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Antimicrobial and drug-synergistic potential of *Alpinia conchigera* Griff.-derived phenylpropanoids against *Mycobacterium smegmatis*

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ABSTRACT

Aims: This study aimed to evaluate the antimicrobial activity of naturally derived phenylpropanoids from *Alpinia conchigera* (*A. conchigera*) Griff. and its synthetic analogues, as well as interactions between selected compounds with first-line tuberculosis (TB) drug, rifampicin, against *Mycobacterium smegmatis,* a potential opportunistic nontuberculous mycobacterium (NTM) and a surrogate organism for TB.
Methodology and results: Twelve phenylpropanoids of *A. conchigera* were evaluated for antimicrobial activity against *M. smegmatis (*ATCC 14468). The phenylpropanoid compound from *A. conchigera* with the lowest minimum inhibitory concentration and bactericidal (MIC, MBC) values were selected for checkerboard tetrazolium microplate assay (TEMA) with rifampicin to determine drug interactions. A majority of the compounds had antimicrobial activity, however, purified natural compound 1'*S*-1'-acetoxychavicol acetate (ACA) showed the highest antimicrobial activity with an MIC value of 62.5 µg/mL against *M. smegmatis.* The combination of ACA and rifampicin produced indifferent interaction with fractional inhibition concentration (FIC) index of 1.5, while the combination of rifampicin and ACA synthetic analogue 4-allyl-2,6-methoxyphenyl isobutyrate produced a synergistic interaction effect with FIC index of 0.5. None of the compounds tested were bactericidal but appear to be bacteriostatic.
Conclusion, significance and impact of study: This study presents the first report on the antimicrobial potential of natural *A. conchigera*-derived ACA against *M. smegmatis* as well as the synergistic interaction of 4-allyl-2,6-methoxyphenyl isobutyrate with rifampicin which warrants further investigation.

Keywords: plant phenylpropranoids, natural products, tuberculosis, non-tuberculous mycobacteria, minimum inhibition concentration assay

INTRODUCTION

Despite being an ancient disease that has plagued humankind since 3000 BC (Barberis *et al*., 2017), tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains among the top 10 causes of disease and death worldwide, resulting in an estimated 1.6 million deaths in 2018. According to the Global Tuberculosis Report 2018 by World Health Organization (WHO), TB can affect all age groups including children. Active pulmonary and extra-pulmonary TB, which infects the lungs or other parts of the body, respectively, is prevalent worldwide with the highest number of cases reported in South-East Asia and Western Pacific regions. The risk of contracting TB increases in conditions of crowding, poverty, and immunocompromised states, and TB is currently the primary cause of death in HIV-infected individuals. This situation challenges efforts to achieve the Sustainable Development Goals (SDGs) to "End TB" by 2030 (Lönnroth and Raviglione, 2015).

Additionally, the emergence of *M. tuberculosis* strains that are resistant to antibiotics has become a global concern. Drug resistance has largely arisen from inappropriate and/or incomplete treatments, among which adverse effects of standard first-line TB drugs such as isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin, is an important factor (Liang *et al*., 2012). It has been estimated by WHO in 2017 that 558,000 patients were infected with MTB strains resistant against rifampicin, a standard first-line drug for TB treatment. However, in the case of multidrug-resistant TB (MDR-TB), which exhibits resistance against both rifampicin and isoniazid — treatment and cure may still be achieved through second-line drugs. A more significant challenge is

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the treatment of extensively drug-resistant TB (XDR-TB), which is defined as MDR-TB that is also resistant to any fluoroquinolone and at least one of three injectable second-line drugs (i.e. amikacin, kanamycin, or capreomycin), and is extremely difficult to cure. In any case of resistance, the risk of toxicity, adverse effects and reduced adherence becomes additional factors for incomplete treatments, which not only increases mortality but further fuels drug resistance.

Hence, one approach to reducing side effects and consequent drug resistance is to identify compounds with possible synergistic interactions, which may result in overall stronger antimicrobial activities compared to using individual drugs (Fazly Bazzaz *et al*., 2018). Such combinations can lead to better drug regimens that can reduce the therapeutic dose of standard drugs, thus reducing the risk of adverse effects while increasing efficacy (Hagihara *et al*., 2011).

Approximately 25-50% of recent pharmaceuticals are derived from plants (Gupta and Birdi, 2017) as they produce various types of secondary metabolites that exhibit antimicrobial activity (Sibanda and Okoh, 2007; Abreu *et al*., 2012; Gupta and Birdi, 2017), as a means of protection against microbes that are pathogenic to them. The many structurally diverse compounds produced by plants further provide opportunities for being harnessed in drug development (Balouiri *et al*., 2016).

