

Pharmacophore Modelling of Phytochemicals from *Clinacanthus Nutans* for Antimicrobial Activity

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ABSTRACT Pharmacophore modelling is incorporated as a part of approach in computer-aided drug design (CADD) which assists high-throughput virtual screening and drug design. It offers information on protein-ligand interaction as well as pharmacophore features responsible for interaction with the protein target. Current studies of phytochemicals isolated from *Clinacanthus nutans* Lindau locally known as *belalai gajah* are rich of flavonoids and phenolic compounds which able to induce antimicrobial activity. However, essential information of compounds activity against target protein is still inadequate. Hexane, ethyl acetate and methanol fractions of *C. nutans* showed no inhibition zone against Gram-negative bacterial strain namely *Escherichia coli* and Gram-positive bacterial strains namely *Staphylococcus aureus* and *Salmonella typhimurium*. Pharmacophore model generated from selected broad-spectrum antibiotics (cefixime, ampicillin, amoxicillin and ciprofloxacin) revealed features of hydrogen bond donor and acceptor, hydrophobic and aromatic ring. Six C-glycosyl flavones reported from literature have more similar features and higher fit-value to generated pharmacophore model. We recommend further study to determine minimum inhibitory concentration of *C. nutans* using various solvent extracts to justify claim of antimicrobial activity of this plant. Synergic effect and concentration of bioactive flavonoids against antimicrobial activity also need to be considered in future studies.

KEYWORDS: Pharmacophore modelling; *Clinacanthus nutans*; antimicrobial; computer-aided drug design; ligand-based.

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INTRODUCTION

Pharmacophore modelling emerged as an essential approach in computer-aided drug design process which assists in drug discovery over these years. This approach is useful to conduct virtual screening on potential compounds to predict their biological activity. Two types of pharmacophore modelling are ligand-based virtual screening (LBVS) and structure-based virtual screening. LBVS is incorporated into this research to evaluate active features of test compounds against generated pharmacophore model (Ferreira *et al.*, 2015). Generally, ligand-based approach is based on hypothesis that similar compound structures have more probability to exert similar bioactivity (Taboureau *et al.*, 2012). As more compounds are identified from various plants, virtual screening acts as a fast and cost-effective method to evaluate biological activity of reported compounds in supporting the biological assay report.

Clinacanthus nutans Lindau is one of the plants which known well for its medicinal benefits. It is locally known as Sabah snake grass or *belalai gajah*. It is native to countries of South East Asia, particularly in Malaysia and Thailand. It is traditionally used to treat oxidative stress-related diseases, fever, skin rashes, snake bites, and skin lesion caused by herpes simplex virus and varicella-zoster virus (P'ng *et al.*, 2012; Siang *et al.*, 2013). Much attention has been given to *C. nutans* especially from Thailand as most studies reported the anti-oxidative, anti-inflammatory, anti-viral

and analgesic effect but few on antimicrobial properties (Siang *et al.*, 2013). Thus, this research aims to screen antimicrobial activity of *C. nutans* fractions against Gram-negative and gram-positive bacteria assisted by ligand-based pharmacophore modelling as virtual screening.

The antimicrobial screening was done by Kirby-Bauer disc diffusion method based on the published protocol (Ghatage *et al.*, 2014). Gram-negative bacteria used was *Escherichia coli* and Gram-positive bacteria were *Staphylococcus aureus* and *Salmonella typhimurium*. Hexane fraction, ethyl acetate fraction and methanol fractions of *C. nutans* extract were tested in antimicrobial assay. Ligand-based pharmacophore modelling was executed using LigandScout 4.10 OMEGA software. Training set comprised of 4 antibiotic drugs; cefixime, ampicillin, amoxicillin and ciprofloxacin. Test set comprised of 17 phytochemicals from *C. nutans* as reported in literatures were orientin, vitaxin, isoorientin, isovitaxin, isomollupentin 7-O- β -glucopyranoside, shaftoside, stigmaterol, cycloclinoside A1, clinacoside A, cycloclinoside A2, β -sitosterol, lupeol, entadamide A, 2-*cis*-entadamide A, clinamide A, clinamide B and clinamide C (Pittaya *et al.*, 2004; Tu *et al.*, 2014; Teshima *et al.*, 1998).

METHODOLOGY

Sample Collection and Extraction

C. nutans was collected from Kedah, Malaysia and identified by Plant Science Department, UNIMAS. The dried leaves were ground and macerated in methanol (MeOH) for three days. Solvent removal was done using rotary evaporator and crude extract were stored in cool temperature.

