Research Article

Molecular Docking of Putative Compounds in Aqueous *Muntingia calabura* L. Leaf Extracts with Cytochrome P450 Proteins

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Submission Date: 12-02-2022; Revision Date: 13-03-2022; Accepted Date: 15-04-2022.

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ABSTRACT

Studies claim that *Muntingia calabura* L. (*M. calabura*) exhibits antibacterial, antipyretic, antidiabetic, antioxidant, and anti-inflammatory properties. Despite these numerous claims, limited studies have shown its hepatoprotective property. Thus, we investigated the hepatoprotective property of aqueous *M. calabura* L. leaf extracts (AMCLE) by inhibiting salient CYP450 enzymes associated with hepatotoxicity, CYP3A4 CYP2E1, CYP1A2, and CYP2D6. Aqueous leaf extracts were subjected to phytochemical screening to identify potentially active compounds. A literature search was done to determine the specific metabolites. The identified candidates were docked with CYP450 enzymes virtually. The phytochemical screening revealed that AMCLE contains phenols, tannins, saponins, alkaloids, and flavonoids. The docking experiment showed that galangin, a flavonoid, has the highest binding affinity to the CYP450 enzymes compared to all the putative metabolites tested. Also, galangin outranked most known enzyme inhibitors, except for ritonavir and α-naphthoflavone, inhibitors of CYP3A4 and CYP1A2, respectively. These data suggest that the CYP450-associated hepatoprotective property of AMCLE may be attributed to galangin. Hence, further studies are warranted to support these findings.

Key words: Hepatoprotection, Hepatotoxicity, Cytochrome P450, Molecular Docking, *Muntingia calabura.*

INTRODUCTION

An estimated two million deaths annually are due to liver complications such as hepatitis, cirrhosis, and hepatocellular carcinoma.[1] In 2015, liver cirrhosis was responsible for at least 9.5/100 thousand individuals.[2] Fatty liver disease has been attributed to increases in triglyceride and free cholesterol accumulation and dysregulated cholesterol metabolism, which are physiological hallmarks of an obese demographic.[3] The scarcity of treatment options contributes to the grave nature of liver disease prevalence. However, this kind of treatment could trigger drug-induced liver damage, which has been reported to cause 13% of acute liver damage cases in the United States.[4] The drug-induced liver damage phenomenon has prompted people to look at alternative measures, such as antioxidants and herbal medicine.[5,6] Herbal medicine exists as teas, capsules, and extracts. One plant recently studied for medicinal properties is *M. calabura* L., commonly known as Jamaican berry. Despite their medicinal importance, *M. calabura* L. trees are cultivated as ornamental trees in the Philippines. Several studies claim that it has antioxidant, cytotoxic, antibacterial, antiproliferative, antiplatelet aggregation, antihypertensive, cardioprotective, anti-inflammatory properties, immune response, and mineral homeostasis.[7,8]

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DOI: 10.5530/ajbls.2022.11.18

CYP450 enzymes are a family of monooxygenase enzymes responsible for the oxidative metabolism of lipophilic drugs and xenobiotics.[9] CYP450 enzymes, CYP3A4 and CYP2D6 exhibit the highest range of drug metabolism by metabolizing more than 50 % of clinically used drugs.[10] Each constituent of the CYP450 family also specializes in metabolizing certain kinds of drugs. CYP2D6, for instance, is known to metabolize antidepressants, angiotensin II antagonists, non-steroidal anti-inflammatory drugs, and hypoglycemics.[11] CYP3E1, on the other hand, is responsible for the bioactivation of substrates and exhibits a notable role in ethanol-induced hepatotoxicity and procarcinogen metabolism.[12] One of these low molecular weight substrates is acetaminophen, known for its hepatotoxic tendencies in an overdose. In the metabolism of acetaminophen, CYP2E1, CYP1A2, CYP2D6, and CYP3A4 are the primary constituents of the CYP450 system, which metabolize acetaminophen. CYP1A2 is known to contribute around 30-56% to its metabolism and is only known to exhibit activity during an overdose.[13] CYP3A4, on the other hand, contributes at least 1-20% to acetaminophen metabolism and is active at lower doses.[14] CYP2E1 is the main contributor to acetaminophen metabolism and contributes around 30-78% to metabolism.[15] Double knockout studies suggest that mice without an active CYP2E1 and CYP1A2 are protected against hepatotoxicity and glutathione depletion despite an Acetaminophen (APAP) overdose.[16] CYP2D6 contributes 4-22% to APAP metabolism, with varying expression levels observed between individuals.[13]

This study predicts the hepatoprotective property of putative metabolites in aqueous *M. calabura* L. leaf extracts (AMCLE) through *in silico* molecular docking with CYP450 enzymes.

