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Diptoindonesin D, a Potent Antibacterial Activity Against Gram-positive Bacteria, an Inhibitor of Penicillin-binding Protein 2a from the Stem Bark of *Shorea roxburghii* G.Don

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Abstract

The emergence and spread of antimicrobial resistance (AMR) is a serious threat to public human health. The challenge now is an urgent need to new potent antibiotics combating AMR which can be an effective way to solve the crisis. A new potent antibacterial activity of diptoindonesin D, together with four known compounds as hopeafuran, α-viniferan, hopeahainol A and hopeahainol C were isolated from the stem bark of *Shorea roxburghii* G. Don in Khon Kaen province, Thailand. Diptoindonesin D were screened for antimicrobial activity and possesses significant activity minimum inhibitory concentrations (MIC) values of 4.68 and 9.37 μg/mL against *Staphylococcus epidermidis* and *Staphylococcus aureus*, respectively. The absolute configuration of diptoindonesin D were evaluated by circular dichroism (ECD) calculations. The ECD results indicated that the possible configuration of diptoindonesin D should be R-configuration. Furthermore, reverse docking was performed to identify the protein target of diptoindonesin D based on four scoring functions using GOLD program. This investigation is the first time that reveals interaction between diptoindonesin D and its binding with cell wall synthesis protein targets. The results highlighted that, penicillin-binding protein 2a (PBP2a), an antibacterial cell wall, might be the potential target for the binding of diptoindonesin D. The obtained

results can be useful as a guideline for the development of effective diptoindonesin D as a PBP2a inhibitor.

Keywords: antimicrobial resistance, *Shorea roxburghii* G. Don, target identification, cell wall target, molecular docking, ECD calculations

1. INTRODUCTION

Dipterocarpaceae are a family of 17 genera. Shorea is the largest genera with 196 species of mainly tropical lowland rainforest trees which are found in Southeast Asia, particularly in Thailand. The genus Shorea is a rich resource of various resveratrol (3,5,4'-trihydroxystilbene) and stilbene-based phytochemical [1]. They are found to be substantially helpful for human health owing to its significant antiviral effects from oligostibenoid compounds [2] such as hepatoprotective activity [3] anticancer [4, 5] antitumor effects [6] antioxidant activity [7, 8], antidiabetogenic in olive oil-loaded mice and pancreatic lipase inhibitory activity [9], an acetylcholinesterase (AChE) inhibitory activity [10, 11], immunosuppressive [12], anti-hyperuricemic and anti-inflammatory effects [13]. In Thailand, the previous studies on *S. roxburghii* in Phatthalung province located in the southern part of Thailand also found some resveratrol compounds.

In recent years, the antimicrobial resistance (AMR) is now spreading faster than ever as one of the most an emergent has become a serious global health concern and major challenging current trends worldwide [14]. Multidrug-resistant superbugs causing 700,000 deaths each year are predicted to kill over 10 million people worldwide by 2050 and will cost the global economy about 100-trillion US dollars between 2015 and 2050. So, AMR tended to effect the human higher than cancer [15]. In addition, a substantial percentage of hospital-acquired infections are caused by highly resistant phathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA). Resistance to the most first-line antibiotics to treat infections caused by *S. aureus* is widespread over the world [16]. MRSA is one of the most multidrug-resistant pathogens worldwide, the emergence of methicillin-resistant PBP2a specifically plays vital role on crosslinked network between peptidoglycan chains of the cell wall, whereas other PBPs are inhibited by β-lactam antibiotics [17].

The need for new antibiotics has arisen due to the serious resistance to current drugs [18]. In order to overcome this emerging crisis, the finding new potent compounds are needed for the development of new antibiotic resistance and it is one of the major global challenge [19]. Antibiotic resistance can essentially be acquired through four different pathways and expressed by four different mechanisms [20]. One of the potential targets of novel antibiotics that could be effective against resistant bacteria is the bacterial cell membrane [21]. The classification of antibiotics is based on their mechanism of action and the main mechanisms include the inhibition in cell wall synthesis, the inhibition in protein synthesis, the inhibition in nucleic acids synthesis and antimetabolites. Antimicrobials acting at the cell wall level are the most selective, being bactericidal and presenting a high therapeutic index. The inhibition of peptidoglycan synthesis also leads to cell lysis [22]. Therefore, it is reported that the new inhibitors for bacterial cell wall biosynthesis are currently an attractive anti-bacterial target [23]. Biosynthesis of the cell wall consists of three stages which are the cytoplasmic stage (Stage I), the membrane-associated stage (Stage II) and the exocytoplasmic stage (Stage III) [24]. In each stage, the protein targets involving that stage were reported [25 – 29]. The enzyme targets in stage I involve in cytoplasm. Firstly, UDP-N-acetyl glucosamine (UDP-GlcNAc) is synthesized from the glycolytic intermediate D-fructose-6-phosphate (fructose-6P) and catalyzed sequentially

