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ฤทธิ์การต้านมาลาเรียในหลอดทดลองและความเป็นพิษต่อเซลล์ของสารสกัดหยาบสมุนไพรไทย 20 ชนิดต่อเชื้อพลาสโมเดียม ฟาลซิพารัม สายพันธุ์ TM267

In Vitro Antimalarial Activity and Cytotoxity of 20 Ethanolic Crude Extracts from Thai Herbs Against *Plasmodium falciparum* TM267

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บทคัดย่อ:

วัตถุประสงค์: เพื่อศึกษาฤทธิ์การต้านการเจริญเติบโตต่อเชื้อ *Plasmodium falciparum* (*P. falciparum*) สายพันธุ์ TM267 ที่ดื้อต่อยา chloroquine ของสารสกัดที่สกัดโดยเอทานอลที่ได้จากพืชสมุนไพรไทย 20 ชนิดเพื่อเป็นข้อมูลเบื้องต้นในการพัฒนายาต้านมาลาเรีย และนำไปศึกษาการจำลองการจับกันเชิงโมเลกุลกับเอนไซม์เป้าหมาย *Plasmodium falciparum* dihydrofolate reductase (PfDHFR)

รับต้นฉบับวันที่ 12 ตุลาคม 2559 รับลงตีพิมพ์วันที่ 6 มกราคม 2560

วัสดุและวิธีการ: ศึกษาฤทธิ์การต้านการเจริญเติบโตของเชื้อมาลาเรีย *P. falciparum* TM267 ในหลอดทดลองโดยการตรวจวัด parasite lactate dehydrogenese และทดสอบความเป็นพิษต่อเซลล์โดยใช้ Vero cell ด้วยวิธี 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide หาค่า 50% inhibitory concentration (IC_{50}) และ 50% cytotoxicity concentration จากกราฟความสัมพันธ์ระหว่างปริมาณสารและการตอบสนองต่อสารนั้นๆ และนำไปวิเคราะห์การจำลองการจับกันเชิงโมเลกุลกับโครงสร้างคริสตอลเชิงซ้อนของเอนไซม์ PfDHFR-thymidylate synthase กับ pyrimethamine, nicotinamide adenine dinucleotide phosphate และ deoxyuridylate

ผลการศึกษา: จากสารสกัดทั้งหมดพบว่าสารสกัดจากรากเจตมูลเพลิงแดงมีฤทธิ์ต้านการเจริญเติบโตของมาลาเรียสูงที่สุด ให้ค่า IC_{50} เท่ากับ 3.7 ไมโครกรัมต่อมิลลิลิตร และมีพิษต่อ Vero cell น้อยที่สุด ตามด้วยสารสกัดจากเปลือกมะกรูด รากคนทีสอ ใบกะเพราแดง หัวกระเทียม และลำต้นกำแพงเจ็ดชั้น ตามลำดับ จากสารที่ออกฤทธิ์ทั้งหมด 7 ชนิดที่เคยมีรายงานว่าสามารถสกัดได้จากพืชที่กล่าวมานั้นพบว่ามีคะแนนสูงจากการทำนายการจับกันกับ PfDHFR ยิ่งไปกว่านั้นสาร Citrusoside C ที่ได้จากมะกรูดมีคะแนนในการทำนายการจับกันสูงสุด

สรุป: เจตมูลเพลิงแดง คนทีสอ และมะกรูด น่าจะมีสารที่มีความสามารถในการยับยั้งเชื้อมาลาเรียได้ด้วยการจับที่บริเวณเร่งปฏิกริยาของ PfDHFR นอกจากนี้สาร Citrusosides จากมะกรูดยังเป็นสารที่น่าสนใจเพื่อที่จะนำไปศึกษาเพิ่มเติมต่อไป

คำสำคัญ: มาลาเรีย, พลาสโมเดียม ฟาลซิพารัม, สมุนไพรไทย

Abstract:

Objective: To determine the antimalarial activity of ethanol crude extracts from 20 Thai herbs against *Plasmodium falciparum* (*P. falciparum*) chloroquine-resistant strain TM267. Molecular docking of the active compounds from the selected Thai herbs were analyzed with *Plasmodium falciparum* dihydrofolate reductase (PfDHFR).

