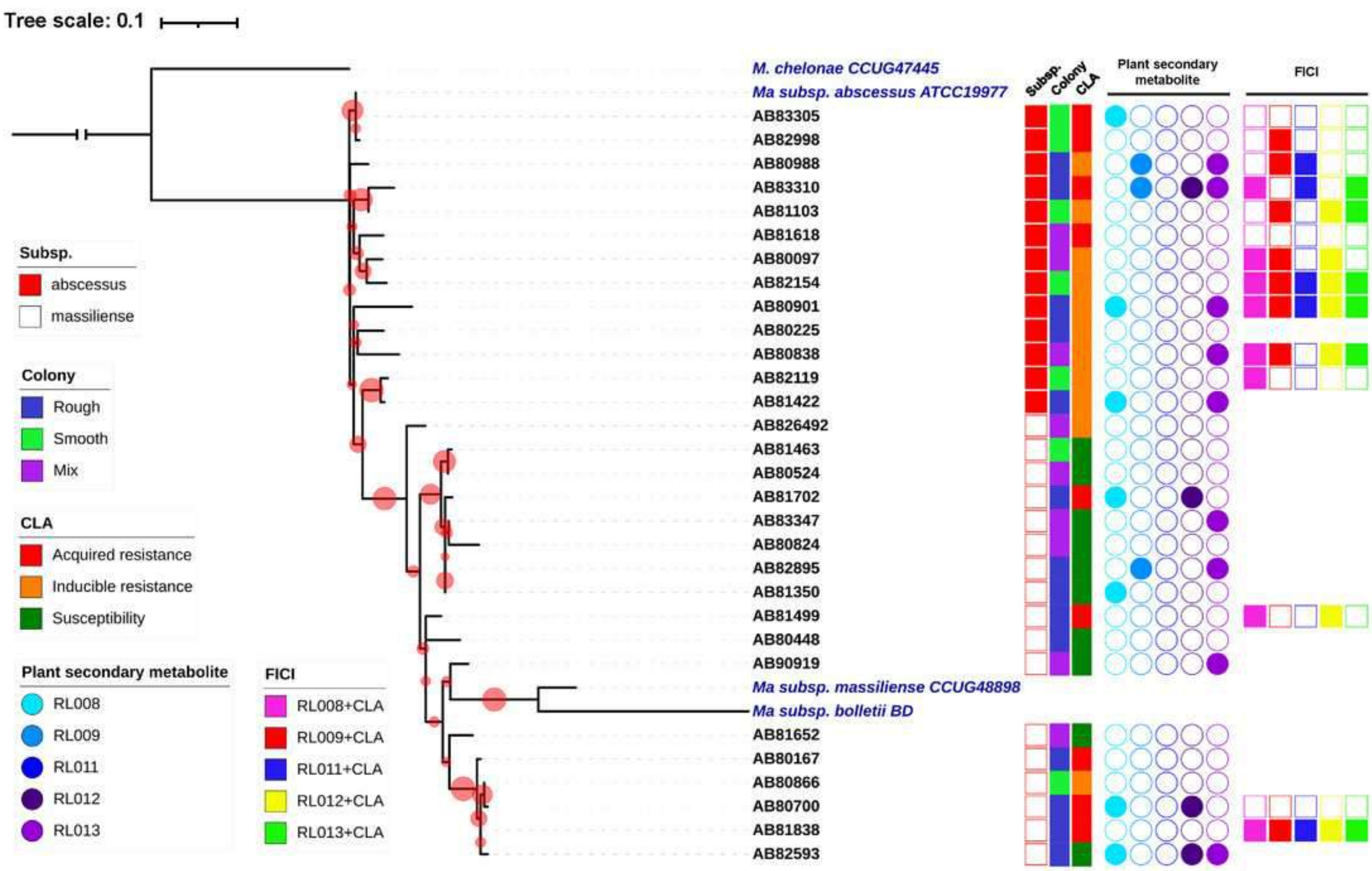

Tree scale: 0.1
M. chelonae CCUG47445
Ma subsp. abscessus ATCC19977
AB83305
AB82998
AB80988
AB83310
AB81103
AB81618
AB80097
AB82154
AB80901
AB80225
AB80838
AB82119
AB81422
AB826492
AB81463
AB80524
AB81702
AB83347
AB80824
AB82895
AB81350
AB81499
AB80448
AB90919
Ma subsp. massiliense CCUG48898
Ma subsp. bolletii BD
AB81652
AB80167
AB80866
AB80700
AB81838
AB82593
Subsp.
Colony
CLA
Plant secondary metabolite
FICI
Subsp.
abscessus
massiliense
Colony
Rough
Smooth
Mix
CLA
Acquired resistance
Inducible resistance
Susceptibility
Plant secondary metabolite
RL008
RL009
RL011
RL012
RL013
FICI
RL008+CLA
RL009+CLA
RL011+CLA
RL012+CLA
RL013+CLA

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 ± SD.

