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Anti-mycobacterial activity of two natural Bisanthraquinones: (+)-1,1'-Bislunatin and (+)-2,2'-Epicytoskyrin A

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Abstract. Tuberculosis is considered one of the leading death causes in the world by a single infectious agent, *Mycobacterium tuberculosis* (*Mtb*). The search for a new biologically active compound for the treatment of Tuberculosis is an urgent need due to the rise of multidrug-resistant phenomena. Endophytic fungi were known for a vast reservoir for bioactive secondary metabolites. Endophytic fungi, *Diaporthe* sp. GNB-10 associated with gambier plant *Uncaria gambier* Roxb was able to produce two natural bisanthraquinone, (+)-1,1'-Bislunatin (Bis) and (+)-2,2'-Epicytoskyrin A (Epi), possessing strong antibacterial activities against the various pathogen. In this study, the biological activity screening of (Bis) and (Epi) will be extended by evaluating their activity against *Mtb* H37Rv. The in-vitro assay was conducted via determination of Minimum Inhibitory Concentration (MIC), nutrient-starvation *Mtb*, Biofilm *Mtb* formation, and *Mtb*- infected macrophage assay. Meanwhile, the in-silico screening was done by docking and Adsorption, Distribution, Metabolism, and Excretion (ADME) studies. Bis and Epi showed promising anti-tubercular activity, as shown by the MIC value of Bis, which is the same as rifampicin (standard drug) at 0.422 μ M. Meanwhile, Epi carried out the same MIC value as isoniazid (standard drug) at 0.844 μ M. Further investigation on in-vitro assay resulted in Epi and Bis, which were observed for having the ability to combat nutrient-starvation and biofilm of the *Mtb* model with relatively moderate activity in bacterial reduction with log reduction between 1-2 folds. The cytotoxicity study revealed that Bis possessed high toxicity at all tested concentrations, while the toxicity of Epi decreased as the tested concentrations decreased. Both compounds could reduce the number of *Mtb* infected into macrophages in 2 folds log reduction. The in-silico result via docking study shows that both compounds have good affinity with pantothenate kinase (PanK) enzyme with a glide score of -8.427 kcal/mol and -7.481 kcal/mol for Epi and Bis, respectively. This study suggests that Epi displayed a potency to be developed further as an anti-mycobacterial compound as it is shown by in-vitro assay, cytotoxicity, and in-silico study. Despite possessing good anti-mycobacterial activity, further studies such as QSAR (Quantitative-Structure Activity Relationship) is needed to improve the physicochemical properties of Epi while maintaining its biological properties.

1. Introduction

Tuberculosis (TB) remains a great health concern as it was estimated in 2016, over 10.4 million people developed active TB disease and this resulted in 1.7 million deaths [1]. This disease is caused by a single pathogen named *Mycobacterium tuberculosis*. Some challenges have emerged in combating *M.*



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tuberculosis because this bacteria has drug resistance mechanisms that make most antimicrobials ineffective. Its unique lipid-rich cell envelope structure has low permeability to most clinical antibiotics [2]. Moreover, the persistent phase of infection is one of the characteristics of Tuberculosis, when the bacteria are thought to be in a slow-growing or non-growing state [3]. The treatment TB disease is a long process that involves complicated drug regimens, with disadvantage effects and interactions, and is associated with poor patient fulfillment [4]. Approximately, around 4% of TB patients have been treated with the first-line TB drug, such as isoniazid and rifampicin developed the multidrug-resistant (MDR-TB), causing the resistance over current TB drugs [5, 6]. Although first-line drugs have successfully treated around 85% of TB diseases, the treatment-related adverse effects, including hepatotoxicity, allergic skin reactions, gastrointestinal and neurological disorders, contribute to significant morbidity and reduce to effective therapy [7]. The current drug development pipeline is still insufficient to eradicate causative bacteria from TB patients completely. There remains an essential need to discover and develop new anti-TB drugs, particularly to target drug-resistant and dormant strains of *M. tuberculosis*, as well as providing a more effective and shorter duration of treatment [8, 9].

Natural sources represent a vast reservoir of chemically-diverse molecules that can provide bioactive compounds for therapeutic agents. A variety of compounds isolated from natural products have been reported to possess substantial antibacterial activity. In recent years, there has been a renewed interest in the investigation of the natural sources for the identification of novel anti-tubercular agents [1, 2]. One of the natural resources that can be widely explored for producing secondary metabolites with anti-tubercular activity is endophytic fungus since it contains a large pool of active compounds [2]. From 360 morphologically distinct endophytic fungi, extracts from 92 isolates were found to inhibit the growth of *M. tuberculosis* H37Rv (MIC of 0.0625–200 µg/ml) [10]. The recent study revealed that anthraquinone metabolites isolated from the ant pathogenic fungus of *Cordyceps morakotii* BCC 56811, i.e., morakotins C–D, lunatin, rheoemodin, YM187781, bislunatin have potent as an anti-bacterial, anti-fungal, anti-cancer and anti-tubercular agent in vitro testing [11].