Fractionation

Solvent partition was carried out on the crude extract using solvent with increasing polarity starting with hexane, ethyl acetate (EtOAc) and MeOH. 50 ml of MeOH crude (10 grams) was partitioned with hexane (100 ml) and the fractions was separated accordingly. The MeOH fraction was again partitioned two times with hexane. The three hexane fractions were combined, concentrated and labelled as hexane fraction. The MeOH fraction was concentrated and added dropwise into EtOAc (200 ml) while stirring to yield EtOAc fraction and insoluble brown solid as MeOH fraction. All fractions were evaporated to dryness and dissolved with MeOH solvent in concentrated form for antimicrobial assay.

Disk Diffusion Method

This assay was conducted in laminar airflow hood to ensure sterile condition. About 100 μ L of pure culture of *E. coli*, *S. aureus* and *S. typhimurium* were pipetted into sterilized nutrient broth followed by incubation at 37 °C for 2 hours. After 2 hours, 1.0 mL of cultured broths were tested for its optical density (OD) at 600 nm by using UV/Vis spectrophotometer. The value was adjusted to 0.168 OD for standard used in antimicrobial assays. About 18.0 g of nutrient agar was dissolved in 500 mL of distilled water. It was stirred and autoclaved at 121 °C for 2 hours. Sterile agar was poured into petri dish and left to solidify at room temperature.

Each bacterial strain was swabbed using sterile cotton bud throughout the agar plates uniformly. Each concentrated fraction sample was loaded onto sterile 6.0 mm filter paper disc and placed gently onto the agar plate. Five replicates were done for each fraction of each strain. The plates were sealed with parafilm and incubated at 37 °C for 24 hours. Zone of inhibition was recorded after 24 hours.

Ligand-based Virtual Screening

Three-dimensional (3D) pharmacophore model was generated by using LigandScout 4.10 OMEGA software. Two-dimensional (2D) chemical structures of training set and test set were drawn using ChemDraw 12.0 software. They were saved as MDL Molfile. format to be imported to LigandScout. All the chemical structures were referred as ligands in LigandScout.

Pharmacophore Hypothesis Generation

The first step in pharmacophore model generation was minimizing the energy to create the conformational space for each ligand which represented the flexibility of the ligand in space. Each ligand was displayed as standard 3D pharmacophore structure. Next was to cluster ligands in order to align them followed by generation of pharmacophore model. The model was generated based on alignment and overlap of valid merged pharmacophore of training set ligands.

Pharmacophore Model Validation

Pharmacophore validation was used to identify every ligand that fitted into pharmacophore model and shared the common chemical features. Each pharmacophore of test set and training set was merged into the pharmacophore model thus their common chemical features were observed in the merged pharmacophore model. The chemical feature is referred as pharmacophore feature in LigandScout. The built-in pharmacophore features are hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), hydrophobic (H) and aromatic ring (AR).

RESULT AND DISCUSSION

Antimicrobial Activity

Hexane fraction, EtOAc fraction and MeOH fraction tested against *E. coli*, *S. aureus* and *S. typhimurium* with five replicates each. All samples showed no inhibition zone around 6mm disc. This is a qualitative test as no minimum inhibitory concentration (MIC) was determined because the exact concentration of sample adhered to disc was unknown. This method has been suggested suitable only for test of pure compounds (Ncubes *et al.*, 2008).

Ligand-Based Virtual Screening

The pharmacophore model was generated by superimposition and merging all pharmacophores as according to their respective flexible alignment. Four HBA domains were observed as red sphere, one HBD domain as green arrow, one H domain as yellow sphere and one AR domain as blue ring inside H domain. All these features represented the shared pharmacophore features of ligands. These features are likely to impart the antimicrobial activity of ligand through cellular binding interaction with bacteria. Ampicillin, ciprofloxacin, cefixime and amoxicillin are broad-spectrum antibiotics. The suggested pharmacophore model is displayed as Figure 1.

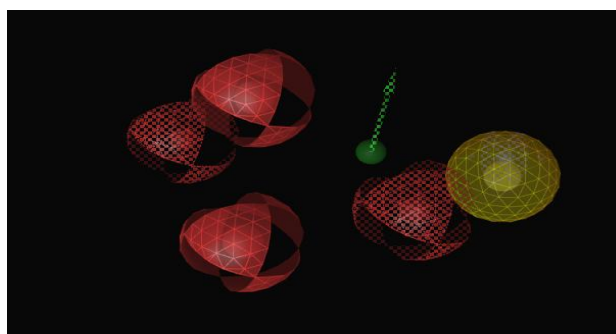


Figure 1. Pharmacophore model with seven pharmacophore domains of HBA, HBD, H and AR.