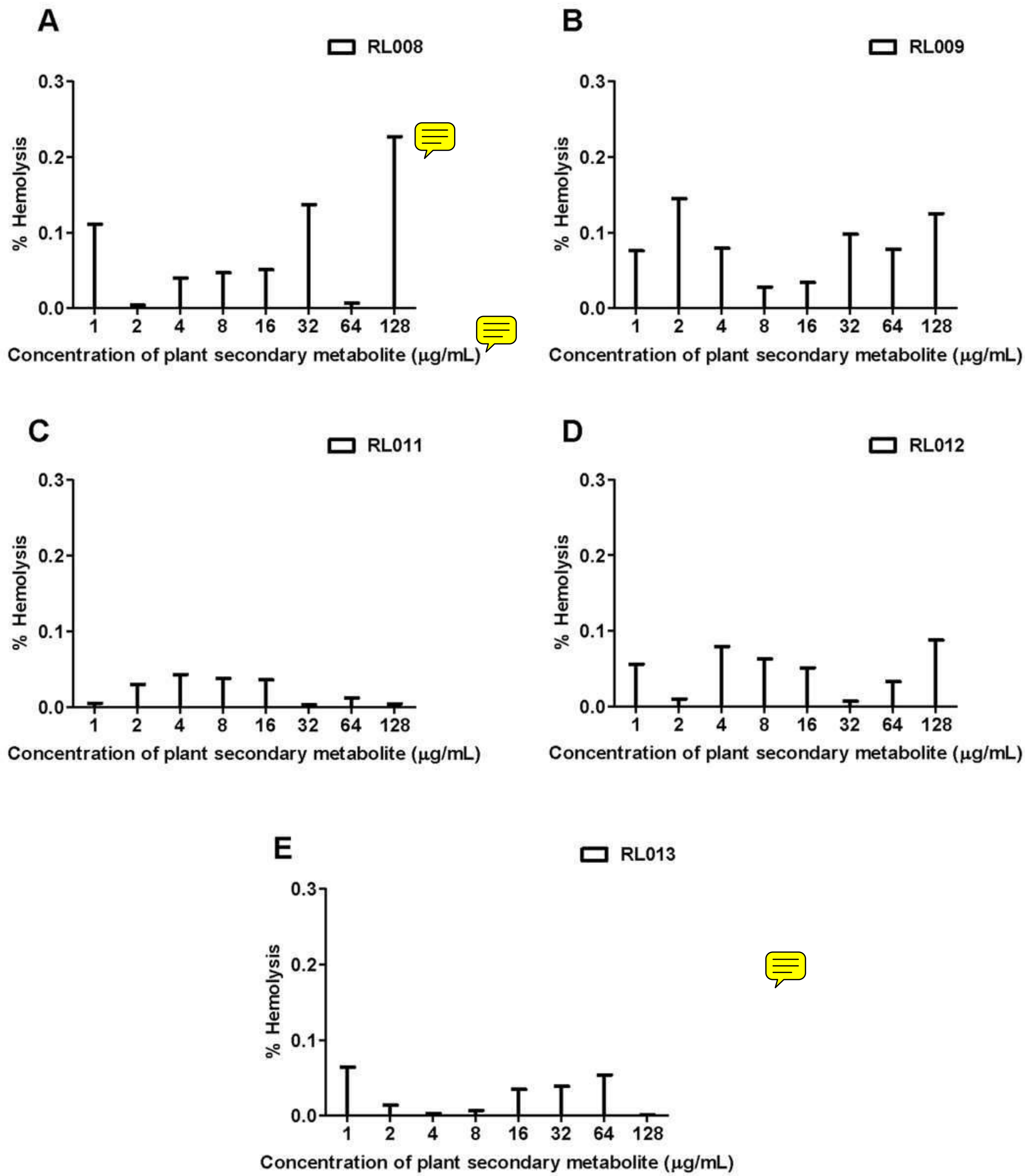

A
RL008
% Hemolysis
0.0
0.1
0.2
0.3
1
2
4
8
16
32
64
128
Concentration of plant secondary metabolite (µg/mL)
B
RL009
% Hemolysis
Concentration of plant secondary metabolite (µg/mL)
C
RL011
% Hemolysis
Concentration of plant secondary metabolite (µg/mL)
D
RL012
% Hemolysis
Concentration of plant secondary metabolite (µg/mL)
E
RL013
% Hemolysis
Concentration of plant secondary metabolite (µg/mL)

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 µg/mL of RL013 (showing some shrinkage due to osmotic effects).