In this study, two bisanthraquinones are produced by endophytic fungus *Diaporthe* sp. GNB-10 associated with Gambier plant (*Uncaria gambier* Roxb) (+)-1,1'-Bislunatin (Bis) and (+)-2,2'-Epicytoskirin-A (Epi). They were evaluated for their activity against *Mycobacterium tuberculosis* (*Mtb*) H37Rv. The previous data show promising antibacterial and antioxidant activities of both Epi and Bis for several pathogen microorganisms, as reported by Agusta et al. [12] and Praptiwi et al. 2015 [13]. Despite the potential antimicrobial activity of Bis and Epi, we observe that there is no study revealed the antimycobacterial activity of both compounds in-vitro and in-silico testing. In the previous study, the acute oral toxicity of Bis and Epi evaluated by testing in mice (*Mus musculus*) indicated that both Bis and Epi were included as low toxicity compounds [13]. To the best of our knowledge, this is the first study reporting the anti-mycobacterial activity of Bis and Epi by in-vitro and in-silico studies.

To confirm the anti-tubercular activity, we conducted a biological evaluation of tested compounds (Bis, Epi, and standard Tb drugs) via determination of Minimum Inhibitory Concentration (MIC) value for identification of minimum concentration needed to kill the replicating *Mtb*. In vitro assay of nutrient-starved (dormant) *Mtb* was for investigating the effect of tested compounds to non-replicating *Mtb* cells. Moreover, the biofilm *Mtb* model assays and *Mtb* infected macrophages assays were also conducted. In the investigation of the safety of the compounds, the cytotoxicity test was also performed. Furthermore, to preliminary investigate the potential targets and to confirm the experimental activity of tested molecules as anti-tubercular drugs, we performed the docking study by using different enzymes and receptors enzymes involved with cell wall synthesis, cell growth, and DNA replication.

2. Materials and methods

2.1. Biological Evaluation of anti-tubercular activity

The tested compounds, (+)-1,1'-Bislunatin (MW 570.462 g/mol) and (+)-2,2-Epicytoskirin-A (MW: 574.494 g/mol) were isolated from endophytic fungus *Diaporthe* sp. GNBP-10⁹ [14]. The stock solutions were made by diluting the proportional amount of tested compounds with DMSO (Dimethyl Sulfoxide, Sigma Aldrich) to form 10 mmol of stock solutions.

2.1.1. MIC (Minimal Inhibitory Concentration).

The MIC values were identified against Mtb H37Rv using Alamar blue as a fluorescent dye. The Middlebrook 7H9 (HiMedia Laboratories) and OADC (oleic acid, albumin, dextrose, and catalase) (HiMedia Laboratories) with a 9:1 ratio were used as growth media. Serial dilutions technique, as written in detail by Collin et al. [15], was used for providing tested compounds in each well from the concentration of 200 μ M- to 0.105 μ M. Serial two-fold dilution of each drug was prepared directly in a sterile 96-well microtiter plate using 100 μ l 7H9S. Rifampicin and isoniazid were used as drug standards. A growth control just contained media and Mtb without tested compounds, and a sterile control containing growth media were also prepared only on each plate. Bacteria (100 μ l) were added to all wells except for sterile control. Sterile water was added to all perimeter wells to avoid evaporation during the incubation period. The plate was incubated for 37°C in a normal atmosphere for seven days incubations. After incubations, 50 μ l Alamar blue solution (Resazurin-based solution (Sigma Aldrich) was added to all wells [16].

2.1.2. Cytotoxicity.

The Epi and Bis were further evaluated for the toxicity in the mouse macrophage cell line RAW 264.7 that was obtained from The National Centre for Cell Science (NCCS), Pune, India. The cytotoxicity was conducted at the concentration 100, 10, 1, 0.1, and 0.01 μ M. The mouse macrophage cell line was grown in RPMI-1640, a (HiMedia Laboratories) medium supplemented with 10% Fetal flasks. After cells achieved 80-90% of cell confluency, cells were scraped and seeded into well, i.e., 5000 cells per well in poly-L-lysine coated plates. The plates were incubated at the temperature 37°C, 5% CO₂, 95% air, and 100% relative humidity for 24 h before the addition of Epi and Bis at previously mentioned concentrations. After 48h of exposure, viability was assessed based on cellular conversion of MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich) into formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay. The absorbance was then read at the wavelength of 595 nm using Perkin Elmer Victor X3 microplate reader against the blank [17].

2.1.3. In vitro of Mtb dormant state assay (nutrient starvation model).

The Mtb culture in the Middlebrook 7H9 medium supplemented with OADC was starved for six weeks at 37 °C by the formation of pellet and washing with PBS (Phosphate Buffer Saline, HiMedia Laboratories) was used to wash and resuspend the pellet. The starved cultures were treated with standard drugs (isoniazid and rifampicin) along with tested compounds Bis and Epi for seven days at the concentration of 10 μ M. The treated cell suspensions were diluted 10-fold up to 10⁻⁶ using Middlebrook 7H9 medium supplemented with OADC, and 100 μ l of each dilution was plated in 48 well plates in triplicates along with 900 μ l of Middlebrook 7H9 (HiMedia Laboratories) with OADC (HiMedia Laboratories). The microplates were incubated at 37 °C for 21 days without agitation. Wells with visible bacterial growth were counted as positive, and MPN (Most Probable Number) values were calculated using standard statistical methods. Control was designated by the addition of growth media by nutrient-starved Mtb without the addition of tested compounds [18].

2.1.4. Mtb biofilm assay.

Biofilm was developed by adding Sauton's media (HiMedia) to each well in a 12 well sterile plate with the Mtb culture within six weeks of incubation at 37 °C. Tested compounds were added to the matured biofilm. Test compounds were added at 30 μ M, swirled (n = 4), and sealed. After seven days incubation, Tween-80 (0.1% v/v) was added by mixing at the room temperature for about fifteen min.