One example of a plant commonly used for medicinal purposes in Malaysia is *Alpinia conchigera* Griff., locally known as lengkuas ranting, lengkuas kecil, lengkuas padang, lengkuas genting or cengkenam (Burkill, 1996; Aziz *et al*., 2013). This species belongs to the family Zingiberaceae, and the rhizomes of this species are commonly used as a spice or ginger substitute for flavoring food, while the shoots are used in vegetarian dishes (Ibrahim *et al*., 2007). The rhizomes are also reportedly used as a post-partum treatment, treatment of fungal infections and other treatments as part of traditional medicine (Ibrahim *et al*., 2007). In particular, Aziz *et al.* (2013) reported that extracts from *A. conchigera* rhizome demonstrated antibacterial, antidermatophytic and anticandidal activity especially for treating skin infections. Many of the active compounds from these traditional medicinal plants are phenylpropanoids, which are extensively studied since they reportedly possess antimicrobial properties and other potential pharmacological applications (Aslam *et al.*, 2009; Goyal *et al*., 2012). These secondary metabolites are synthesized from primary metabolites, phenylalanine or tyrosine, and can be divided into five groups, including flavonoids, monolignols, phenolic acids, stilbenes, and coumarins (Deng and Lu, 2017). However, mass production of bioactive compounds such as phenylpropanoids requires standardization and optimization which may be better achieved using characterized natural compounds and/or synthetic analogues of the active compounds, instead of crude extracts. Furthermore, anti-tubercular potential of *A. conchigera*-derived phenylpropranoids has not been evaluated in previous studies.

Hence, in this study, naturally-derived and synthetic chemical analogues of phenylpropanoids from *A. conchigera* Griff. were screened to first determine their bacteriostatic and bactericidal potential against *Mycobacterium smegmatis,* a non-pathogenic and fast-growing non-tuberculous mycobacterium (NTM), often used as a surrogate organism for *M. tuberculosis*. *M. smegmatis* and other NTM have also gained more interest in recent years due to their potential to become opportunistic pathogens among immunocompromised individuals (Wu *et al.*, 2018). Drug interaction was assessed for combinations of rifampicin and selected compounds against *M. smegmatis* to determine whether a synergistic interaction could be utilized for the future development of new NTM and TB treatment regimens.

MATERIALS AND METHODS

Isolation of phenylpropanoid compounds of *A. conchigera* and production of its synthetic analogues

Figure 1 denotes the 12 phenylpropanoid compounds of *A. conchigera* that were evaluated. Among these, two compounds 1'*S*-1'-acetoxychavicol acetate, ACA (compound 1) and *trans*-*p*-coumaryl diacetate, TPCA (compound 2) were isolated from *n*-hexane crude extract of *A. conchigera*. The rhizome of *A. conchigera* Griff. (specimen no: KL5049) was cultivated in Ulu Langat, province of Selangor, West-coast of Peninsular Malaysia. This species was identified and deposited in the herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur. The remaining seven phenylpropanoid compounds evaluated were ACA derivatives, which were synthesized with various functional group modification as described in detail in earlier publications (Anuar, 2018; Mohammad Taib *et al*., 2020).

Briefly, the hydroxyl group of α-vinylbenzyl alcohol was acetylated with acetic anhydride to obtain compound 3; replacement of hydroxyl group in 4-allyl-2-methoxyphenol with an acyl chloride produced structure compound 10; butyryl chloride and 2-furoyl chloride afforded compounds 4 and 5, respectively; while compounds 6-9 were prepared from 4-allyl-2,6-dimethoxyphenol, i.e. the reaction of compound 11 and respective acyl chloride with the addition of 4-dimethylaminopyridine (DMAP) and triethylamine. In addition, compound 12 was commercially available and obtained from Sigma-Aldrich Co. without further purification.

Bacterial culture

Mycobacterium smegmatis ATCC 14468 purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) was maintained frozen in glycerol at –20 °C, then sub-cultured on Middlebrook 7H10 agar and stored at 4 °C for regular use as a stock culture. A fresh culture was prepared from the stock culture for the studies. *M. smegmatis* was cultured on Middlebrook 7H10 agar for