by glucosamine-6-phosphate synthase (GlmS), phosphoglucosamine mutase (GlmM) and the bi-functional enzyme glucosamine-1-phosphate acetyltransferase/ N-acetylglucosamine-1-phosphate uridyltransferase (GlmU). Then, UDP-GlcNAc produced an initial precursor, UDP-N-acetylmuramic acid (UDP-MurNAc). UDP-MurNAc is catalyzed by MurA (UDP-*N*-acetyl-glucosamine-enolpyruvyltransferase), MurB (UDP-*N*-acetyl enolpyruvyl glucosamine reductase), MurC (UDP-N-acetylmuramoyl:L-alanine ligase), MurD (UDP-N-acetylmuramoyl-L-alanine:D-glutamate ligase), MurE (UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--L-lysine ligase**)** and MurF (UDP-N-acetylmuramoyl-tripeptide-D-alanyl-D-ananine ligase), respectively. The last two step in Stage I is the synthesis of D-Ala residues by Alr (alanine racemase) and Ddl (D-Ala-D-Ala ligase), an enzyme that catalyzes the D-ala-D-ala dipeptide formation in UDP-MurNAc pentapeptide. MurI (Glutamate Racemase) is also found to be essential in *E. coli* for the production of D-Glu during the MurD step. In stage II, the mechanism consists of MraY (phospho-N-acetylmuramoyl-pentapeptide translocase) transferred UDP-MurNAc pentapeptide to an undecaprenyl pyrophosphate and MurG (UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase) added the GlcNAc to produce GlcNAc-MurNAc(pentapeptide) phosphoryl undecaprenol (lipid II). Next, stage III involves in both transglycosylase and transpeptidases. Transglycosylase catalyzes the formation of the linear peptidoglycan chains and these are crosslinked by transpeptidases which consist of an essential family of enzymes, known as penicillin-binding proteins (PBPs).

PBPs are classified by mass into high-molecular-weight PBPs, which are transpeptidases that form peptidoglycan cross-links, and low-molecular-weight PBPs, which are typically hydrolases [30]. The high-molecular mass PBPs are further divided into Class A and Class B PBPs. The low-molecular mass PBPs, sometimes called Class C PBPs. The one exception is *S. aureus* PBP4, low-molecular-weight transpeptidase but acts as cross-links peptidoglycan activities [31].

The enzymes involving in this stage are PBPs including PBPa, PBP2a PBP2x and PBP4 [32], which enzymes involved in the assembly of the bacterial cell wall, are beta-lactamase, serine-β-lactamase [33], metallo-β-lactamase [34], D-alanyl-D-lactate ligase (VanA) [35], Putative multidrug export ATP-binding/permease protein SAV1866 (SAV1866) [36], peptidoglycan transglycosylase and D-Ala-D-Ala dipeptide (VanX) [28].

In this work, the compounds from *S. roxburghii*, found in Khon Kaen province, northern part of Thailand is isolated. Then, the antimicrobial properties of extracted compounds are investigated in order to propose the new activity of the compounds from the Genus Shorea. In addition, the selected extracted compound is identified for the protein targets in the cell-wall target by using molecular docking technique.

2. MATERIALS AND METHODS

2.1 Chemicals and Equipments

All solvents such as ethanol, methanol, acetone, ethyl acetate, chloroform, were analytical grade and were purchased from Nanjing Chemical Reagent Co., Ltd., Nanjing. Acetone and chloroform deuteriated solvents for NMR measurement were purchased from Merck, Inc. Fourier transform NMR spectra were recorded at 500 MHz for ^{1}H and at 125 MHz for ^{13}C on a Bruker DRX-500 NMR spectrometer with tetramethylsilane (TMS) or solvent signals as internal references, at 25°C . The semipreparative HPLC was performed using a 250 x 10mm, 5 μm, Hypersil ODS column (Thermo Fisher Scientific, USA) on a Hitachi HPLC system consisting of a L-7110 pump (Hitachi) with a L-7420 UV/Vis Detector (Hitachi). Analytical TLC was performed on GF254 (10-20 mm) plates with 0.2 mm layer thickness. Column chromatography, Sephadex LH-20 was purchased by Pharmacia Biotech, Uppsala, Sweden and Silica gel (200-300 mesh) was produced by Qingdao Marine Chemical Factory,

Qingdao, People's Republic of China. The Jasco J-815 CD spectrometer was applied to obtain the CD spectra of diptoindonesin D.

2.2 Extraction and Isolation of Dipterocarpaceous Barks

The stem-bark of *S. roxburghii* was collected from the Sam Sung District, Khon Kaen Province, Thailand. The stem-bark was separated and then left air-dried for 2 weeks and cut into small pieces before being ground into powder. To preparation of crude ethanolic extract, Ground samples were macerated in ethanol for 7 days. The slurry was filtered and the obtained ethanolic extracts were dried with a rotary evaporator under reduced pressure at 40 °C. The dried extracts were tested for biological activities in order to select bioactive extracts for further isolation. One kilogram of dried powdered bark was macerated in ethanol (2.5 L) for 7 days. The slurry was filtered and the obtained ethanolic extract was dried with a rotary evaporator under reduced pressure at 40 °C. The dried extract (98 g) was prepared by grinding and dissolved in methanol, mixed with silica gel (118 g), then dried in hot air oven at 60 °C for 1 day. The sample was separated using wet column chromatographic technique. The column was packed with 1 kg silica gel (200-300 mesh) and was eluted with gradient mixtures of $CHCl_3$ and MeOH (100:0 to 0:100), to give 8 major fractions (A – H). Fraction D was subjected to sephadex column chromatography by elution with $CHCl_3$ and MeOH (1:1). Using the same methodology, fraction F1 – F6 obtained. Fraction F4 was subjected to RP-18 HPLC-UV using MeOH/H_2O solution (50:50). Fraction E was isolated compounds using reverse phase column chromatography, eluted with MeOH/H_2O solution (40:60). Fraction G was isolated compounds using sephadex column chromatography, eluted with 100% MeOH. The obtained were left for 2-3 day at 25 °C to allow crystallization.