The contents of each well were centrifuged at 4000 rpm for ten minutes at room temperature, and the pellet was suspended in 5 ml of washing buffer (PBS with 10% glycerol and 0.05% Tween-80) and repeated for three times. The pellet was then resuspended in 5 ml of washing buffer and mixed overnight at room temperature. The mixture was homogenized through the syringe passage five times. The homogenized biofilm mixture was diluted 10-fold up to 10^{-6} using Middlebrook 7H9 medium supplemented with OADC. Then, 100 μ l of each dilution was plated in 48 well plates in triplicate along with 900 μ l of Middlebrook 7H9 medium supplemented with OADC.. The plate was incubated at 37° C for three weeks. The presence of bacteria in the biofilm form was determined by comparing plates with positive control plates by the MPN method [19, 20]. Control was designated by the addition of growth media by biofilm-form of Mtb without the addition of tested compounds.

2.1.5. *Mtb* infected macrophage model.

The active compounds from the in-vitro anti-TB assays were evaluated for the Mtb infected macrophage model. The mouse macrophage primary cell line extracted from balb/c mice was cultured in RPMI-1640 media supplemented with 10% FBS. Mtb H37Rv cultured in 7H9 Middlebrook broth was then added at a multiplicity of infection of 10:1 for macrophages, and then incubated for four h. Epi and Bis were added to the cells at 10 μ M final concentration, and the plates were incubated for 48 h. Macrophage cells were lysed using 1 ml of 1 $\times$ PBS containing 0.1% Triton X 100. Mtb-infected macrophages were suspended in 7H9 Middlebrook broth. Serial dilutions were then performed, and cells were plated and incubated at 37 °C for three weeks. The Mtb-infected macrophage in growth medium without the addition of tested compounds was designated control. The analysis of this data was used by calculating the Most Probable Number (MPN) using the standard statistical method [21].

2.2. Molecular docking and ADME (Absorption, Distribution, Metabolism, and Excretion) studies

2.2.1. Ligand preparation.

ChemBio3D Ultra 12.0 (www.cambridgesoft.com) was used to draw the chemical structures of Bis and Epi. Both chemical structures were saved in the mol version for further ligand preparation. Ligand candidates were energy minimized and used as a single file using Ligprep Module on Schrodinger (LigPrep v2.2, Schrodinger, LLC, New York, NY). Figure 1 shows the molecular structures of (+)-1,1'-Bislunatin and (+)-2,2-Epicytoskirin-A.

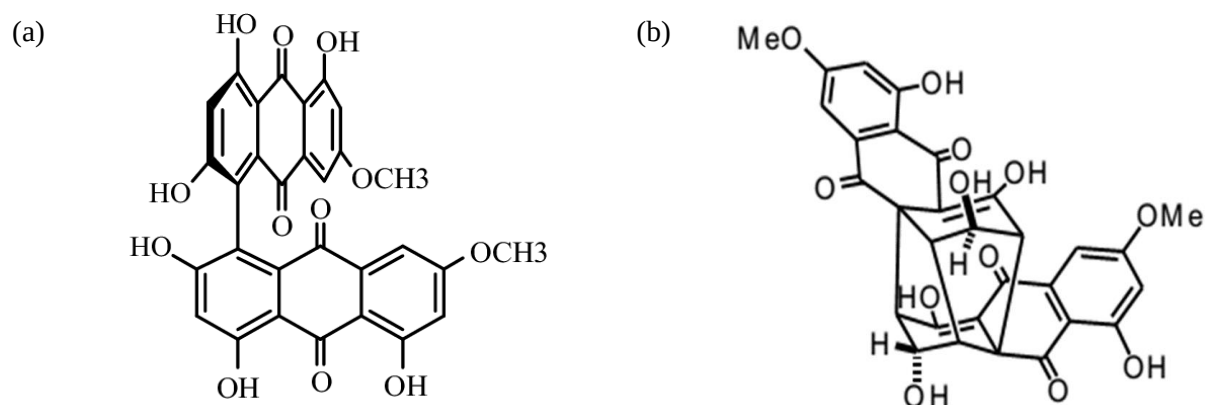


Figure 1. (a) 1,1 Bislunatin (Bis) (MW: 570.462 g/mol); (b) (+)-2,2-Epicytoskirin-A (Epi) (MW: 574.494 g/mol).

The three-dimensional crystal structure of all proteins reported (Table 1) in this study was downloaded from Protein Data Bank (PDB), and the in-silico models were subjected to the Protein Preparation Wizard workflow implemented in the Schrodinger software. The proteins were pre-processed by the addition of hydrogen, assigning the bond order, identifying overlaps, missing atoms,

creating zero-order bonds to metals, and creating disulfide bonds. The co-factors, unwanted water molecules, and chains were removed. The proteins were optimized, and energy was minimized using the optimized potential for liquid simulations (OPLS) force field. The prepared proteins were then saved in the uncompressed version (maegz) for further analysis. Table 1 shows the protein targets for docking studies.

2.2.2. Grid generation.

Grid box parameter was determined by centroid workspace ligand selected in the receptor tab for co-crystal ligand with the maximum space generated was 20Å^o length. All of the detailed parameters for grid generation were followed from the Maestro package module.

Table 1. Proteins targets, grid generation settings, and their role in *Mtb* growth.