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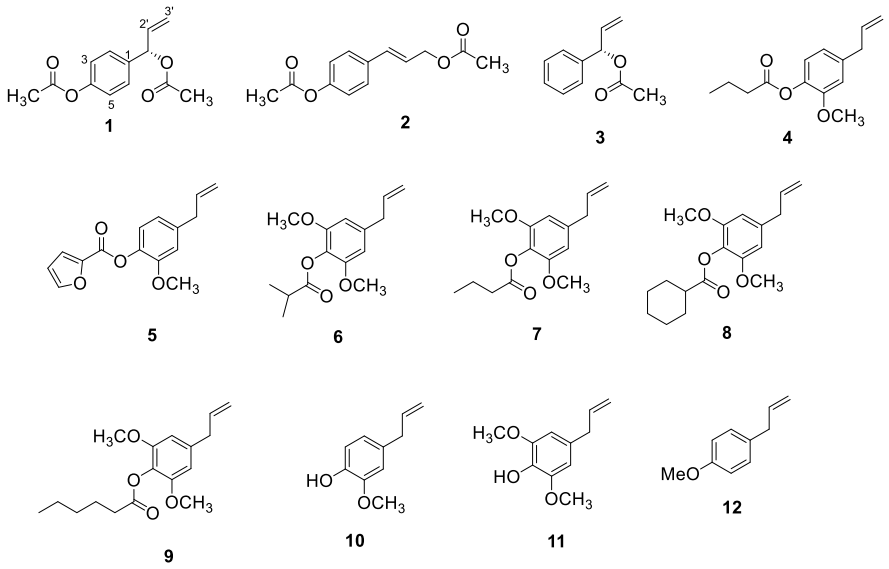

Figure 1: Structures of ACA and its analogues used in this study.

two days at 37 °C under anaerobic conditions (4% CO_2). Then, bacterial suspensions were prepared by culturing colonies from Middlebrook 7H10 agar into sterile Middlebrook 7H9 broth, then grown to log phase at 37 °C under anaerobic conditions for two days before assays were conducted.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays

The bacterial suspension was centrifuged (4000 rpm, 1 min), washed in phosphate buffer saline (PBS) and adjusted to optical density (OD) 0.75–0.85 (approximately 3×10^7 CFU/mL) using spectrophotometer read at 600 nm (OD_{600}). The bacterial suspension was then diluted in sterile distilled water to obtain the final cell density of 6×10^6 CFU/ mL (Belanger *et al*., 1999; Zgoda and Porter, 2003). The compound stock solutions were prepared with 5% dimethyl sulfoxide (DMSO) and diluted into working concentrations of 8000 µg/mL in sterile distilled water immediately before use.

The MIC of the compounds against *M. smegmatis* were performed using tetrazolium microplate assay (TEMA) based on methods by Caviedes *et al.* (2002), with some modifications, in sterile 96-wells microplates. Each compound was prepared in serial two-fold dilutions with 7H9 broth, which produced volumes of 100 µL/well with different final concentrations starting with 4000 µg/mL, 2000 µg/mL, 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL and 31.25 µg/mL, and compared against positive control with no drugs or compounds added (only broth and bacteria) and the negative control containing only broth and rifampicin, with starting concentration at 128 µg/mL, as the *M. smegmatis* is known to be inherently resistant against rifampicin.

Compounds were tested in triplicates. A volume of 100 µL of prepared inoculum was transferred to all wells except for negative control wells to achieve a concentration of 5.0×10^5 cells/mL (Božić *et al.*, 2014; Fazly Bazzaz *et al*., 2018). The culture microplate was sealed with parafilm and incubated under anaerobic conditions at 37 °C for 2 to 3 days (Li *et al*., 2004). The MIC of samples was determined by adding 50 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and incubating at 37 °C for 30 min (Eloff, 1998; Balouiri *et al*., 2016). The MIC values were determined based on the well containing the lowest concentration of the compound which did not reduce the yellow coloured dye to purple colour.

The compounds with observable MIC values were further screened to evaluate their minimum bactericidal concentration (MBC) values. The MBC was performed directly after bacterial incubation (without the addition of MTT), whereby wells containing equal to and greater concentration of antimicrobial agents with MIC are subcultured onto nutrient agar by using sterile inoculating loop. Then, the plates were incubated at 37 °C under anaerobic conditions for two days. After incubation, the plates were observed for the growth of the bacterial colonies and the plates with the lowest concentration having an absence of bacterial growth were recorded as the MBC value. The MBC/MIC ratio was calculated, whereby a ratio of >2 is considered to indicate bactericidal activity (Balouiri *et al*., 2016).

Drug interaction checkerboard assay

In this study, a drug combination test was conducted to determine the type of interaction between combinations of the compound and the selected drug against *M. smegmatis*. Drug combination testing was done using a checkerboard assay based on methods published by Bonapace *et al*. (2002) with slight modification. Briefly, the phenylpropanoid compounds with the lowest MIC values

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and/or highest MBC/MIC ratio (1, 6) were combined with rifampicin as the standard drug for *M. smegmatis*. The working concentrations of compound and drugs tested were three folds above and below their MIC values, to enable observation of different potential interactions and to fit a 6 × 6 well configuration on the microtiter plate, and a serial dilution was performed for both rifampicin and compound. The working concentration of rifampicin, 1 and 6 were between 128 µg/mL–4 µg/mL, 62.5 µg/mL–0.98 µg/mL, and 4000 µg/mL–125 µg/mL, respectively. Rifampicin alone, negative control (no bacteria), and positive control (no antibiotic/compound) were included on each microplate. Microplates were inoculated with 100 µL of bacteria suspension into all wells except negative control well. Plates were sealed with parafilm and incubated for 2 days at 37 °C under anaerobic conditions. After incubation time, 50 µL of MTT solution was added into all wells whereby a visible color change from yellow to purple indicated bacterial growth. The MIC was defined as the lowest concentration of drug/compound combination without bacterial growth and was used to calculate the fractional inhibitory concentration (FIC) index. The FIC index was calculated using a formula as illustrated below (Fazly Bazzaz *et al*., 2018):