Material and Method: An *in vitro* study of antimalarial activity against *P. falciparum* TM267 was done using a parasite lactate dehydrogenese assay, and the cytotoxic effects of extracts were tested against Vero cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The 50% inhibitory concentration (IC_{50}) and 50% cytotoxicity concentration were calculated from the dose-response curves. Molecular docking and post-docking were analyzed with the x-ray crystal structure of PfDHFR-thymidylate synthase complexed with pyrimethamine, nicotinamide adenine dinucleotide phosphate and deoxyuridylate.

Results: Of these, the *Plumbago indica* L. root extract showed high antimalarial activity, with an IC_{50} value of 3.7 µg/ml and less cytotoxicity when tested against Vero cells, followed by the *Citrus hystrix* DC. fruit extract, *Vitex trifolia* Linn. root extract, *Ocimum sanctum* L. leave extract, of *Allium sativum* L. bulb extract and *Salacia chinensis* L. stem extract, respectively. All 7 active compounds reported from these herbal extracts had high docking scores against PfDHFR. The Citrusoside C from *Citrus hystrix* DC. had the highest docking score.

Conclusion: It could be purposed that there were active compounds in *Plumbago indica* L., *Vitex trifolia* Linn. and *Citrus hystrix* DC. which are potential inhibitors against malaria that could bind to the active site of PfDHFR. However, the active Citrusosides from *Citrus hystrix* DC. should be further investigated for their effectiveness against malaria.

Keywords: malaria, *P. falciparum*, Thai herbs

Introduction

Malaria is one of the most common and important parasitic diseases. Nearly 500 million people suffer from malaria each year. Of these, 1–2% have complicated clinical symptoms of severe malaria. In Thailand, 15,446 malaria cases were reported in 2015.[1] *Plasmodium falciparum* (*P. falciparum*) is responsible for the most severe manifestation due to its virulence and the ability to resist most available anti-malarial drugs.[2] The resistance of *P. falciparum* to the commonly used antimalarial drugs, including the newly introduced artemisinin, has resulted in a resurgence in treatment failures.[3,4] The dihydrofolate reductase (DHFR) domain of *P. falciparum* known as dihydrofolate reductase-thymidylate synthase (DHFR-TS), is one of the validated targets for antimalarial therapy.[5,6] Molecular docking is one of the most frequently used methods in structure-based design of a new drug, as it can predict the binding conformation of an inhibitor when bound to the target molecule forming a stable complex.[7] Therefore, new highly effective and affordable antimalarial agents that target *Plasmodium falciparum* dihydrofolate reductase (PfDHFR) are urgently needed.

For centuries, local plants used in traditional medicine have been a good source for drug development. For example, quinine and artemisinin were derived from *Cinchona pubescens* Vahl (cinchona tree bark) and *Artemisia annua* (sweet wormwood), respectively[2], and have been successfully used against resistant strains of malaria parasites. Antimalarial activities of many local Thai plants have been reported, for example the Siamese neem tree, green tea extracts, *Garcinia mangostana* Linn., *Phyllanthus emblica*, *Annona squamosa*, *Centella asiatica* and *Ipomoea pes-caprae*.[8-11] The objective of this study was to determine the antimalarial activity against the *P. falciparum* strain TM267 of ethanol crude extracts from 20 local Thai herbs, *Azadirachta indica* A., *Saccharu mofficinarum* L., *Curcuma longa* L., *Curcuma xanthorrhiza* Roxb., *Zingiber montanum*, *Citrus hystrix* DC., *Hibiscus sabdariffa* L., *Ocimum sanctum* L., *Ocimum basilicum* L., *Allium sativum* L., *Quercus infectoria*, *Myristica fragrans* Houtt., *Strychnos nux-vomica* L., *Salacia chinensis* L., *Maclura cochinchinensis* Lour., *Pentace burmanica* Kurz., *Mammea siamensis* Kosterm., *Vitex trifolia* Linn., *Plumbago indica* L. and *Dracaena loureiri*. Interestingly, this study also examined potential antimalarial drug prediction from PfDHFR docking.