No	Protein Name	PDB	Role
1	Enoyl-ACO-Reductase (InhA)	1BVR	Involve in the biosynthesis of mycolic acids
2	3-oxoacyl-[acyl-carrier-protein] reductase (MaBa)	1UZN	Catalyzes the condensation reaction of fatty acid synthesis by the addition to an acyl acceptor of two carbons
3	Pantothenate Kinase (PaNK) <i>Mtb</i>	3AF3	Responsible for fatty acid derivatives that are key precursors to mycolic acid
4	PknI serin threonine kinase	5M06	Signaling protein
5	Arabinosyl transferase (EmbC)	3PTY	Responsible for the polymerization of arabinose into the arabinan of arabinogalactan
6	Protein Kinase PknG	2PZI	Transferase, a regulatory protein
7	Transpeptidase type II	3VAE	Cell wall and cell processes
8	RNA polymerase Chain D	6C06	Sigma factors are initiation factors that promote the attachment of RNA polymerase to specific sites
9	Glutamate Racemase	5HJ7	Provides the (R)-glutamate required for cell wall biosynthesis
10	DprE1 (Decaprenylphosphoryl-beta-D-ribose oxidase)	4FDO	The arabinose donor for mycobacterial cell wall biosynthesis
11	Mycolic Acid Synthase	1LE1	Lipid metabolism
12	UDP-Galactopyranose mutase	1V0J	cell wall and cell processes
13	Dihydrofolate Reductase (DHFR)	4M2x	Key enzyme in folate metabolism
14	Dihydropteroate synthase	4B6C	Catalyzes the condensation of para-aminobenzoate (pABA)
15	Cyclopropane mycolic acid synthase 2	1KPI	Cyclopropanated mycolic acids are key factors participating in cell envelope permeability,
16	DNA gyrase-B-sub unit	4B6C	catalyzes the ATP-dependent negative super-coiling of double-stranded closed-circular DNA

2.2.3. Glide XP (Extra-Precision) docking.

Grid box parameter was determined by centroid workspace ligand selected in the receptor tab for co-crystal ligand with the maximum space generated was 20Å^o length. All of the detailed parameters for grid generation were followed from the Maestro package module.

2.2.4. In silico prediction of physicochemical properties.

In-silico prediction of Absorption, Distribution, Metabolism, Excretion (ADME) was done using the Qik-Prop feature in the maestro module of Schrodinger software. The criteria of the filter included molecular weight 160-480, number of heavy atoms 20-70, lipophilicity 40-130, number of hydrogen bonding donor 4-7, number of hydrogen bonding acceptor 8-12, percentage of human oral absorption, solubility, cell permeability, etc. [22].

3. Results and discussion

3.1. Biological evaluation of anti-tubercular activity

Two natural bisanthraquinones, Bis and Epi were purely isolated from endophytic fungi *Diaporthe* sp. GNPB-10 associated with gambier plant *Uncaria gambier* Roxb as described in detail by Augusta et al., 2006 [14]. Minimum Inhibitory Concentration (MIC) evaluation was conducted by the Microplate Alamar Blue Assay (MABA). The color transformation of Resazurin solution (blue) to pink indicates the presence of Resorufin (reduced form of Resazurin). In viable cells, the presence of NADPH can reduce Resazurin (blue) to become Resorufin (pink) [23]. MIC investigation showed that Bis and Epi exhibited strong activity against *Mtb* H37Rv with MIC values were 0.422 μ M and 0.844 μ M, respectively, as shown in Table 2. The MIC values of Bis and Epi exhibited similar activity to the first-line *Mtb* drugs, isoniazid and rifampicin. Besides, this result also triggered for further investigation of Bis and Epi for combating nutrient-starved and biofilm *Mtb*. This investigation shows that both Epi and Bis have a strong activity to combat the replicating *Mtb*. The ability of Bis and Epi to inhibit *Mtb* added the scientific feature of these compounds as previously revealed that they have strong anti-bacterial activity against various bacterial pathogens. Epi and Bis have reported to alteration of the bacterial cell membrane with an increase of cation efflux [12, 24]. This study suggested that Epi and Bis have a good ability to penetrate the replicating *Mtb* cell membrane as it could inhibit the *Mtb* growth at the concentration as same as the standard TB-drugs. Epi and Bis have the potency to be developed for an anti-mycobacterial agent, although it needs further study for understanding their role as an anti-mycobacterial agent.

Table 2. The Minimum Inhibitory Concentration (MIC) values of Bis, Epi, and standard Tb-drugs.

No	Tested compounds	MIC (μ M)	Classification [25]
1	(+)-1,1'-Bislunatin (Bis)	0.422	strong
2	(+)-2,2-Epicytoskirin-A (Epi)	0.844	strong
3	Isoniazid (INH) (standard drug)	0.844	strong
4	Rifampicin (Rif) (standard drug)	0.422	strong

The in-vitro anti-mycobacterial screening was generally just established by the determination of MIC value. Further study of Epi and Bis as an anti-mycobacterial agent was conducted by investigating the ability of both compounds to inhibit the dormant/nutrient-starved *Mtb*. The nutrient-starved *Mtb* assay (dormant *Mtb*) was conducted to understand the ability of tested compounds to eradicate the non-replicating *Mtb* (dormant *Mtb*). It is known that the dormant *Mtb* causes latent TB disease and is able to persist in its human host despite extensive chemotherapy [26]. All classes of antibiotics require bacterial replication for their action. The non-replicating state (dormant state) is thought to render *Mtb* phenotypically resistant to otherwise bactericidal antibiotics [27]. The lengthy *Mtb* treatment is caused by the dormancy induced drug-tolerance developed by *Mtb* during infection in response to conditions within the host [28]. The *Mtb* bacteria was made nutrient-starved by removing growth nutrition and incubated for six weeks in the PBS. The starved bacteria were then treated with tested compounds and standards. The effect of Bis and Epi at the concentration of 10 μ M to reduce the number of nutrient-deficiency *Mtb* is shown in Figure 2. Bis and Epi exhibited relatively similar activity to standard drugs (INH) in reducing the *Mtb* dormant model with a log reduction value was approximately 1.14-1.42 fold. This assay showed Epi and Bis were not able to eradicate the latent/nutrient starved *Mtb*, so they were not proper to be developed as for the 2nd-line anti- TB compound combating MDR of TB. The ability of the standard drugs to eradicate the dormant *Mtb* was evaluated to be slightly active due to the utilization of first-line Tb drugs that were not designated for combating the dormant *Mtb*.