FIC index = FIC_a + FIC_b = [MIC_a combination/MIC_a individual] + [MIC_b combination/MIC_b individual]

where A and B are drug and compound, respectively. The interaction between the combination of compound and drugs was determined using calculated FIC index values and interpreted as: synergistic (≤0.5), additive (0.5 < X <1.0), indifferent (1.0≤ X ≤2.0), or antagonistic (>2.0).

RESULTS

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

In this study, twelve phenylpropanoid compounds of *A. conchigera* were prepared and tested against *M. smegmatis*. The positive and negative control was as expected with no contamination observed. A majority of the compounds (1–12) were active and resulted in bacterial inhibition on MIC assay. However, compounds 11 and 12 lacked antimicrobial activity as no inhibition was observed even at the highest concentration of 4000 µg/mL. Among these, purified natural compound ACA (compound 1) showed the highest antimicrobial activity with an MIC value of 62.5 µg/mL against *M. smegmatis* (Table 1). Among the synthetic analogues, compound 9 had the lowest MIC; however, this compound did not have an observable MBC value. Instead, the synthetic analogue compound 6, which had an MIC of 1000 µg/mL, and an MBC of 2000 µg/mL consequently had the highest MBC/MIC ratio. However, none of the phenylpropranoid compounds recorded MBC/MIC ratios >2, and thus these compounds are considered to not possess appreciable bactericidal activity.

Drug interaction

The compound that exhibited the lowest MIC value, i.e. 1'S-1'-acetoxychavicol acetate, ACA (compound 1) and the synthetic analogue with the lowest MIC and highest MBC/MIC ratio 4-alyll-2-methoxyphenyl butyrate (compound 6) were selected for drug-compound combination study with the first-line drug rifampicin against *M. smegmatis*.

The combination of compound 1 and rifampicin produced indifferent interaction with FIC index value of 1.5, as the MIC of compound 1 reduced by two-fold while the antimicrobial activity rifampicin was unchanged. Conversely, the combination of rifampicin and 6 produced a synergistic interaction effect, whereby the MIC of rifampicin and compound 6, reduced by four-fold resulting in an FIC index of 0.5 (Table 2). The positive and negative control was as expected with no contamination observed.

DISCUSSION

Phenylpropanoids have been hypothesized to interact with many cellular processes, which directly influence human health as they can act as antioxidants due to multiple hydroxyl groups, unsaturated double bonds and cinnamic acid derivatives present in their structure (Tonari *et al*., 2002; Korkina, 2007). The hydroxyl group and the unsaturated double bonds may react with oxidative ions and radicals in the cell, while the benzene and phenol ring structure in the phenylpropanoid ease their transport across the cellular membrane. This study reports the anti-tubercular potential of naturally-derived and synthetic analogues of phenylpropanoids from Malaysian *A. conchigera* Griff, colloquially known as lengkuas ranting, a rhizome that is used both for its traditional medicine and nutritional properties in local Malaysian cuisine.

Using a combination of MIC, MBC, and drug interaction microplate assays, different inhibition activities of phenylpropanoid towards *M. smegmatis* were observed, likely due to different chemical structures of the compound (Figure 1). While, all the phenylpropanoid compounds have the same parental structure based on the ACA (1), the difference in the value of MIC of the compounds may lie in the differences of the functional group. The presence of carbonyl ester moiety attached directly to the benzene ring at carbon position 4 (C-4) may contribute to higher antimicrobial activity against the test organism. The higher the alkyl chain attached to the carbonyl ester, the lower the activity of antimicrobial against the test organisms because increasing alkyl side chain length result in loss of biological activity of the compound towards the organisms (Silva *et al*., 2019). Indeed, the 1'*S*-1'-acetoxychavicol acetate, ACA which had the lowest MIC, consisted of carbonyl ester moiety attached directly to the benzene ring at C-4 with a 2'-3'-double bond also found in the structure.

Furthermore, several previous studies reporting high antimicrobial activities of phenylpropanoids using natural products or extracts instead of the synthetic version

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Table 1: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of phenylpropanoid compounds against *Mycobacterium smegmatis*.