Material and Method

Approval for this study was obtained from the Ethics Committee, Huachiew Chalermprakiet University, Samutprakarn, Thailand (EC No. อ.326/2558). All participants involved were informed of the objectives of study and signed consent forms.

Preparation of crude extracts

The twenty Thai herbs to be studied were purchased from a folk-medicinal store in Bangkok, Thailand. All herbs were botanically authenticated by Lecturer Benjawan Somboonsuk (Wat Pho Thai Traditional Medical School, Wat Phra Chetuphon Vimolmangklararm Rajwaramahaviharn, Thailand). These herbs were preserved at the Faculty of Science, Kasetsart University. To begin the extract preparation, five hundred milliliters of 95% ethanol were used to soak 100 g of plant materials separately at room temperature for 72 h. The supernatant fractions were filtered with 10 nm Whatman filter paper, and concentrated by a rotary evaporator at 50 °C until dry and kept at −20 ºC until use. The extract yield (%w/w) was determined for each extract by using the formula:

$$\text{Yield (\%)} = \frac{\text{weight of evaporated extract}}{\text{weight of dried plants}} \times 100$$

The crude extracts were dissolved in 100% dimethyl sulfoxide (DMSO) to a concentration of 250 mg/ml as a stock solution. The stock solution of artemisinin (the reference drug) was similarly prepared in sterile water. All stock solutions were stored at –20 °C until use.

Cultivation of *P. falciparum*

The *P. falciparum* chloroquine-resistant strain (TM267) was a generous gift from Professor Dr.Srisin Khusmith (Department of Immunology and Microbiology, Faculty of Tropical Medicine, Mahidol University). After we received it, the *P. falciparum* strain TM267 was continuously cultured according to standard methods[12] with some modifications in a gas mixture of 5% CO_2 , 1% O_2 in N_2, in a medium composed of RPMI 1640, buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), gentamicin (2.5 µg/ml), sodium bicarbonate (25 mM) and supplemented with 1% Albumax II. The parasites were cultured in 3–5% haematocrit of blood group O cells collected from normal healthy donors. The parasite growth was monitored daily by examination of Giemsa-stained thin blood films until isolates reached the pigmented trophozoite stage. The parasite cultures were synchronized to the ring stage by treatment with 5% sorbital.[13]

***In vitro* antimalarial activity using the pLDH assay**

The pLDH activity was used to measure parasite viability.[14] Briefly, one hundred and ninety-nine microliters of *P. falciparum*-infected erythrocyte suspension was prepared with 2% parasitemia and 2% haematocrit and seeded into each well of a test plate. Then, one microliter of the crude extracts was added into each well. The final concentrations of the crude extracts ranged from 0.002–2.5 mg/ml. The highest concentration of DMSO that the parasites were exposed to was 1%, which was shown to have no measurable effect on parasite viability. All conditions were performed in duplicate. Artemisinin (Sigma) and DMSO were used as the positive and negative controls, respectively. The tested plates were incubated in a CO_2 incubator at 37 ºC for 72 h. Then, the plates were frozen at –20 ºC for 30 min and thawed in a 37 ºC incubator for 30 min, and this was repeated three times to obtain complete hemolysis of the *P. falciparum*-infected erythrocytes. The pLDH activity from both the test samples and controls were evaluated. Briefly, one hundred microliters of Malstat reagent were added into each well in a new 96-well plate. Then 20 µl of each well from the tested plate were added followed by addition of 20 µl of nitroblue tetrazolium/phenazine ethosulphate (NBT/PES) solution. The plates were incubated in the dark for 1 h. The development of the blue color was measured at 620 nm using an enzyme-linked immunosorbent assay (ELISA) reader. The percentage of growth inhibition and the IC_{50} values were calculated from the dose-response curves, using non-linear dose-response curve fitting analyses with GraphPad Prism 6.