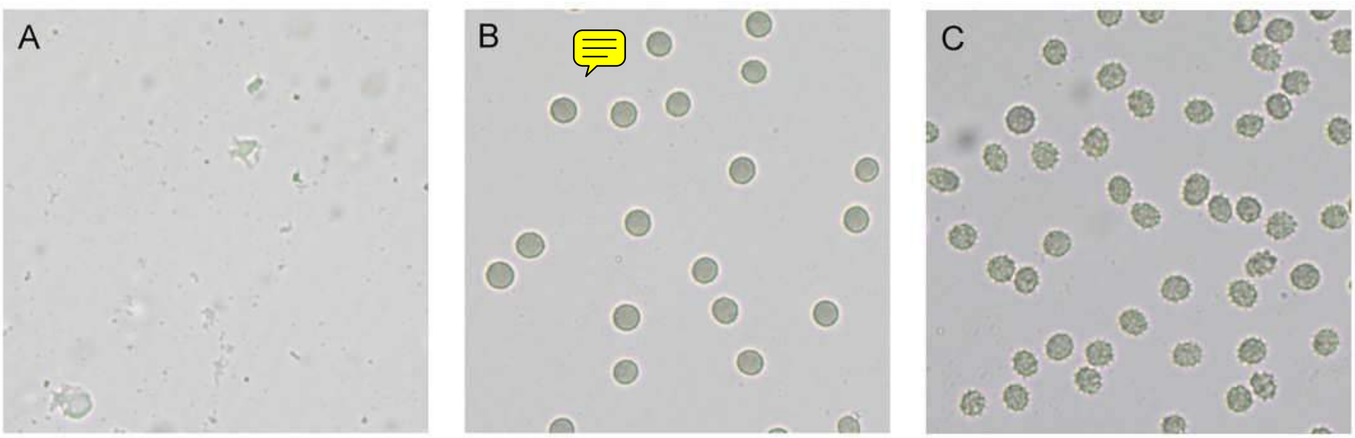
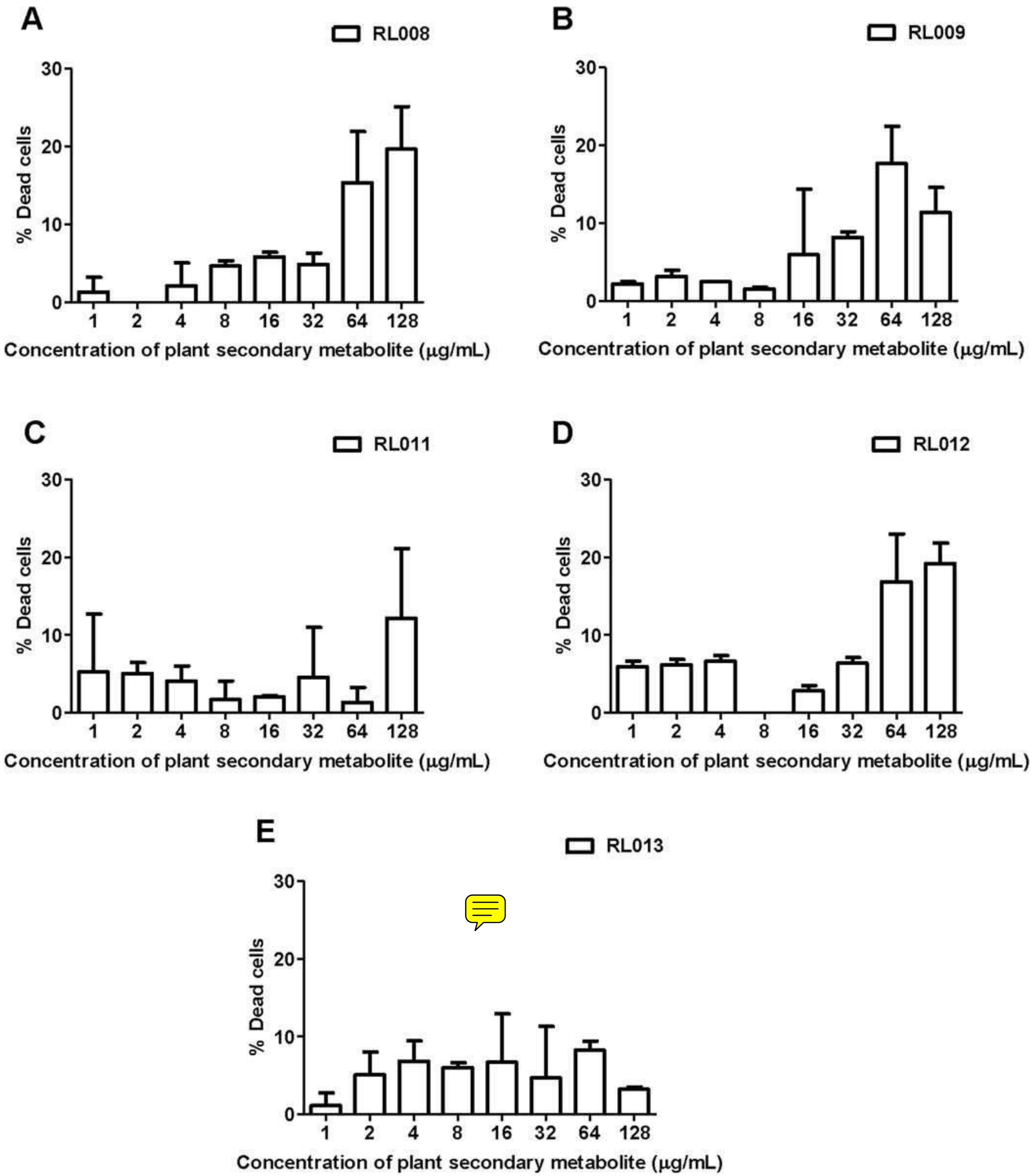


Figure 4

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 ± SD.


A
RL008
30
20
10
0
% Dead cells
1
2
4
8
16
32
64
128
Concentration of plant secondary metabolite (μg/mL)
B
RL009
C
RL011
D
RL012
E
RL013