Name of the compound/drug	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC ratio
1'*S*-1'-acetoxychavicol acetate, ACA (1)	62.5	62.5	1
trans-p-coumaryl diacetate, TPCA (2)	1000	1000	1
α-vinylbenzyl acetate (3)	2000	2000	1
4-allyl-2-methoxyphenyl butyrate (4)	4000	NB	NA
4-allyl-2-methoxyphenyl furan-2-carboxylate (5)	2000	4000	2
4-allyl-2,6-methoxyphenyl isobutyrate (6)	1000	2000	2
4-allyl-2,6-dimethoxyphenyl butyrate (7)	2000	NB	NA
4-allyl-2,6-dimethoxyphenyl cyclohexane carboxylate (8)	4000	4000	1
4-allyl-2,6-dimethoxyphenyl hexanoate (9)	250	NB	NA
4-allyl-2-methoxyphenol (10)	4000	4000	1
4-allyl-2,6-dimethoxyphenol (11)	NI	NB	NA
4-allyl-4-methoxyphenol (12)	NI	NB	NA
Rifampicin	64	NT	NA

NI: No inhibition showed even at the highest concentration of 4000 µg/mL; NB: No bactericidal activity observed; NA: Not applicable or able to be calculated; NT: Not tested.

Table 2: Drug combination study using checkerboard assay.

Combination	Average individual MIC (µg/mL)	Average combination MIC (µg/mL)	Change in antimicrobial activity (Fold)	Average individual FIC index	FIC index	Interaction
1 + rifampicin	62.5/64	31.25/64	+2/1	0.5/1	1.5	Indifferent
6 + rifampicin	1000/64	250/16	+4/+4	0.25/0.25	0.5	Synergistic

FIC Index values are interpreted as: synergistic (≤ 0.5), additive (0.5 < X< 1.0), indifferent (1.0 ≤ X ≤ 2.0), or antagonistic (>2.0).

(Rigano *et al*., 2007; Kamal *et al.*, 2012; Aziz *et al*., 2013). This is because the biological activity of a natural product is higher than in synthetic products due to the limitation in chemical scaffolds of synthetic products (Harvey, 2000; Stratton *et al.*, 2015; Guo, 2017). Chemical scaffolds enable natural products to exhibit stronger binding to the targets and increased interactions with biological molecules such as proteins (Carlson *et al.*, 2010; Guo, 2017). In this study, ACA was the main phenylpropanoid compounds isolated from n-hexane crude extract, and as expected this natural compound exhibited better antimicrobial activity compared to the synthetic analogues evaluated. Albeit none of the compounds tested could be deemed bactericidal, both natural compounds ACA and TPCA had MBC/MIC ratio of 1 while two synthetic compounds 5 and 6 had MBC/MIC ratio of 2, indicating the latter's higher bactericidal potential.

Besides determining the individual antimicrobial potential of a compound, drug interaction studies are useful for identifying compounds that have the potential to enhance the effects of standard drug or chemicals, which can be useful in improving treatment regimens and

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reduce the emergence of multi-drug resistant microbes. Hemaiswarya and Doble (2010) previously reported synergistic interaction between phenylpropanoids and antibiotics against Gram-negative and Gram-positive microbes, in particular highlighting the role of the hydrophilic groups to exert damage on bacterial membrane. In this study, drug interactions of ACA (compound 1) and compound 6 with first-line TB drug rifampicin were assessed. The results suggest that combination of compound 1 with rifampicin and compound 6 with rifampicin against *M. smegmatis* produced indifferent (FIC index: 1.5) and synergistic (FIC index: 0.5) interactions, respectively. Synergism refers to positive interactions which result in a greater enhancement in the activity of two agents when combined compared to when used separately, whereas indifference denotes a lack of difference in activity of two agents used in combination compared to when used separately (Kumar *et al*., 2012).