MATERIALS AND METHODS

Plant Collection and Drying

Fresh *M. calabura* L. leaves were collected in San Miguel, Manila, Philippines. Leaf specimens were identified and authenticated at the Institute of Biology, Jose Vera Santos Memorial Herbarium, College of Science, University of the Philippines - Diliman. The fresh leaves were washed with water to remove dirt particles and dust. They were air-dried under shade until brittle before pulverization, weighing, and storage in airtight containers lined with black plastic bags and silica desiccants. They were kept in a cool and dry environment away from direct sunlight.

AMCLE Preparation and Storage

The aqueous leaf extraction protocol was adapted from a previous study with few changes.[17] Aqueous *M. calabura* L. leaf extract (AMCLE) concentrations of 1%, 2%, 4%, and 10% (%w/v) (10, 20, 40, and 100 g/L dH_2O, respectively) were prepared by varying the amount of pulverized dried leaves used in one liter of boiling distilled water. They were steeped in boiling water for five minutes with occasional stirring, left for 24 hr, filtered, and placed in separate amber bottles for storage at 4°C until use.

Phytochemical Screening of AMCLE

The extracts were subjected to a qualitative phytochemical screening for carbohydrates, proteins, and secondary metabolites, such as phenols, tannins, flavonoids, alkaloids, and saponins, at the Department of Biology Laboratory, University of the Philippines Manila. The protocol was adapted from a previous experiment but modified.[18]

Ligand-Enzyme Virtual Screening via AutoDock Tools

After the data from the phytochemical screening was obtained, a literature review was conducted to identify specific putative phytochemical candidates present in AMCLE. The three-dimensional structures of these phytochemicals were obtained from chemical molecule databases PubChem (https://pubchem.ncbi.nlm.nih.gov) and Protein Data Bank (www.rcsb.org/pdb). The protein structures of the cytochrome P450 enzymes of interest were obtained from Protein Data Bank. The following enzymes were chosen based on their association with hepatotoxicity according to previous studies: CYP3A4 (PDB ID: 5VC0), CYP2E1 (PDB ID: 3T3Z), CYP1A2 (PDB ID: 2HI4), and CYP2D6 (PDB ID: 3TBG).[19-24]

In silico molecular docking was performed using AutoDock Tools v1.5.6. The method for the procedure was based on a similar molecular docking study.[25] The enzyme was prepared by deleting water molecules and merging non-polar hydrogen molecules. Gasteiger charges were added afterward. The ligand was then uploaded into AutoDock Tools v1.5.6. A covalent map was then set up with the energy barrier height and half-width set to 1000 and 5.0 Angstrom, respectively. Then, a grid box with 60 points in the X, Y, and Z axes and spacing of 0.200 Angstrom was set with the target residue centered on the grid box. The grid box configuration was then saved. A grid map was generated in preparation for the docking procedure. Genetic algorithm parameters were also set with the number of

GA runs set at 25, whereas default settings were used for other parameters. The output for docking was set with the Lamarckian GA (4.2) setting and was saved as a configuration file.

RESULTS

Phytochemical Screening of Crude Extracts

All concentrations of AMCLE were subjected to different qualitative phytochemical tests, which are presented in Table 1. AMCLE tested positive for phenols, tannins, saponins, alkaloids, and flavonoids. Positive results exhibited either a change in the color of the solution or precipitation, or both. Also, the results show that varying concentrations of AMCLE did not affect the phytochemicals detected. Thus, ample amounts of the said metabolites are present even at a 1% concentration of AMCLE.

Table 1: Phytochemical screening of varying concentrations of AMCLE.

Phytochemical Test	Compound	AMCLE (1%)	AMCLE (2%)	AMCLE (4%)	AMCLE (8%)
Fehling	Reducing Sugars	-	-	-	-
IKI	Starch	-	-	-	-
Molisch	Carbohydrates	-	-	-	-
Ninhydrin	Ammonia	-	-	-	-
Biuret	Proteins	-	-	-	-
5% $FeCl_3$	Phenols	+	+	+	+
10% $FeCl_3$	Tannins	+	+	+	+
Foam Layer	Saponins	+	+	+	+
Mayer	Alkaloids	+	+	+	+
Lead Acetate	Flavonoids	+	+	+	+

Lead-likeness of the Different Putative Metabolites in AMCLE

Lipinski's Rule of Five (Ro5) assesses the bioavailability of oral drugs based on their molecular weight, log P value, and the number of H-bond acceptors and donors. As seen in Table 2., none of the specific phytochemicals tested violated any criteria. Their molecular weights (g/mol) were within 154.12 to 270.24. Log P values ranged from 0.7 to 2.3. They had around 4 to 5 H-bond acceptors and around 3 to 4 H-bond donors.