Cytotoxicity activity on Vero cells using MTT assay

The cytotoxicity of extracts on host cells was determined against Vero cells using an MTT assay.[15] Vero cells were thawed and cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. Briefly, Vero cells (10^4 cells/ml, 100 µl/well) were seeded into 96-well flat-bottom tissue culture plates in complete medium. After 24 h of seeding, 100 µl of various concentrations of crude extracts or drug solutions were added into each well which was then incubated for 48 h in 5% CO_2 at 37 °C. DMSO was used as a negative control. All conditions were done in duplicate. After incubation,

MTT solution (5 mg/ml) was added into each well and gently mixed and incubated for another 2 h. Then the supernatant was removed and 100 µl of DMSO was added. The formation of formazan was measured with an ELISA reader at 540/620 nm. The CC_{50} values were calculated by analysis of the dose–response curves. The therapeutic index was calculated as a ratio of the CC_{50} of the Vero cells divided by the IC_{50} of *P. falciparum.*

Molecular Docking of active compounds to PfDHFR

The molecular docking and post-docking analyses were assessed by GOLD 5.3.0.[16] The x-ray crystal structure of PfDHFR-TS complexed with pyrimethamine, NADPH, and dUMP was obtained from the Protein Data Bank (accession number 1J3J) with 2.3 Å resolution. For docking with GOLD, hydrogen atoms were added into the protein structure using the "*protonation* and *tautomers*" function in the configuration option of the *Gold Setup* window of the GOLD package. To prepare the ligands for docking into PfDHFR, the 3D coordinate files of 7 active compounds from Thai herbs (*Citrus hystrix* DC., *Vitex trifolia* Linn. and *Plumbago indica* L.) were taken from PubChem in MOL format. A total of 34 chemical structures were found and used in the computational docking procedure with PfDHFR. The automatic GA parameter setting was used in all of the GOLD docking calculations. One hundred percent search efficiency was applied, with a minimum of 10,000 and a maximum of 123,000 operations per ligand. The binding site was defined to include all amino acid residues within a 6 Å radii from the center of the pyrimethamine; all of the water molecules were removed. The Gold scoring function was applied in all the docking calculations. The protein-ligand interactions were analyzed and visualized by Discover Studio 2016.[17]

Results

Twenty ethanolic crude extracts from local Thai herbs belonging to several families were evaluated for their antimalarial activity. Of these, eleven were commonly used in Thai traditional medicine, and the other nine were commonly used for daily cooking. Scientific names, Thai local names, the used part(s) and the % extraction yield of the herbs are shown in Table 1.

Preliminary screening of antimalarial activities was performed at the concentration of 2.5 mg/ml. Among the t ested herbs, the six ethanol extracts of *Plumbago indica* L., *Citrus hystrix* DC., *Ocimum sanctum* L., *Salacia chinensis* L., *Allium sativum* L., and *Vitex trifolia* Linn. had high anti-malarial activities as shown by their high inhibition percentages (56.34%±3.29%; 54.79%±6.21%; 53.82%±4.22%; 48.89%±1.13%; 47.83%±0.50%; 46.83%±4.15%, respectively) as shown in Figure 1. For the positive control, artemisinin was tested against *P. falciparum* TM267, which had an IC_{50} value of 4.2 µg/ml. These results became the basis for the next assay, which was designed to identify antimalarial activities using IC_{50} as a parameter. For estimating the potential of a given extract to inhibit the parasite's growth without host toxicity, a selectivity index was calculated. The higher the selectivity index, the higher the selective antimalarial activity of the given extract.[18] The IC_{50} value of the crude extract of *Plumbago indica* L. root indicated that it was the most potent of the tested herbs, greater than artemisinin and the crude extracts of *Citrus hystrix* DC fruit., *Vitex trifolia* Linn. root, *Ocimum sanctum* L. leave, *Allium sativum* L. bulb and *Salacia chinensis* L. stem. For the toxicity assay against Vero cells, only the ethanolic crude extracts *Plumbago indi*ca L. root had no cytotoxicity (SI>10). The other four extracts from the above list were less toxic based on their selectivity index (SI>3) and the extracts of *Salacia chinensis* L. stem could not identify by the MTT assay (Table 2).