2.3 Antimicrobial Assay

Antimicrobial activity was evaluated against seven bacteria strains: three Gram-negative, four Gram-positives and two fungal strains with reference drugs. Gram-negative bacteria used were (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimunium*), Gram-positive (*Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Bacillus cereus*), and fungal strains (*Candida albicans and Cryptococcus albidus*) were used in this study. The antimicrobial activities were determined by the minimum inhibitory concentration (MIC) in accordance with NCCLS guideline M38-P and M7-A6 for testing of conidium-forming filamentous fungi and bacteria respectively [37]. Pre-cultures of the tested bacteria were made by inoculating 25 mL of Nutrient broth (NB) and incubating for 18-24 h at 37 °C. The tested conidium-forming filamentous fungi, *C. albicans* and *C. albidus*, were made by grown on Potato dextrose agar (PDA) or Sabouraud dextrose agar (SDA) for more than three days at 28 °C. The colonies were harvested, suspended in sterile saline, and adjusted to concentration that yielded an absorbance similar to that of a 0.5 McFarland standard, the equivalence of 1-2 x 10^8 cfu/mL. Then the samples were further diluted 1 : 10,000 in NB or SDAto 1 x 10^4 cfu/mL For the test from a stock solution of the compounds of 4 mg/mL in DMSO, 10 μL tested solution was pipette into the wells in column 2 in 96-well plate. Using the multipipettes, 100 μL medium with microorganism were dispensed into wells from column 3 to column 11. Another 200 μL broth bacteria dilution was dispensed into column 2, then being mixed up and down 6-8 times. The 100 μL mixtures from column 2 and add this to column 3 were withdrawn. This made column 3 a two-fold dilution of column 2. The procedure down to column 11 was repeated. Then, the 100 μL from column 11 was discarded rather than putting them in column 12. The compounds were diluted two fold concentration from 200 μg/mL to 0.39 μg/mL, from column 2 to column 11. The 100 μL of sterile medium

into column 1, and medium with microorganism was put into column 12 as control groups. After incubating another 24 h for bacteria or 48-72 h for fungi, the lowest concentration of compounds that inhibited the visible growth of organism was considered as MIC_{80}.

2.4 ECD Calculations and Molecular Docking

The possible conformation of R- and S- configuration of diptoindonesin D were built and then optimized at M062X/6-31g(d) level of theory using Gaussian09 (http://www.gaussian.com). The frequency calculations and PCM model with methanol as solvent were also applied. From all conformations in each configuration, the conformations showing the relative free energy within 5 kcal/mol including the Boltzmann weight were considered for the ECD calculations. The ECD spectra were calculated to the selected conformation using TD-CAM-B3LYP/6-311++G(d,p) method including PCM model with methanol in Gaussian09. The Boltzmann weight was applied to generate the calculated ECD spectra and the similarity factor between experimental and calculated ECD spectra were determined to select the configuration of diptoindonesin D for using in the further docking step.

In order to identify the potential antibacterial cell wall protein target of the diptoindonesin D, molecular docking technique using GOLD v5.2 program [38] was applied. The antibacterial cell wall targets were selected and taken from the Protein Data Bank (https://www.rcsb.org). The available protein structures of the proteins in Stage I, Stage II and Stage III in the biosynthesis of cell wall are shown as follows. For stage I, the structures of GlmU (PDB code 4AA7), MurB (PDB code 2Q85), MurC (PDB code 2F00), MurD (PDB code 2Y66), MurF (PDB code 4CVL), Alr (PDB code 6G59), Ddl (PDB code 2I80) and MurI (PDB code 2VVT) are used. For stage II, the structures of MraY (PDB code 5JNQ) and MurG (PDB code 3S2U) are used. For stage III, the structures of PBP2a (PDB code 1MWU), PBP2x (PDB code 5OJ0), PBP4 (PDB code 5TY7), PBPa (PDB code 3UPO), Beta-lactamase (PDB code 1GHM), Serine β-lactamase (PDB code 2X71), Metallo β-lactamase (PDB code 6F2N), VanA (PDB code 1E4E), Sav1866 (PDB code 2ONJ) and peptidoglycan transglycosylase (PDB code 3HZS) are used.

In the docking calculation, the radius of the binding site was set to 10 Å. The GoldScore, Chemscore, ChemPLP, and ASP score fitness functions were applied. GoldScore is a force field-based scoring function. Chemscore and ChemPLP are empirical scoring functions and ASP is based on knowledge-based scoring function.

The ga run was set to 100 and the other parameters were set to default.The selected conformation was based on the highest fitness score (the lowest-energy) representing the most favorable orientation. The inhibitors complexed in each complex structure were also redocked back to the protein for comparing the GoldScore. The protein structure showing the fitness score of diptoindonesin D higher than that of its inhibitor from the PDB was selected for insight investigation of the binding.