Rifampicin is a polyketide that inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerases and is used to treat a range of bacterial infections, most prominently TB and *Mycobacterium avium* complex. The breakpoint for resistance in drug-sensitive MTB such as MTB H37Rv ATCC 27294 is MIC higher than 2 µg/mL (CLSI, 2018). However, MIC of rifampicin against *M. smegmatis* is typically much higher, as observed here with an MIC of 64 µg/mL, as this NTM is naturally more resistant against rifampicin and has a drug sensitivity profile that matches more closely with MDR-TB (Chaturvedi *et al.*, 2007). While ACA had the lowest MIC, it does not appear to influence the activity of rifampicin against *M. smegmatis*. Conversely, when compound 6 was combined with rifampicin, the MIC value reduced from 64 µg/mL to 16 µg/mL; while the MIC for compound 6 reduced from 1000 µg/mL to 250 µg/mL. The change in antimicrobial activity for both agents increased by four-folds when combined, showing enhancement in the antimicrobial activity against *M. smegmatis*. Rifampicin inhibits DNA-dependent RNA polymerase activity of *M. tuberculosis* through the formation of a stable complex with the enzyme, which suppresses the initiation of RNA synthesis (Wehrli, 1983; Campbell *et al*., 2001). Specifically, rifampicin interacts with the β subunit of RNA polymerase (RNAP) as changes in RNAP structure caused by the mutation has been attributed to the emergence of resistance towards rifampicin. The synergistic interaction of compound 6 and rifampicin suggests that compound 6 may disrupt the bacterial membrane allowing the rifampicin to target the RNAP, and/or it also directly targets RNAP or its associated proteins, allowing each to interact with RNAP more effectively to then inhibit the growth of the *M. smegmatis*. Although the mechanism for phenylpropanoids against mycobacteria is yet to be described, studies evaluating antimicrobial activity of organic compounds such as acetamide suggests that its bactericidal activity against *M. tuberculosis* and *M. smegmatis* hinges on targeting the mycobacterial membrane protein Large 3 (MmpL3), a relatively conserved protein, which translocates mycolic acids across the inner membrane (IM) (Shetty *et al.,* 2018). Additionally, the IM of *M. smegmatis* was characterized to contain an unusual lipid, the diacyl phosphatidylinositol dimannoside, which produces a bilayer environment resulting in low fluidity and consequently reduces the influx of drugs (Bansal-Mutalik and Nikaido, 2014). Together, these reports suggest that the enhanced activity of rifampicin in the presence of compound 6, likely relates to the action of the latter on the membrane scaffold, thus allowing more effective entry into the mycobacterium. In future studies, the mode of action of compound 6 alone and in combination with different antibiotics, as well as the extent of damage on mycobacteria, including *M. tuberculosis*, should be investigated.

The following are caveats to the interpretation of the findings of this study. Firstly, the surrogate organism used, *M. smegmatis*, grows significantly faster compared to the *M. tuberculosis*; the doubling time of *M. smegmatis* is approximately 3-4 hours while *M. tuberculosis* doubles approximately every 24 hours (Andreu *et al*., 2004; Sao *et al.*, 2018). This difference in growth rate was suggested by Stephan *et al.* (2005) as arising from the presence of porin-mediated influx of nutrients in *M. smegmatis*, the absence of which for other mycobacteria such as *M. tuberculosis*, becomes a determinant for slower growth. Although *M. smegmatis* is a well-described non-pathogenic model for *M. tuberculosis* (Chaturvedi *et al.*, 2007), the difference in incubation time and the different mechanisms of resistance against rifampicin, i.e. inherent versus acquired, differs between the two mycobacteria may affect extrapolation of these results for *M. tuberculosis*. The fact that the compound has antimicrobial and synergistic effect on the inherently resistant *M. smegmatis,* however, suggests that it may have more pronounced effect on reducing the acquired rifampicin resistance seen in *M. tuberculosis*, which has been reported in a previous study (Chaturvedi *et al.*, 2007).

Secondly, being natural compounds or synthetic analogues of these, technical issues of dissolvability of the compounds in different buffers may affect final concentrations that actually reach the test microbes, suggesting that the antimicrobial effects may be greater if full solubility was achieved. Thirdly, the fact that dissolved rifampicin is orange in colour may have influenced the colour changes of the light-sensitive bacterial growth indicator tetrazolium (MTT). Although this limits the accuracy of the compound MIC values recorded in the presence of rifampicin, it is not expected to significantly influence the results of drug interaction studies as the FIC index values are ratios.

CONCLUSION

This study has demonstrated that Malaysian *A. conchigera*-derived purified phenylpropanoid 1'*S*-1'-acetoxychavicol acetate (ACA) possesses good antimicrobial potential against *M. smegmatis* with an MIC

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value of 62.5 µg/mL, which was superior compared to the other natural and synthetic phenylpropanoid compounds tested here and comparable to that of the standard drug rifampicin. However, a synergistic interaction was observed when the synthetic analogue 4-allyl-2,6-methoxyphenyl isobutyrate was combined with rifampicin against *M. smegmatis* with FIC index of 0.5, indicating that compound 6 can enhance the effect of rifampicin and *vice versa*. The findings from this study suggest that these phenylpropanoids have the potential to be further investigated as candidate antimicrobial agents in TB drug discovery as well as potential use in treating *M. smegmatis* infections.

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DECLARATIONS

The authors declare no conflicts of interest.

REFERENCES

Abreu, A. C., McBain, A. J. and Simões, M. (2012). Plants as sources of new antimicrobials and resistance-modifying agents. *Natural Product Reports* **29, 1007**

Andreu, N., Soto, C. Y., Roca, I., Martín, C. and Gibert, I. (2004). *Mycobacterium smegmatis* displays the *Mycobacterium tuberculosis* virulence-related neutral red character when expressing the *Rv0577* gene. *FEMS Microbiology Letters* **231, 283-289.**

Anuar, N. (2018). Chemical constituents from *Alpinia conchigera* Griff., synthesis of 1'-acetoxychevicol acetate analogues and their antimicrobial evaluation. Msc. Thesis. Universiti Sains Malaysia, Malaysia.