M. tuberculosis is not only able to stay in the dormant state and being reactivated again when suppression of the immune system happens, this bacteria is also competent to reside in the biofilm form. The biofilm formation in the pathogen bacteria is important since it makes bacteria to be more

tolerant of antibiotic and evokes the different immune responses [29]. The biofilm formation of the bacterial pathogen reduces antibiotic permeability that led to the decrement of antibiotic activity [30]. To observe the effect of Bis and Epi in the biofilm-form of *Mtb*, we used the Sauton media to help the formation of biofilm *Mtb*. Figure 3 shows the number of bacteria treated with tested compounds at a particular concentration (30 μ M). Both compounds were evaluated to reduce the number of biofilm of *Mtb* cells. Epi does not exhibit any activity for reducing *Mtb* in the biofilm form as the log cell/ml of Epi is observed same with control. Bis exhibited 1 fold log reduction of cells whilst standard drugs RIF and INH showed 1.5 and 1.2 fold of reduction, respectively.

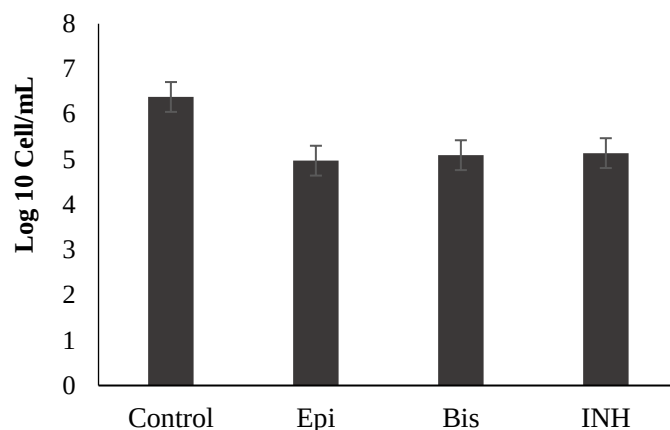


Figure 2. Activity profile of tested compounds and standard TB drugs in reducing the nutrient starvation *Mtb* model. Concentrations of tested compounds and standard drugs were 10 μ M. The bacterial count was estimated using the Most Probable Number assay, and the significance plot was obtained by adopting two way ANOVA ($p < 0.0001$, using GraphPad Prism Software). EPI: (+)-2,2-Epicystoskirin-A ; BIS: (1,1)-Bislunatin; INH: Isoniazid.

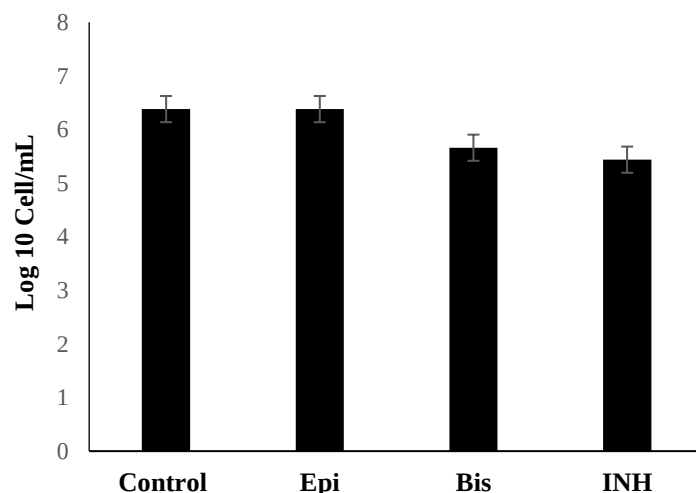


Figure 3. Biofilm inhibitory activity of tested compounds and standard drugs against *Mtb*. Bacterial count estimation (mean $\pm$ S.D., $n = 4$) was conducted using the Most Probable Number (MPN) assay. EPI: (+)-2,2-Epicystoskirin-A ; BIS: (1,1)-Bislunatin; INH: Isoniazid.

The ability of Bis and Epi to penetrate over host-cells was investigated by *Mtb* infected macrophages assays. This assay was conducted to understand whether both compounds remain active and available as anti-TB in the host-cell (macrophages) environment. The bioavailability of therapeutic agents in the biological system is important to ensure that the therapeutic agent is available

in the proper amount to kill the pathogen microorganism. Figure 4 shows the assay result describing Epi and Bis can penetrate through macrophages cell and be active to reduce the number of bacteria in 2 fold log reduction for Bis, 1.8 log reduction for Epi, and 2.6 log reduction for standard drug, isoniazid.