3. RESULTS AND DISCUSSION

3.1 Isolation of Resveratrol from Thai Dipterocapaceous Plant

The ethanolic extract was separated by using column chromatography and repeated purification by sephadex LH-20, reverse phase column chromatography and RP-18 HPLC-UV. Five known resveratrol oligomers were isolated and their structures were compared with literatures and were identified as diptoindonesin D (12.51 mg) (**1**), hopeafuran (9.22 mg) (**2**), α-viniferan (6.07 mg) (**3**), hopeahainol A (5.20 mg) (**4**) and hopeahainol C (5.97 mg) (**5**), as shown in Figure 1, by spectral analysis to determine by comparison of H-NMR data with reported values [37, 39]. These compounds have not been reported from Dipterocarpaceous plants in Thailand. These compounds possessed biological activities including α-viniferan inhibited

murine leukemia P-388 cells. It was reported that hopeafuran showed anti-hyperlipidemic effects of the constituents on pancreatic lipase activity (IC_{50} of 26.6 μM), although their inhibitory activities were considerably less than that of a lipase inhibitor, orlistat (IC_{50} of 56 nM) [9]. For α-viniferin which is an oligostilbene of trimeric resveratrol from *Dipterocarpus verrucosus*, it possessed moderate antibacterial activity against diverse bacteria and antioxidant activity and exhibited high phenolic content [40]. For hopeahainol A, it showed an acetylcholinesterase (AChE) inhibitory with IC_{50} values of 11.28 μM [11]. Hopeahainol C exhibited potent antioxidant and radical scavenger capacity activities have been reported [9 – 10, 41].

Previously literatures also reported that the diptoindonesin D, a new modified stilbene dimer, was isolated for the first time from the acetone extract of the tree bark of *Hopea dryobalanoides* Miq., a species endemic to Indonesia. The diptoindonesin D showed moderately cytotoxic properties against murine leukemia P-388 cells [42]. In this work, antimicrobial activity of diptoindonesin D is focused and its configuration is also examined by using Circular Dichroism.

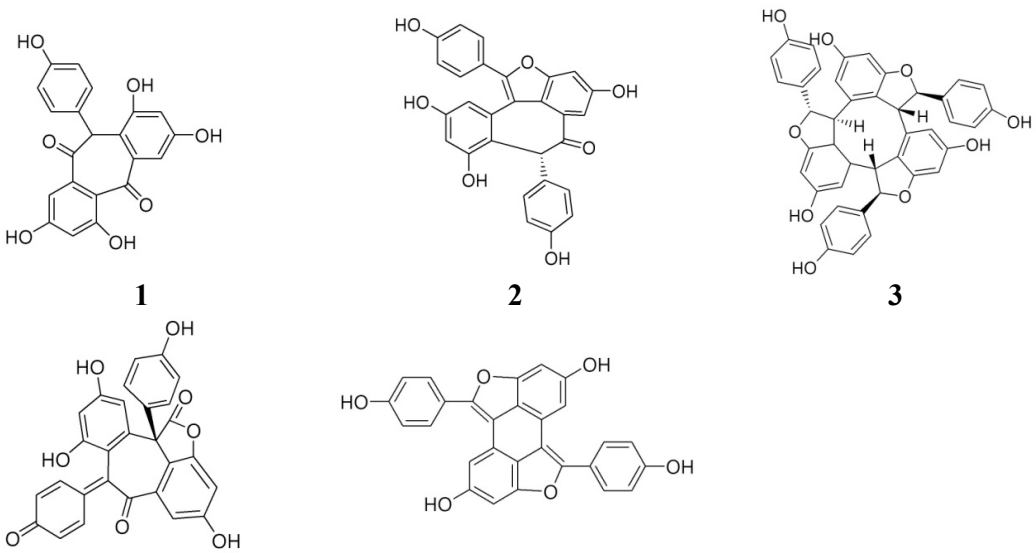

Figure 1 Structures of isolated compounds 1-5 from *S. roxburghii*.

3.2 Antimicrobial Activity

The MIC values against Gram-negative bacteria, Gram-positive bacteria and fungal strains of the diptoindonesin D against these bacterial are presented in Table 1. The known antibiotics (oxacillin, gentamicin, ciprofloxacin and amprotericin) were also tested. Mbaveng et al. classified the level of antimicrobial activity based on the MIC value as follows [43]. The significant activity is defined for Minimum inhibitory concentration (MIC) less than 10 μg/mL. Moderate and low activities are defined for 10 μg/mL < MIC < 100 μg/mL and MIC > 100 μg/mL, respectively. In this study, the results of the MIC determinations displayed noticeable MIC values of diptoindonesin D against all of the tested microbial species. The diptoindonesin D possessed significant antibacterial activity against Gram-positive bacteria, which are *S. epidermidis* (4.68 μg/mL), *S. aureus* (9.37 μg/mL) and *B. cereus* (18.75 μg/mL) but it revealed the low activity to *E. faecalis* (>200 μg/mL). However, the diptoindonesin D showed very low activity

against all of tested Gram-negative bacteria and two fungal strains. According to the experimental data the results indicate the potent activity of the diptoindonesin D against Gram-positive bacteria. Therefore, this study will focus on the binding of the diptoindonesin D to bacterial cell wall protein targets in order to propose the target of diptoindonesin D.

Table 1 MIC values (μg/mL) of diptoindonesin D and reference drugs against Gram-negative bacteria, Gram-positive bacteria and fungal strains.