Aslam, S. N., Stevenson, P. C., Kokubun, T. and Hall, D. R. (2009). Antibacterial and antifungal activity of cicerfuran and related 2-arylbenzofurans and stilbenes. *Microbiological Research* **164, 191-195.**

Aziz, A. N., Ibrahim, H., Syamsir, D. R., Mohtar, M., Vejayan, J. and Awang, K. (2013). Antimicrobial compounds from *Alpinia conchigera*. *Journal of Ethnopharmacology* **145, 798-802.**

Balouiri, M., Sadiki, M. and Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis* **6, 71-79.**

Bansal-Mutalik, R., & Nikaido, H. (2014). Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. *Proceedings of the National Academy of Sciences*, ***111*****(13), 4958-4963.**

Barberis, I., Bragazzi, N. L., Galluzzo, L. and Martini, M. (2017). The history of tuberculosis: From the first historical records to the isolation of Koch's bacillus. *Journal of Preventive Medicine and Hygiene* **58, E9-E12.**

Belanger, A. E. and Hatfull, G. F. (1999). Exponential-phase glycogen recycling is essential for growth of *Mycobacterium smegmatis*. *Journal of Bacteriology* **181, 6670-6678.**

Bonapace, C. R., Bosso, J. A., Friedrich, L. V. and White, R. L. (2002). Comparison of methods of interpretation of checkerboard synergy testing. *Diagnostic Microbiology and Infectious Disease* **44, 363-366.**

Božić, D. D., Milenković, M., Ivković, B. and Ćirković, I. (2014). Antibacterial activity of three newly-synthesized chalcones & synergism with antibiotics against clinical isolates of methicillin-resistant *Staphylococcus aureus*. *Indian Journal of Medical Research* **140, 130.**

Burkill, I. H. (1966). A dictionary of the economic products of the Malay peninsula (2nd ed.). Kuala Lumpur, Malaysia: Ministry of Agriculture and Cooperatives.

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A. and Darst, S. A. (2001). Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* **104, 901-912.**

Carlson, E. E. (2010). Natural products as chemical probes. *ACS Chemical Biology* **5, 639-653.**

Caviedes, L., Delgado, J. and Gilman, R. H. (2002). Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **40, 1873-1874.**

Chaturvedi, V., Dwivedi, N., Tripathi, R.P. and Sinha, S. (2007). Evaluation of *Mycobacterium smegmatis* as a possible surrogate screen for selecting molecules active against multi-drug resistant *Mycobacterium tuberculosis*. *Journal of General and Applied Microbiology* **53, 333-337.**

CLSI. (2018). Performance Standards for Susceptibility Testing of Mycobacteria, *Nocardia* spp, and Other Aerobic Actinomycetes. 1st edn. Clinical and Laboratory Standards Institute, PA, USA.

Deng, Y. and Lu, S. (2017). Biosynthesis and regulation of phenylpropanoids in plants. *Critical Reviews in Plant Sciences* **36, 257-290.**

Eloff, J. N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* **64, 711-713.**

Fazly Bazzaz, B. S., Khameneh, B., Zahedian Ostad, M. R. and Hosseinzadeh, H. (2018). *In vitro* evaluation of antibacterial activity of verbascoside, lemon verbena extract and caffeine in combination with gentamicin against drug-resistant *Staphylococcus*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

aureus and *Escherichia coli* clinical isolates. *Avicenna Journal of Phytomedicine* **8, 246.**

Goyal, S., Lambert, C., Cluzet, S., Mérillon, J. M. and Ramawat, K. G. (2012). Secondary metabolites and plant defence. In: Plant Defence: Biological Control. Mérillon J. M. M. and Ramawat K. G. G. (eds.). Springer, Dordrecht. **pp. 109-138.**

Guo, Z. (2017). The modification of natural products for medical use. *Acta Pharmaceutica Sinica B* **7, 119-136.**

Gupta, P. D. and Birdi, T. J. (2017). Development of botanicals to combat antibiotic resistance. *Journal of Ayurveda and Integrative Medicine* **8, 266-275.**

Hagihara, M., Crandon, J. L. and Nicolau, D. P. (2011). The efficacy and safety of antibiotic combination therapy for infections caused by Gram-positive and Gram-negative organisms. *Expert Opinion on Drug Safety* **11, 221-233.**

Harvey, A. (2000). Strategies for discovering drugs from previously unexplored natural products. *Drug Discovery Today* **5, 294-300.**

Hemaiswarya S. and Doble M. (2010). Synergistic interaction of phenylpropanoids with antibiotics against bacteria. *Journal of Medical Microbiology* **59, 1469-1476.**