Table 1 Description of herbs and their extraction yield used for antimalarial activity screening.

No.	Scientific name	Other name	Thai name	Family	Part	% Yeild of extract
1	*Azadirachta indica* A.	Siamese neem tree	สะเดา	Meliaceae	leaves	5.4
2	*Saccharu officinarum* L.	Sugarcane	อ้อยแดง	Poaceae (Gramineae)	stems	2.0
3	*Curcuma longa* L.	Turmeric	ขมิ้นชัน	Zingiberaceae	rhizomes	10.6
4	*Curcuma xanthorrhiza* Roxb.	-	ว่านชักมดลูก	Zingiberaceae	rhizomes	8.3
5	*Zingiber montanum*	Bengalroot, Phlai	ไพล	Zingiberaceae	rhizomes	5.9
6	*Citrus hystrix* DC.	Leechlime	มะกรูด	Rutaceae	fruit	28.0
7	*Hibiscus sabdariffa* L.	Roselle	กระเจี๊ยบแดง	Malvaceae	flowers	16.9
8	*Ocimum sanctum* L.	Holy basil	กะเพราแดง	Lamiaceae	leaves	12.5
9	*Ocimum basilicum* L.	Sweet basil	โหระพา	Labiatae	leaves	15.8
10	*Allium sativum* L.	Garlic	กระเทียม	Alliaceae	bulbs	1.0
11	*Quercus infectoria*	Nutgall	เบญกานี	Fagaceae	fruit	73.2
12	*Myristica fragrans*	Nutmeg	จันทน์เทศ	Myristicaeae	stems	8.0
13	*Strychnos nux-vomica*	Nux-vomica tree	โกฐกะกลิ้ง	Loganiaceae	seeds	1.3
14	*Salacia chinensis* L.	-	กำแพงเจ็ดชั้น	Celastraceae	stems	7.3
15	*Maclura cochinchinensis* (Lour.)	Cockspur thorn	แกแล	Moraceae	stems	14.2
16	*Pentace burmanica* Kurz	Chaulmoogra	สีเสียดเปลือก	Tiliaceae	bark	11.1
17	*Mammea siamensis* Kosterm.	Negkassar	สารภี	Guttiferae	flowers	4.6
18	*Vitex trifolia* Linn.	-	คนทีสอ	Verbenceae	roots	5.7
19	*Plumbago indica* L.	Rose-colored Leadwort	เจตมูลเพลิงแดง	Plumbaginaceae	roots	20.3
20	*Dracaena loureiri* Gagnep	-	จันทน์ผา	Agavaceae	stems	7.5

Table 2 IC_{50}, CC_{50} and selectivity index (SI) of the six selected ethanolic crude extracts

Ethanolic extract	IC_{50} (mg/ml)	CC_{50} (mg/ml)	SI
Citrus hystrix DC.	0.208	0.630	3.02
Ocimum sanctum L.	0.339	>1.250	>3.68
Allium sativum L.	0.473	>2.500	>5.28
Salacia chinensis L.	2.310	NA*	NA*
Vitex trifolia Linn.	0.320	>1.250	>3.90
Plumbago indica L.	0.004	0.145	48.33
Artemisinin	0.004	NA*	NA*