Compounds	Minimum inhibitory concentration (μg/mL)								
	Gram-negative			Gram-positive				Fungus	
	E. coli	*Ps. Aeruginosa*	*S. typhimunium*	*S. aureus*	*S. epidermidis*	*E. faecalis*	*B. cereus*	*C. albicans*	*C. albidus*
Diptoindonesin D	100	200	>200	9.37	4.68	>200	18.75	200	200
Oxacillin	-	-	-	1.17	0.78	-	25	-	-
Gentamicin	1.56	1.56	0.78	-	-	12.5	0.39	-	-
Ciprofloxacin	2.34	-	0.39	-	-	1.56	0.39	-	-
Amprotericin B	-	-	-	-	-	-	-	0.78	0.78

3.3 Identification of Diptoindonesin D Configuration

From the geometrical optimization, the structures with the intramolecular H-bond between hydroxyl group and carbonyl group revealed the less stable structure with the relative energy about 10 kcal/mol. Two up- and down- puckered conformations of seven membered ring with the intramolecular H-bond between hydroxyl group and carbonyl group showed the comparable energy with the relative energy less than 2 kcal/mol. Therefore, these two conformations of each configuration were selected and performed ECD calculations. Their optimized structures are shown in Figure 2. From the Boltzmann population in R-configurations obtaining from the frequency calculations, conf1 and conf2 are about 90.29% and 9.71%, respectively. For S-configuration, the Boltzmann populations of conf1 and conf2 are about 11.17% and 88.83%, respectively. The UV and ECD spectra including Boltzmann weights were studied using SpecDisc program [44]. From the validation of the UV spectra, the highest best similarity factor between experimental and calculated UV spectra are 0.9342 and 0.9334 for the R- and S- configuration, respectively, with the shift of -11 nm. Therefore, the shift value of ±15 nm was set in order to determine the similarity factor of ECD spectra. The highest similarity factor of ECD spectra are found with the value of 0.8086 and 0.0793 for the R- and S- configuration, respectively, with the shift of -11 nm. The results indicated that the configuration of diptoindonesin D should be R-configuration. Therefore, two conformations of R-configuration were used for the molecular docking step.

3.4 Identification of Possible Antibacterial Target for Diptoindonesin D by Reverse Docking

In order to understand the bacterial cell wall protein targets for R-conf1 and R-conf2, reverse docking studies were performed to identify possible bacterial cell wall protein targets and explain the binding interaction of diptoindonesin D. The fitness scores (GOLD Score, Chemscore, ChemPLP, ASP score) are used for identifying the possible protein target. The fitness scores of the inhibitor in each complex structure are also compared. The docking score results of the inhibitors and

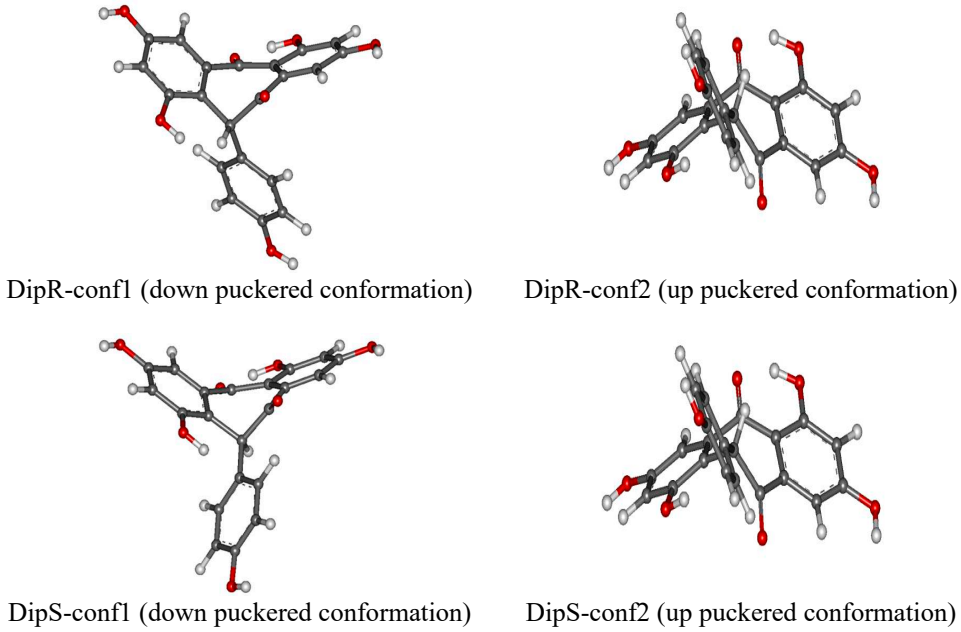

Figure 2 Structures of the selected optimized conformation of R- and S- configurations of diptoindonesin D.

both R-configuration of diptoindonesin D are presented in Table 2.

From the structures in stage I, both R-configurations show the lower docking scores of all scoring functions as compared to those of its inhibitor in almost of the protein target. However, in the GlmU structure, the ChemPLP and ASP scores of diptoindonesin D show the higher scores than those of its inhibitor. The ASP scores of MurB and Alr and the Chemscores of MurC and MurI also show the higher fitness score than its inhibitor.

From the docking of the structures in stage II, only Chemscore of diptoindonesin D in the structures of MurG and MraY reveal the higher fitness scores than that of its inhibitor.

In case of the structures in stage III, four target proteins, i.e. PBP4, PBPa, beta-lactamase and serine-β-lactamase, achieve the better fitness scores of diptoindonesin D in three scoring functions, except GOLDScore, than the fitness scores of its inhibitor. Three target proteins of metallo-β-lactamase, VanA and SAV1866 show the higher fitness scores of diptoindonesin D in some scoring functions. It is also found that, the GOLDScores in metallo-β-lactamase and VanA, ASP score in metallo-β-lactamase, and Chemscore of SAV1866 also exhibit the higher binding score of both conformation than its inhibitor. While focusing the other two target proteins, PBP2x and peptidoglycan transglycosylase, none of scoring functions produced the better fitness score of diptoindonesin D than its inhibitor. PBP2a possesses the highest fitness scores of diptoindonesin D in all scoring functions as compared to its inhibitor. The results can be indicated that PBP2a might be the possible protein target of diptoindonesin D.