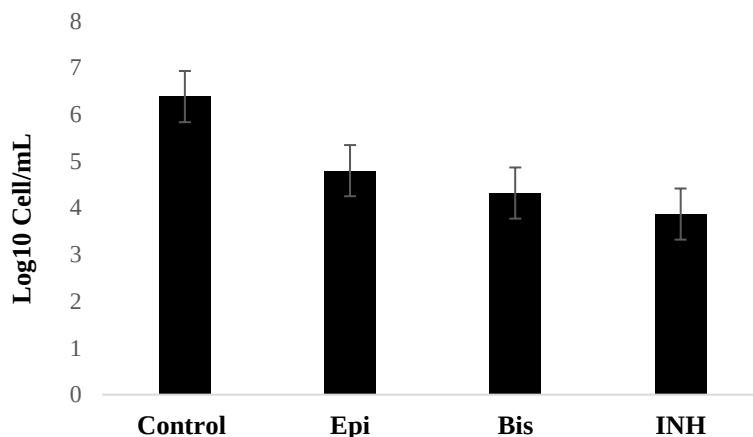


Figure 4. Comparative *Mtb* infected macrophage inhibitory activity plots. Bacterial count estimation (mean $\pm$ S.D., $n = 4$) was conducted using the most probable number assay. EPI: (+)-2,2'-Epicytoskiryn-A ; BIS: (+)-1,1'-Bislunatin; INH: Isoniazid.

The cytotoxicity assays were used to evaluate the safety of these compounds for mammalian cells. The macrophages cells were host cells for *Mtb*, so for the cytotoxicity evaluation, raw cell macrophages lines were used. As presented in Table 3, Epi inhibited 70% of cell growth at a concentration of 100 μ M, and Epi kept inhibiting cell for 4% at cell growth in all tested concentrations.

Table 3. Cytotoxicity of Epi and Bis in all tested concentrations.

Concentration (μ M)	Cytotoxicity		Cell viability	
	Epi	Bis	Epi	Bis
100	79%	69%	21%	31%
10	79%	69%	21%	31%
1	55%	70%	45%	30%
0.1	7%	70%	93%	30%
0.01	4%	69%	96%	39%

3.2. *In silico* molecular docking and pharmacokinetic properties prediction

A molecular docking study was performed to predict the interaction between Bis and Epi with protein targets. Docking result showed that among tested protein targets, Bis and Epi possessed the best affinity with *Mtb* Pantotenate kinase (PanK). Epi showed better binding with PanK protein with docking value of -8.427 kcal/mol, while Bis had a slightly lower binding affinity with a docking value of -7.481 kcal/mol. As shown in Table 4, Among 15 tested proteins target (Table 1, experimental section), Bis and Epi were matched with ten and six protein targets, respectively. The bulky structure of Bis and Epi made them not easily incorporated into the small active binding site of the protein target. Ligand will bind to the targeted protein-based on conformational selection. The ligand stabilizes one of the protein conformations with a sequential shift of the protein population equilibrium [31]. Some features considered to achieve the high receptor binding affinity are the flexibility of protein target and electrostatic aspects [32], [33]. Ligand-protein interaction is involved in many biological processes with consequent pharmaceutical implications, including inhibition of fatty acid

biosynthesis leading to bacterial inactivation [34]. In Table 4, it shows that the protein targets were successfully docked with Epi and Bis.

Table 4. Docking result for Bis and Epi with protein targets.

No	Protein Name	PDB Code	Glide docking score		Interactions with Amino acids*	
			Epi	Bis	Epi	Bis
1	3-oxoacyl-[acyl-carrier-protein] reductase (MaBa)	1UZN	-2.693	-4.459	Arg25, Arg47, Lys107	Asn24, Arg47, Gly90, Ser92
2	Pantothenate Kinase (PanK) <i>Mtb</i>	3AF3	-8.427	-7.481	Lys 103, Tyr 182, Arg 238, Tyr 257	Arg100, Arg 108, Arg 238
3	PknI serin threonin kinase	5M06	-2.378	-5.815	Val92, Gly94, Gln99	Val92, Gln99, Gly94
4	Arabinosyl transferase (EmbC)	3PTY	-1.005	-5.628	Trp926, Pro1013, Thr1044, Gln1061	Arg1055, Trp1057
5	Protein Kinase PknG	2PZI	NI	-5.698	NI	Gly237, Gln238
6	Transpeptidase type II	3VAE	NI	-3.427	NI	Tyr155, Val156, Asp160, Asp241
7	RNA polymerase Chain D	6C0C	-4.058	-5.63	Asp57, Arg84, Arg89	Glu323, Leu324, Arg412
8	Mycolic Acid Synthase	1LE1	NI	-2.107	NI	Leu95, Tyr262
9	Dihydrofolate Reductase (DHFR)	4M2x	no interaction	-5.131	no interaction	Gly18, Arg45, Thr46, Leu127
10	DNA gyrase-B-sub unit	4B6C	no	-2.107	no	Arg82

*Amino Acid abbreviation refers to [35]

NI: no interaction.

The un-docked protein targets with Bis and Epi are not listed in Table 4. All of the tested proteins used in this experiment docked can be viewed in Table 1 (experimental section).

Interaction between protein and both ligands (Bis & Epi) happened in the form of hydrogen bonding and pi interaction. As shown in Figure 5, Epi showed a specific affinity with amino acid residue Lys 103, Tyr 182, Arg 238, Tyr 257. Bis possessed binding affinity with residue Ala100, Arg108, and Arg 238, as pictured in Figure 6. This study can be a preliminary study for the interaction mechanism of both Epi and Bis in killing and inhibiting the growth of replicating *Mtb*. PanK is an interesting target protein for *Mtb* since this enzyme catalyzes the first step in the biosynthesis of the Cofactor Coenzyme A (CoA) by converting pantothenate (vitamin B5) to 4'-phosphopantothenate [36]. Serine/threonine-protein kinases, such as protein kinase B, regulates mycobacterial cell morphology, play an important role in signal transduction pathways, and allow *M. tuberculosis* to grow and survive successfully within the host [8]. Mammals, including humans, contain four isoforms of PanK with wide variation in length, sequence, and tissue-specific expression. PanK protein is an attractive target for inhibition of *Mtb* growth since, naturally, the bacterial and human PanK enzymes appear different [37]. According to the docking study, Epi and Bis are predicted to be a good candidate for anti-tubercular compound. Though, it still needs more experiments and studies to prove this study.