*NA=not available

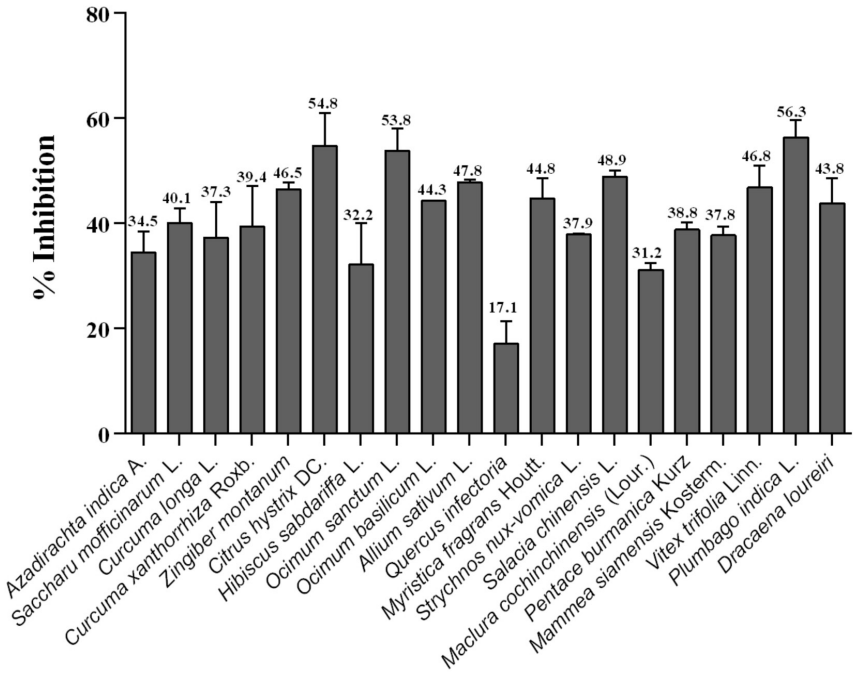


Figure 1 Antimalarial activity against *P. falciparum* TM267 of the 20 ethanol crude extracts based on percentage inhibition

Seven active compounds from three herbs, including *Citrus hystrix* DC., *Vitex trifolia* Linn. and *Plumbago indica* L. were found in the PubChem database (https://pubchem.ncbi.nlm.nih.gov) (Table 3). These compounds were tested for their activity against the PfDHFR using the molecular docking method. All of them had higher docking scores than pyrimethamine, a known inhibitor of PfDHFR. Plumbagin and vitex Trifolin B, the only identified active compounds from *Plumbago indica* L. and *Vitex trifolia* Linn, respectively, contained potential inhibitors against malaria that could bind to the active site of PfDHFR by using hydrogen bonds, π–interactions, and hydrophobic interactions (Figure 2A and Figure 2B). Five active compounds have been reported from Citrus hystrix DC. Among these, Cutrussoside C showed the highest docking score, and all five compounds had scores higher than the compounds from other herbs (Figure 2C). Although *Citrus hystrix* DC. a lower SI, it would be interesting to further investigate the Cutrussosides compounds regarding their SI.

Discussion

The greatest antimalarial activity effect was obtained from the crude extract from *Plumbago indica* L. root (IC_{50}= 3.7 μg/ml), and the same extract was the least toxic (SI=48.3). This is in accordance with previous results of Thiengsusul et al. (2013) who found that the ethanolic extract from of *Plumbago indica* L. had high potency in vitro antimalarial activity.[19] This activity was potentially due to the major constituent of the *Plumbago indica* L. root i.e., plumbagin (5–hydroxy–2–methyl–1,4–naphtho–quinone), which has a wide range of pharmacological activities including antimalarial activity in vitro and in a mouse model with low toxicity.[20] In addition, plumbagin exhibited highly effective biological activities including anticancer, antibacterial, antifungal, anti–leishmania and anti–inflammatory effects.[21] The ethanolic crude extract of *Citrus hystrix* DC. fruit, *Vitex trifolia* Linn. root, *Ocimum sanctum* L. leave, *Allium sativum* L. bulb and *Salacia chinensis* L. stem had little to no antimalarial activity against *P.*

falciparum (IC_{50} ranging from 0.20–2.31 mg/ml). The difference in IC_{50} values could be related to several factors such as the local environment and the period of collection, which can affect the major compounds in herbal extracts.[22] Ethanol is the most commonly used organic solvent for herbal medicinal extraction.[23] In phytochemical studies, the extraction methods are often different due to the type of extracting solvent used, which is generally related to its polarity. Additionally, the results depend on the sensitivity of the laboratory techniques that are used to assay malaria parasite growth, which is demonstrated by previous findings of e.g. different sensitivities from the hypoxanthine incorporation assay (using flow cytometry), the pLDH assay, the Sybr Green plate reader assay and light microscopy.[24]