For the PBP2a protein, the docking scores of both conformation reveal the better scores than methicillin in GOLDScore, Chemscore, ChemPLP and ASP score with the values of 62.50, 22.86, 65.66, and 33.84 for R-conf1 and 69.15, 29.35, 70.13, and 37.53 for R-conf2, respectively. R-conf2 also exhibits the higher scores than its inhibitor and

Table 2 Fitness scores from GOLD docking.

Target	GOLDScore			Chemscore			ChemPLP			ASP score		
	Inhibitor	R-conf1	R-conf2	Inhibitor	R-conf1	R-conf2	Inhibitor	R-conf1	R-conf2	Inhibitor	R-conf1	R-conf2
Stage I: the cytoplasmic stage												
GlmU	45.51	42.50	41.29	19.07	17.50	13.98	39.64	45.49	43.05	19.08	21.54	26.12
MurB	65.99	63.10	58.09	34.01	24.39	25.04	67.01	61.39	58.23	34.74	40.00	45.11
MurC	121.91	58.84	50.56	26.71	23.96	27.12	125.19	56.94	57.58	63.60	33.74	30.36
MurD	82.21	54.35	57.36	37.65	24.53	35.86	87.93	56.95	65.08	40.46	35.41	35.43
MurF	114.41	50.36	58.15	38.92	27.89	30.41	142.68	56.65	56.01	56.91	27.31	30.01
Alr	79.60	60.43	53.34	28.53	21.41	26.43	62.43	56.47	58.79	34.42	38.26	39.23
Ddl	54.08	45.82	45.90	28.70	20.38	17.03	74.88	47.53	45.85	35.62	23.86	24.32
MurI	71.48	49.34	49.57	26.89	25.47	32.39	86.14	58.30	60.15	44.14	31.04	37.14
State II: the membrane-associated stage												
MraY	72.07	56.56	65.40	10.62	24.96	22.91	83.14	64.34	70.76	46.45	33.96	36.38
MurG	117.87	45.74	57.95	17.98	16.59	28.80	95.14	51.74	73.22	62.72	24.30	29.84
Sta te III: the extractytoplasmic stage												
PBP2a	62.06	62.50	69.15	19.73	22.86	29.35	55.87	65.66	70.13	32.36	33.84	37.53
PBP2x	72.92	60.67	61.12	31.56	26.64	28.60	76.26	61.67	63.46	40.31	36.25	37.00
PBP4	62.43	58.53	54.56	21.99	25.15	23.52	56.00	58.25	65.20	28.67	34.17	33.69
PBPa	58.18	54.09	58.84	22.56	23.71	27.81	64.11	55.84	64.40	26.93	31.88	34.09
Beta-lactamase	58.66	55.98	56.25	24.51	26.69	26.65	60.79	67.70	63.58	28.32	36.78	34.59
Serine β-lactamase	62.71	48.16	53.62	22.10	28.31	27.35	64.53	67.22	59.99	31.26	33.08	31.89
Metallo β-lactamase	68.50	72.30	69.18	35.43	29.20	27.95	87.53	68.87	62.93	33.27	38.60	35.90
VanA	62.66	39.21	78.29	41.16	19.75	19.34	90.87	33.89	39.96	49.83	22.77	25.43
Sav1866	106.82	48.24	46.57	12.93	20.32	23.33	63.57	47.97	52.88	51.76	30.51	31.56
Peptidoglycan transglycosylase	64.63	54.31	51.79	0.78	27.03	25.25	114.29	63.30	61.27	57.91	34.06	31.22

R-conf1 in all fitness scores. The results from these scoring functions indicated that are diptoindonesin D might be the candidate to be the inhibitor of the PBP2a protein. Therefore, PBP2a is expected to be an attractive target of diptoindonesin D and is selected for the further analysis of the binding interaction within the binding site of PBP2a. The binding orientation of diptoindonesin D from the molecular docking are shown in Figure 3. In Figure 3A, the docked orientations of R-conf1 obtaining from GOLDScore and Chemscore are located in the similar location, where as those from ChemPLP and ASP score are located in the different orientation. For the docked orientations of R-conf2, all docked orientations are found in the similar location are shown in Figure 3B.

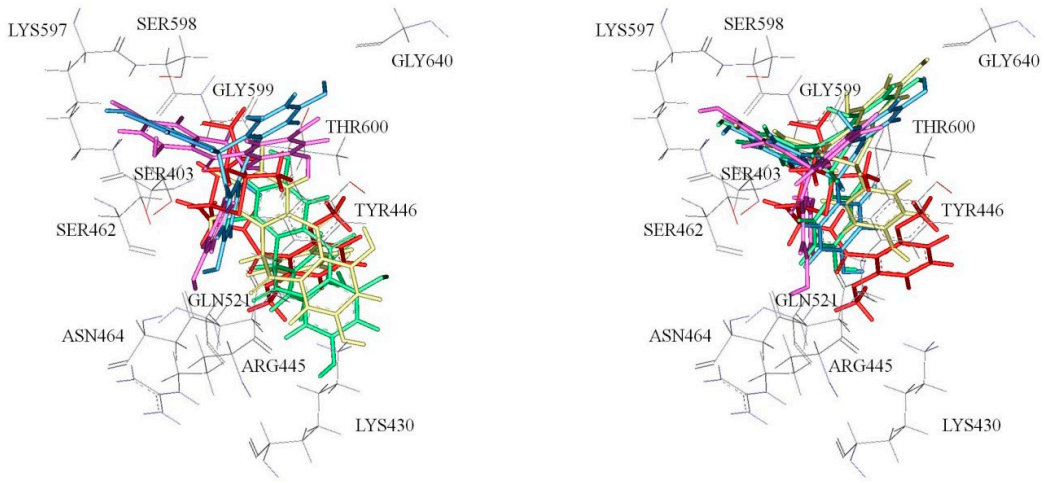

Figure 3 Superimposition of docked R-conf1 (A) and R-conf2 (B) diptoindonesin D in PBP2a as compared with methicillin (Red). Docked orientations of GOLDScore (Pink), Chemscore (Blue), ChemPLP (Green), ASP score (Yellow) are shown in stick model.