Further pharmacophore validation done on each pharmacophore showed not all but ampicillin, cefixime and orientin had all the common features with pharmacophore model. Ciprofloxacin ranked lower than 13 other ligand test sets as it had less features and lower fit-value. LigandScout showed the types of pharmacophore interactions for all ligands in 2D and 3D display. The 2D display shows the interaction in the form of arrow and ring highlighted with the colors corresponding to the 3D display of pharmacophore model. Figures 2-5 show the pharmacophore displays for training sets. Table 1 is the result summary of pharmacophore validation of all ligands.

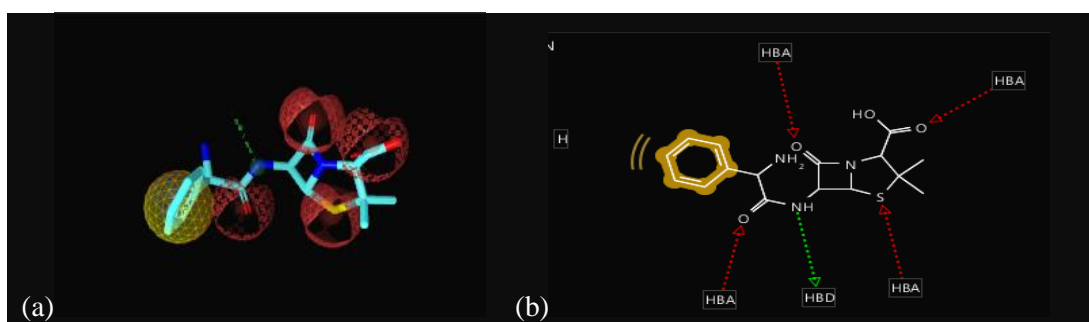


Figure 2. Pharmacophore model of ampicillin in (a) 3D and (b) 2D display.

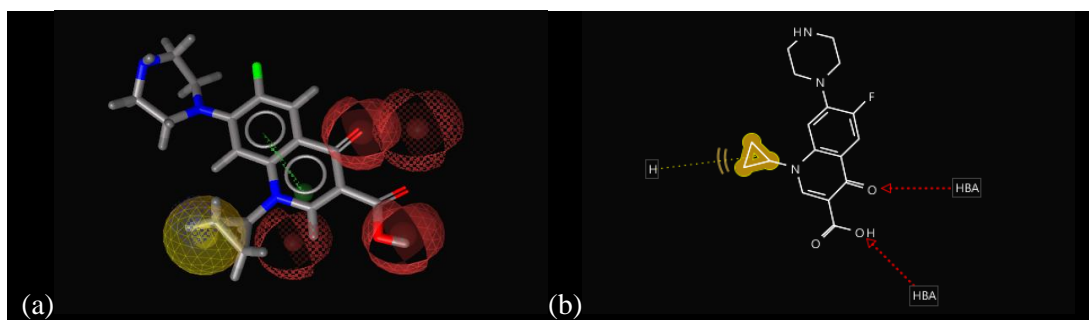


Figure 3. Pharmacophore model of ciprofloxacin (a) 3D and (b) 2D display.

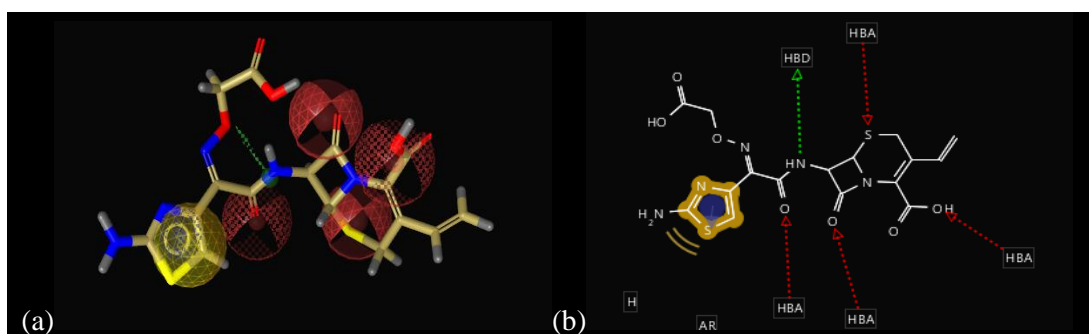


Figure 4. Pharmacophore model of cefixime in (a) 3D and (b) 2D display.