The active ingredients in these herbal medicinal extracts were searched through the PubChem database. Several active compounds could be purposed to inhibitory activity of PfDHFR, which is well-known as a target for antimalarial drugs. The Cutrussoside C from *Citrus hystrix* DC. had the highest docking score, which were presumably to be a potential as an active inhibitor against PfDHFR. Although the crude extract showed some level of toxicity to human cell lines, the active compounds from this herb should be further studied for their effect on malaria and human cell lines.

Table 3 Docking results of all active compounds from three Thai herbs, pyrimethamine and chloroquine.

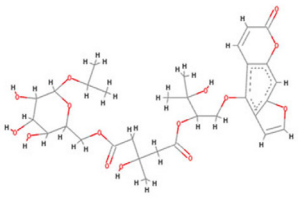
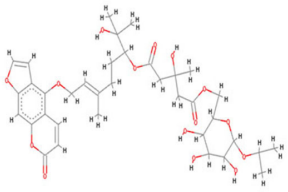
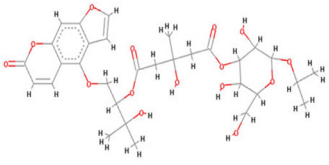
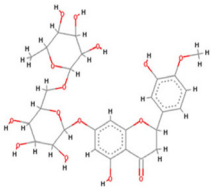
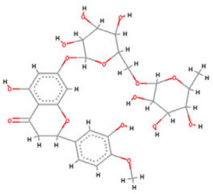
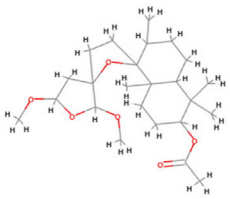
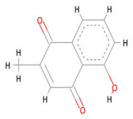
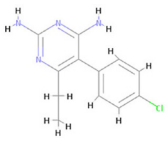
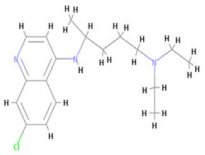
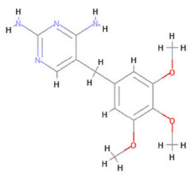
Plant name	Compound	Pubchem compound identification (CID code)	Score	2D structure
Citrus hystrix DC.	Citrusoside C	50901242	76.57	
	Citrusoside B	50901241	75.18	
	Citrusoside D	50901243	73.94	

Table 3 (Continued)

Plant name	Compound	Pubchem compound identification (CID code)	Score	2D structure
	Hesperitin-7-rutinoside	6419939	73.02	
	Hesperetin-7-rhamnoglucoside	3594	68.94	
Vitex trifolia Linn.	Vitex Trifolin B	71579298	46.79	
Plumbago indica L.	Plumbagin	10205	36.20	
-	Pyrimethamine	4993	56.97	
-	Chloroquine	2719	56.34	
-	Trimethoprim	5578	59.92	