The assessment of pharmacokinetics properties was investigated to explore the “drug-likeness” of compounds. The Adsorption, Distribution, Metabolism, and Elimination (ADME) properties of the compounds were predicted and being evaluated based on Lipinski’s “Rule of Five” performed by Qik-Prop feature in the Schrodinger software. Qik-prop efficiently evaluated pharmaceutically relevant properties of tested compounds. Accurate prediction of absorption, distribution, metabolism, elimination (ADME) properties before expensive experimental procedures.

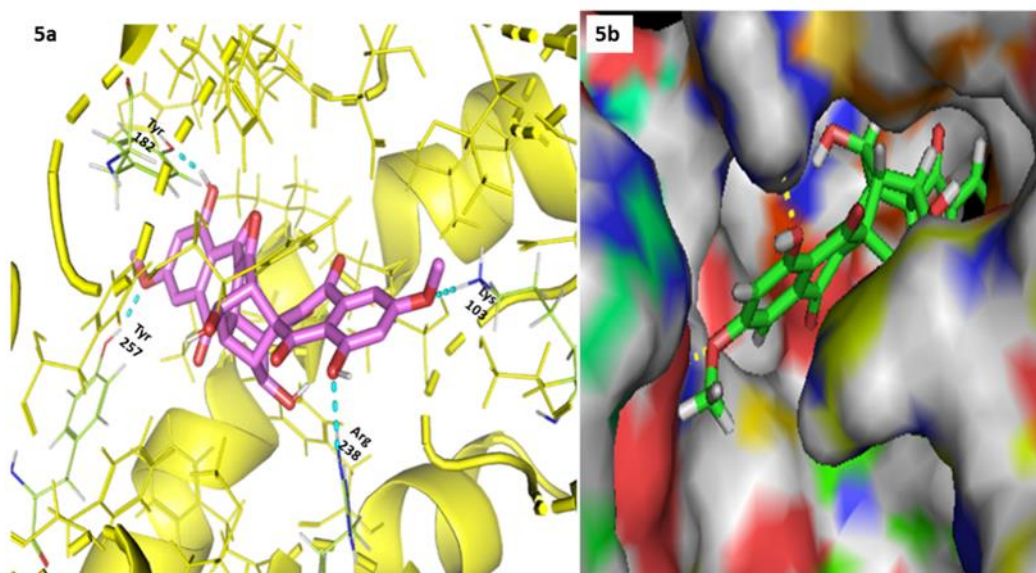


Figure 5 Interaction prediction model of Epi with specific residues in PanK (a). Surface model of Epi with PanK protein (b).

The predicted pharmacokinetic properties for Epi and Bis are shown in Table 5. It demonstrates that both compounds have poor permeability in the Caco-2 model cell. They are not easy to pass the Blood-Brain Barrier (BBB), as shown by their QPP Caco value and QPlogBB, which are out of the range. The bulky structure and structure rigidity might be a reason why both compounds showed poor permeability over Caco cell and BBB [38], [39]. Decrement of drug permeability may contribute to phenotype drug resistance of latent/nutrient-starved Mtb [40]. Furthermore, cell wall alterations and re-modeling in non-replicating cells were suggested to account for the loss of bactericidal activity of Bis and Epi in non-replicating Mtb [40]. The lipophilicity character, presented as Q logO (octanol)/W (water), is one of the important properties to discover the lead compound. Octanol/water partition indicated the lipophilicity of the compound. Bis exhibited higher QPlog O/W than Epi, indicating that Bis carried out more lipophilicity. A recent study stated that lipophilic derivatives compounds were observed more active against replicating mycobacteria than their hydrophilic companion anti-mycobacterial agent [39]. That study correlated with the MIC result (Table 1), in which Bis exhibited stronger activity than Epi in combating replicating Mtb. The aqueous solubility of Bis and Epi were low as indicated by QPlog S value in Table 4, in which the value was out of range for good and optimal aqueous solubilization. The aqueous solubility plays a critical role in the bioavailability of the candidate drugs, and low aqueous solubility might affect their bioavailability in the biological system [41]. The complex chemical structure of Bis and Epi (dimer form) caused them to have low solubility in aqueous solution, as generally possessed by the anthraquinone group [42]. The effect of low aqueous solubility of Bis and Epi on anti-mycobacterial activity was still not fully understood. Therefore, more research studies, such as in vivo, QSAR, pharmacokinetics studies, are needed to have better understanding in this part. The human oral absorption properties show that both compounds are really poor to be absorbed when orally administered. The percentage of oral absorption is below 25% for both compounds, which are categorized as poor absorption. This result indicated that Bis and Epi were better not to be

administered orally. Low human oral absorption and low aqueous solubility might suggest the Bis and Epi were not good to be administered alone. Also, it needed to be coupled with other materials such as an antibody or bio-polymer not only to increase their aqueous solubility but also to accumulate and distribute to the proper target.