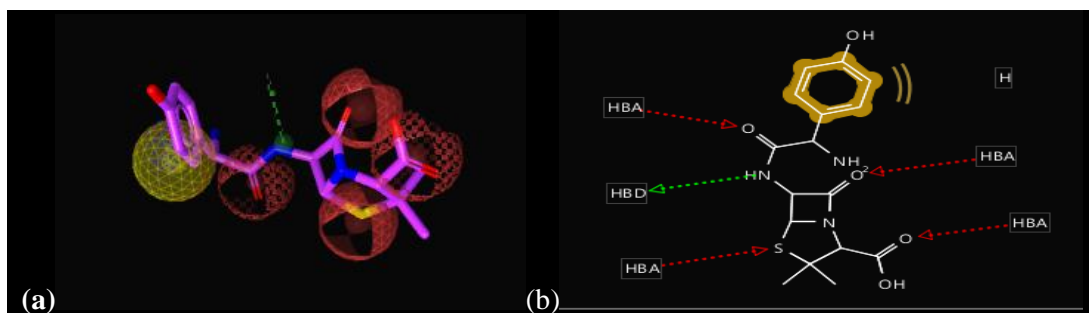


Figure 5: Pharmacophore model of amoxicillin in (a) 3D and (b) 2D display.

Ligand	Set type	AR	H	HBA	HBD	Fit-Value
Cefixime	training	1	1	4	1	65.12
Ampicillin	training	1	1	4	1	59.68
Orientin	test	1	1	4	1	59.36
Amoxicillin	training	-	1	4	1	58.99
Vitaxin	test	1	1	3	1	51.19
isoorientin	test	-	1	3	1	50.36
Isovitexin	test	-	1	3	1	48.70
Cycloclinoside A1	test	-	-	4	1	48.50
Cycloclinoside A2	test	-	-	4	1	48.50
Isomollupentin 7-O- β -glucopyranoside	test	-	1	3	-	44.35
Entadamide A	test	-	1	2	1	43.63
Shaftoside	test	-	1	3	-	43.39
2-cis-entadamide A	test	-	1	3	-	43.34
Clinacoside A	test	-	-	3	1	42.62
Clinamide A	test	-	1	2	1	42.09
Clinamide C	test	-	-	4	-	41.42
Ciprofloxacin	training	-	1	2	-	36.65
Clinamide B	test	-	-	3	-	34.92
β -sitosterol	test	-	1	1	1	31.49
Lupeol	test	-	1	1	1	31.49
Stigmasterol	test	-	1	3	-	31.12

Table 1. Summary of pharmacophore validation of all ligands.

β -sitosterol, lupeol and stigmasterol had less antimicrobial potential as they had small fit-value and less shared features. The six C-glycosyl flavones (orientin, vitaxin, isoorientin, isovitaxin, isomollupentin 7-O- β -glucopyranoside, shaftoside) showed fit-value close to cefixime and shared more features to the model. Flavanoids reported in *C. nutans* was also reported in other plants which function to induce antibacterial response. However the phytochemical content concentration affects the bioactivity of each plant. *M. candidum* which had higher quercetin, gallic acid and catechin level displayed stronger antimicrobial activity unlike *C. nutans* extract (Fai et al., 2013). Thus the synergic effect of phytochemicals especially of flavonoids in *C. nutans* needs to be considered for its antimicrobial properties. Also, previous researches reported the antimicrobial activity by petroleum ether extract and chloroform extract but unlikely for MeOH fraction. This is possibly due to various phytochemicals present in the less-polar fractions than in methanolic fraction. Although we reported no inhibition zone for all three fractions, the absence of antimicrobial properties of *C. nutans* cannot be concluded yet. Using serial dilution test, the MIC of *C. nutans* methanolic extract against *E. coli* and *S. aureus* was reported to be more than 50mg/ml (Fai et al., 2013). MIC of ethyl acetate extract against *E. coli* and *S. typhimurium* were both reported to be more than 100 mg/ml (Arupallan et al., 2014). This indicate possible antimicrobial activity at higher concentration considering as those reported in other literatures. Determination of MIC of *C. nutans* using various extract should be done as recommended in the current antimicrobial protocol to avoid much variation in assay (NCCLS, 2000).

CONCLUSION

Hexane, ethyl acetate and methanol fraction of *C. nutans* extract shows no zone of inhibition using Kirby-Bauer disk diffusion method. The ligand-based pharmacophore modelling shows all phytochemicals have shared pharmacophore features as model generated from selected broad-spectrum antibiotics (cefixime, ampicillin, ciprofloxacin and amoxicillin) but with different hit-values and number of shared pharmacophore features.

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