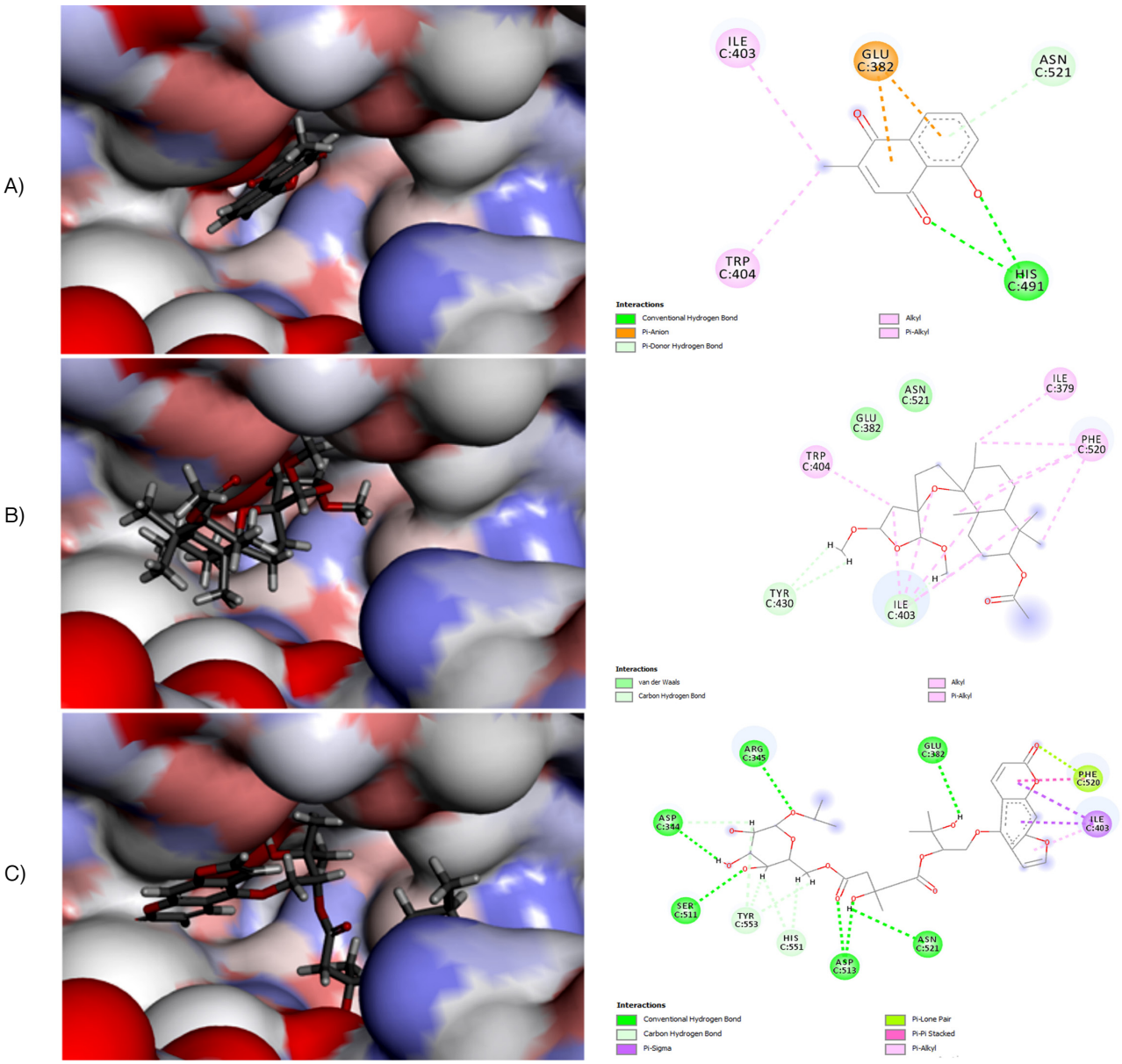


Figure 2 Docking interactions of A) Plumbagin, B) Vitex Trifolin B, and C) Citrusoside C at the active site of PfDHFR

Conclusion

Ethanol crude extract of *Plumbago indica* L. root had higher antimalarial activity than the extracts from *Citrus hystrix* DC., roots of *Vitex trifolia* Linn and artemisinin. The molecular docking identified plumbagin and vitex Trifolin B as the only identified active compounds from *Plumbago indica* L. and *Vitex trifolia* Linn, respectively., which could bind at the active site of PfDHFR. Our results provide preliminary data for using select Thai herbs for potential anti-malarial drug development.

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