Insight analysis of the binding interaction will be shown in the next part.

3.5 Binding Interaction Between R-diptoindonesin D in PBP2a

The hydrogen bonding analysis between diptoindonesin D and the amino acids in the PBP2a binding pocket are shown in Table 3. The binding interactions of all scoring functions of the R-conf 1 in the PBP2a binding pocket are shown in Figures 4-5. From the crystal structure, methicillin, which is covalently formed the bond to SER403, revealed the H-bond to SER403, ASN464, SER598, GLY599, THR600, and ALA601. The docked R-conf1 and R-conf2 also show the similar H-bond interactions as found in the binding of methicillin. According to the explanation in the previous part, docked R-conf1 orientations obtained from four scoring functions reveal two possible locations in the binding pocket.

Table 3 H-bond interactions between R-diptoindonesin D and amino acids in the PBP2a protein.

Structure	H-bond interaction			
	GOLDScore	Chemscore	ChemPLP	ASP score
R-conf1	SER461, TYR446, ASN464, THR582, LYS597, SER598. GLY599	TYR446, SER462, ASN464, LYS597, SER598, THR600, GLY640	LYS430, ARG445, TYR446	ARG445, TYR446, ASN464
R-conf2	SER403, GLU460, SER462, LYS597, SER598, GLY599, THR600	GLU460, ASN464, THR600, GLY640	GLU460, ASN464, THR600, GLY640	GLU460, SER462, SER598, GLY599

The H-bond interactions between R-conf1 in both locations and the amino acids in the PBP2a protein are found to form some H-bonds to the similar amino acids. All orientations of docked R-conf1 show the strong H-bonds between hydroxyl group and amino group of TYR446. The H-bonds to ASN464 are also found in three orientations, except the orientation from ChemPLP. In case of docked R-conf2, it is found that the scores of R-conf2 are higher than those of R-conf1 indicating the tighter binding to the PBP2a. More H-bond interactions to the amino acids in the binding pocket are also found as compared the H-bond found in docked R-conf1. Both conformations of docked R- diptoindonesin D revealed the possible H-bonds to ASN464, SER598, GLY599 and THR600 which are similar to the H-bond between methicillin and PBP2a. The other H-bonds are also found with LYS430, ARG445, GLU460, SER461, SER462, TYR446, THR582, LYS597, and GLY640. The results may indicate the possibility to be the PBP2a inhibitor of. R- diptoindonesin D.