Ibrahim, H., Khalid, N. and Hussin, K. (2007). Cultivated gingers of peninsular Malaysia: Ultilization profiles and micropropagation. *The Gardens' Bulletin Singapore* **59, 71-88.**

Kamal, T., Muzammil, A. and Omar, M. N. (2012). Evaluation of antimicrobial activity of *Artocarpus altilis* on pathogenic microorganisms. *Science Series Data Report* **4, 41-48.**

Korkina, L. G. (2007). Phenylpropanoids as naturally occurring antioxidants: From plant defense to human health. *Cell Molecular Biology* **53, 15-25.**

Kumar, S. N., Siji, J. V., Nambisan, B. and Mohandas, C. (2012). Activity and synergistic interactions of stilbenes and antibiotic combinations against bacteria *in vitro*. *World Journal of Microbiology and Biotechnology* **28, 3143-3150.**

Li, X. Z., Zhang, L. and Nikaido, H. (2004). Efflux pump-mediated intrinsic drug resistance in *Mycobacterium smegmatis*. *Antimicrobial Agents and Chemotherapy* **48, 2415-2423**.

Liang, L., Wu, Q., Gao, L., Hao, Y., Liu, C., Xie, Y., Sun, H., Yan, X., Li, F., Li, H., Fang, H., Ning, N., Cui, Y. and Han, L. (2012). Factors contributing to the high prevalence of multidrug-resistant tuberculosis: A study from China. *Thorax* **67, 632-638.**

Lönnroth, K. and Raviglione, M. (2015). The WHO's new end TB strategy in the post-2015 era of the sustainable development goals. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **110, 148-150.**

Mohamad Taib, M.N.A, Anuar, N., Mohd Hanafiah, K., Al-Shammary, A.A.K., Saaid, M. and Awang, K. (2020). Chemicals constituents isolated from cultivate *Alpinia conchigera* Griff. and antimicrobial activity. *Tropical Life Sciences Research* **31(1), 159-178.**

Rigano, D., Formisano, C., Basile, A., Lavitola, A., Senatore, F., Rosselli, S. and Bruno, M. (2007). Antibacterial activity of flavonoids and phenylpropanoids from *Marrubium globosum* ssp. *libanoticum*. *Phytotherapy Research* **21, 395-397.**

Sao, C., Williams, M., Helden, P., Taylor, M., Carolis, C., J. Wiid, I. and Baker, B. (2018). Generation and characterization of thiol-deficient *Mycobacterium tuberculosis* mutants. *Scientific Data* **5, 180184.**

Sibanda, T. and Okoh, A. (2007). The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents. *African Journal of Biotechnology* **6, 2886-2896.**

Silva, R. H., Andrade, A., Nóbrega, D. F., Castro, R. D. D., Pessôa, H. L., Rani, N. and de Sousa, D. P. (2019). Antimicrobial activity of 4-chlorocinnamic acid derivatives. *BioMed Research International* **3941242.**

Shetty, A., Xu, Z., Lakshmanan, U., Hill, J., Choong, M. L., Chng, S. S., Yamada, Y., Poulsen, A., Dick, T. and Gengenbacher, M. (2018). Novel acetamide indirectly targets mycobacterial transporter MmpL3 by proton motive force disruption. *Frontiers in Microbiology **9***, **2960**.

Stephan, J., Bender, J., Wolschendorf, F., Hoffmann, C., Roth, E., Mailänder, C., Engelhardt, H. and Niederweis, M. (2005). The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. *Molecular Microbiology* **58, 714-730.**

Stratton, C. F., Newman, D. J. and Tan, D. S. (2015). Cheminformatic comparison of approved drugs from natural product versus synthetic origins. *Bioorganic & Medicinal Chemistry Letters* **25, 4802-4807.**

Tonari, K., Mitsui, K. and Yonemoto, K. (2002). Structure and antibacterial activity of cinnamic acid related compounds. *Journal of Oleo Science* **51, 271-273.**

Wehrli, W. (1983). Rifampin: Mechanisms of action and resistance. *Reviews of Infectious Diseases* **5(Supplement_3)**, **S407-S411.**

WHO, World Health Organization. (2018). High levels of antibiotic resistance found worldwide, new data shows. WHO: https://www.who.int/mediacentre/news/releases/2018/antibiotic-resistance-found/en/ [Retrieved on 24 October 2019]

Wu, M. L., Aziz, D. B., Dartois, V. and Dick, T. (2018). NTM drug discovery: Status, gaps and the way forward. *Drug Discovery Today* **23, 1502-1519.**

Zgoda, J. R. and Porter, J. R. (2003). A convenient microdilution method for screening natural products against bacteria and fungi. *Pharmaceutical Biology* **39(3).**