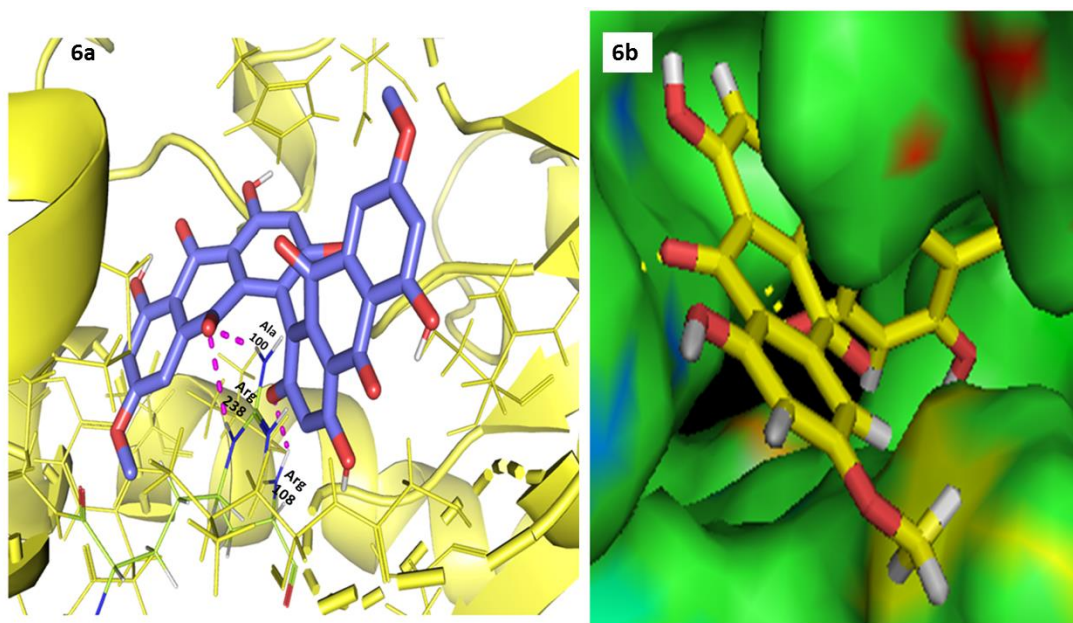


Figure 6 Interaction prediction model of Bis with specific residues in PanK (a). Surface model of Bis with PanK protein (b).

Table 5. Calculated physicochemical properties.

	QPPCaco*	QLogBB*	QLogO/W*	QLogS	% Human oral absorption	Rule of five violations
(+2,2)-Epicitoskyrin-A	7.398	-3.138	1.005	-4.161	9.802	3
1,1-Bislunatin	4.59	-3.709	2.002	-5.388	11.64	3
Range/standard [41]	<25 poor, >500 great	-3 to 1.2	-2 to 6.5	4<PlogS <0 optimal	>80% high, <25% poor	concern above 1

*Parameter Range indicates a preferable value for a drug-like compound. QPPCaco- predicted apparent Caco-2 cell (model for gut-blood barrier) permeability in nm/s. QLogBB – predicted brain/blood partition coefficient. QLogO/W – Predicted octanol/water partition co-efficient log p. QLogS- predicted solubility.

In-silico investigation by the molecular docking and ADME study presented the preliminary study for prediction of the protein target and physicochemical properties of both compounds. Further studies, such as QSAR study, and further target identification modeling, are needed to have a better understanding regarding the inhibition growth mechanism of *Mtb* by Epi and Bis. Also, the availability of Structure-Activity Relationship data can lead to the development of a novel anti-mycobacterial agent inspired by the structure of Epi and Bis. More studies in the biological assays are also needed to prove the ADME prediction for a better understanding of Bis and Epi mechanism as anti-*Mtb*.

4. Conclusion

This study presents the anti-mycobacterial activity of secondary metabolites isolated from endophytic fungi *Diaporthe* sp. GNBP-10 derived from a gambier plant *Uncaria gambier* Roxb, (+)-

1,1'Bislunatin (Bis) and (+)-2,2'-Epicytoskirin-A (Epi). Bis and Epi showed promising anti-tubercular activity against replicating *Mtb* H37Rv, as shown by MIC value 0.422 μ M and 0.844 μ M, respectively. Epi and Bis showed the inhibition against the dormant and biofilm model with relatively similar activity as first-line TB-drug isoniazid. The cytotoxicity study revealed that Bis possessed high toxicity at all tested concentrations, while the toxicity of Epi decreased as the concentrations decreased. According to cytotoxicity results, Epi was observed to be less toxic than Bis. The in-silico study exhibited that both Bis and Epi showed the best binding affinity with the *Mtb* PaNK enzyme, as shown by their glide score after the docking process at and -7.481 kcal/mol and -8.427 kcal/mol respectively. ADME studies predicted that Bis and Epi had low permeability over Caco-2 cell and BBB. Good lipophilicity of Bis and Epi suggested that they could interact well with the *Mtb* cell wall membrane, which was mostly mycolic acid. ADME prediction displayed Epi and Bis to have low aqueous solubility, and they might not be proper to be administered orally. Epi shows promising candidates to be developed as an anti-mycobacterial agent in the future as it shows strong inhibition over replicating *Mtb*, low toxicity, and is predicted to have a good binding affinity with one of *MTb* protein target. Although Epi shows promising anti-myobacterial activity features, more studies such as Quantitative Structure-Activity Relationship (QSAR) and toxicity studies are needed to improve the biological and physicochemical properties. In the future, we would also like to suggest the in-vivo investigation of Epi as anti-mycobacterial agent.

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