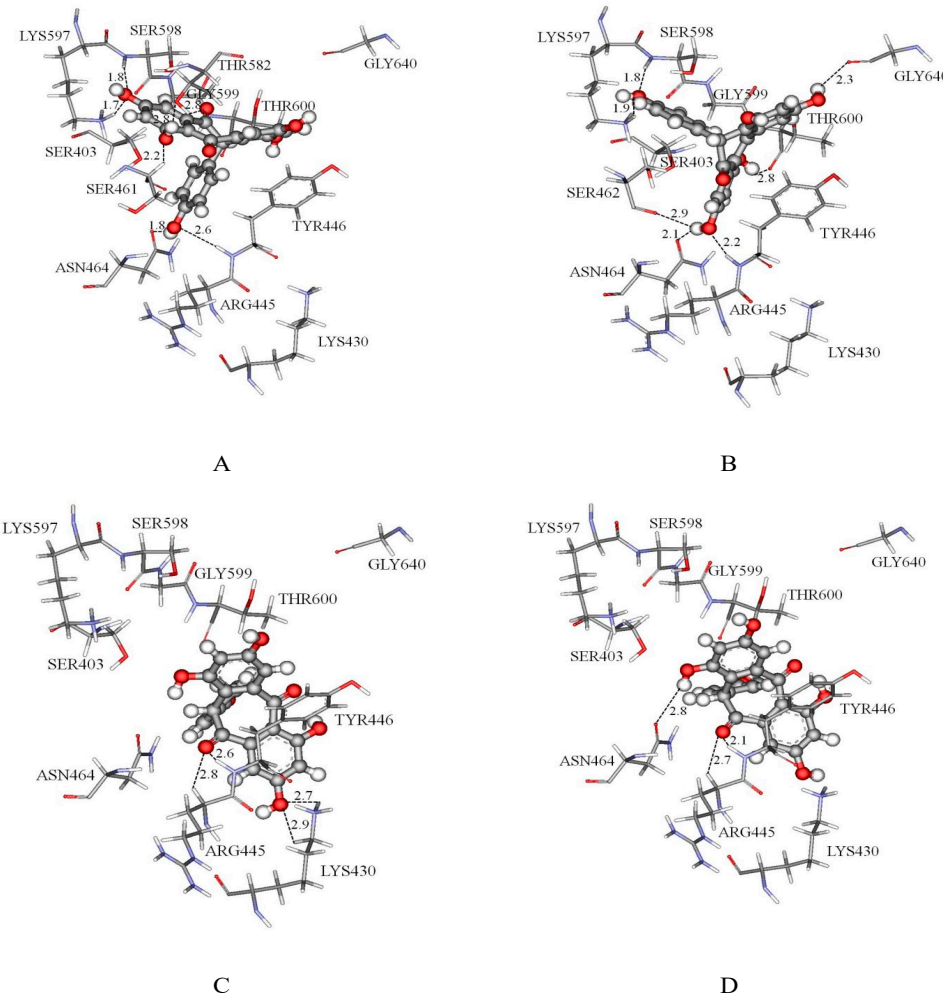

Figure 4 Binding interaction of docked R-conf1 (ball-and-stick model) in the PBP2a's binding pocket obtaining from GOLDScore (A), Chemscore (B), ChemPLP(C), and ASP score (D), as shown in ball-and-stick model. Distances are shown in Angstrom (Å).

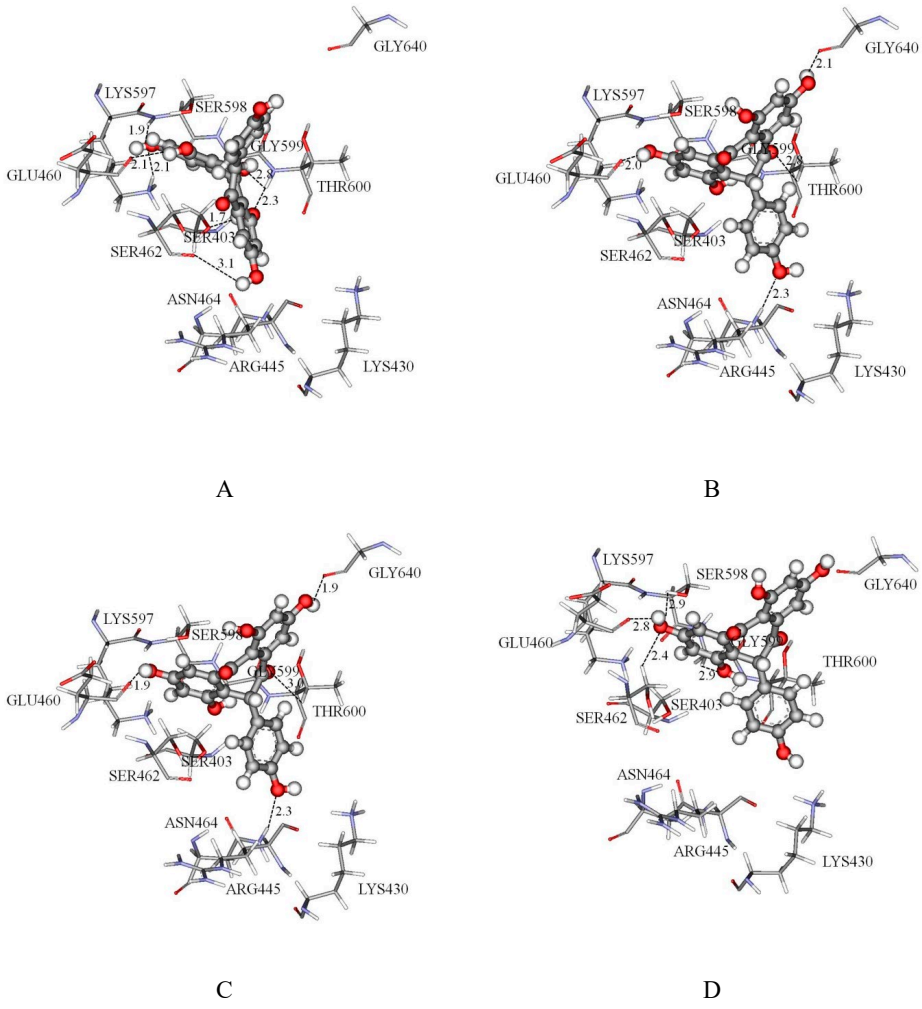

Figure 5 Binding interaction of docked R-conf2 (ball-and-stick model) in the PBP2a's binding pocket obtaining from GOLDScore (A), Chemscore (B), ChemPLP(C), and ASP score (D), as shown in ball-and-stick model. Distances are shown in Angstrom (Å).

4. CONCLUSIONS

Five known resveratrol oligomers, which are diptoindonesin D, hopeafuran, α-viniferan, hopeahainol A and hopeahainol C, were isolated from stem bark of *S. roxburghii*. Moreover, we also reported the first time of the potent antimicrobial activity of diptoindonesin D against Gram-positive bacteria especially *S. epidermidis* and *S. aureus* with MIC values 4.68 and 9.37 µg/mL, respectively. To understand the effect of strong antibacterial activity, molecular modelling techniques, i.e. quantum chemical calculations and molecular docking, were used to identify the configuration of diptoindonesin D and propose the possible protein targets of diptoindonesin D. Two conformations (up- and down- puckered conformation) were found. The ECD results also indicated that the possible configuration of diptoindonesin D should be R-configuration. Then, molecular docking was applied. In this study, enzymes in cell wall and protein synthesis targets were first investigated in order to understand how the diptoindonesin

D interact with microbial enzyme targets and explain the inhibition of diptoindonesin D with these enzymes. The docking results proposed that PBP2a protein might be the possible target for the binding against Gram-positive bacteria. The overall results of this investigation lead to the bioactive compound which can be candidate for improving effective inhibition with increasing attention against Gram-positive bacteria indicating its usefulness as antibacterial agent.

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