On the other hand, Table 3 shows that three out of four controls had no criteria violations. The controls used were α-Naphthoflavone (PubChem CID: 11790), Quinidine (PubChem CID: 441074), 1H-Indazole (PubChem CID: 9221), and Ritonavir (PubChem CID: 392622). Only ritonavir had violated two of the criteria. Their molecular weights (in g/mol) ranged from 118.14 to 720.9. Only ritonavir violated the criterion for molecular weight by weighing 720.9 g/mol. Their log P values ranged from 1.8 to 6; Ritonavir violated this criterion as it has a log *P* value of 6.0. They had around 1 to 9 H-bond acceptors and around 0 to 4 H-bond donors.

Table 2: Lead-likeness assessment of the phytochemicals following Lipinski's Rule of Five (Ro5).

Target Enzymes	Ligands	Mass (g/mol)	Log P	H-bond acceptor	H-bond donor	RO5 violation
		(<500)	(< 5)	(< 10)	(< 5)	(< 2)
CYP1A2, 2D6, 2E1, 3A4	Caffeic Acid	180.16	1.2	4	3	0
CYP1A2, 2D6, 2E1, 3A4	Gallic Acid	170.12	0.7	5	4	0
CYP1A2, 2D6, 2E1, 3A4	Methyl Gallate	184.15	0.9	5	3	0
CYP1A2, 2D6, 2E1, 3A4	Protocatechuic Acid	154.12	1.1	4	3	0
CYP1A2, 2D6, 2E1, 3A4	Galangin (Flavonoid)	270.24	2.3	5	3	0

Table 3: Lead-likeness assessment of the known inhibitors following Lipinski's Rule of Five (Ro5).

Target Enzymes	Known Inhibitor	Mass (g/mol)	Log P	H-bond acceptor	H-bond donor	RO5 violation
		(< 500)	(< 5)	(< 10)	(< 5)	(< 2)
CYP 1A2	α-Naphthoflavone	272.3	4.8	2	0	0
CYP 2D6	Quinidine	324.4	2.9	4	1	0
CYP 2E1	1H-Indazole	118.14	1.8	1	1	0
CYP 3A4	Ritonavir	720.9	6	9	4	2

Virtual Molecular Docking

The docking score of the putative AMCLE compounds and known Cytochrome P450 inhibitors with CYP3A4, CYP2E1, CYP1A2, and CYP2D6 were shown in Table 4. In CYP3A4, galangin shows the lowest free energy but fails to outrank ritonavir, the known inhibitor. Likewise, galangin, methyl gallate, and caffeic acid were the best binders of CYP2E1, outranking the known inhibitor, 1H-Indazole. The compound galangin was comparable with α-Naphthoflavone, the inhibitor of CYP1A2. Both compounds were the strongest binders of CYP1A2. Further, galangin appears to be the strongest binder of CYP2D6, even higher than its inhibitor, quinidine.

DISCUSSION

The aqueous *M. calabura* L. leaf extracts (AMCLE) tested positive for phenols, tannins, saponins, alkaloids, and flavonoids. A similar experiment used ethanolic *M. calabura* L. leaf extracts and ethanolic *M. calabura* L. stem extracts.[26] The classes of phytochemicals present in the leaf extracts were similar to those found in AMCLE. They also reported that glycosides and sterols were present in their extracts. However, glycosides and sterols were not tested in this experiment. Another source indicated that various leaf extracts of *M. calabura* L. contained saponins, tannins, triterpenes, steroids, and flavonoids.[8] A previous study listed specific phytochemicals present in various parts of the *M. calabura* L. plant.[7] Most of the phytochemicals were either phenols or flavonoids. Moreover, a previous study could not detect triterpenes in their ethanolic *M. calabura* L. leaf extracts.[26] Its presence in AMCLE was also not tested. These studies highlight the importance and usefulness of secondary plant metabolites, particularly those belonging to the phenol and flavonoid classes.

Table 4: Docking score (kcal/mol) of the ligands with Cytochrome P450 proteins.

Ligands	Cytochrome P450 proteins			
	CYP3A4	CYP2E1	CYP1A2	CYP2D6
Cytochrome P450 Inhibitors				
Ritonavir	-12.68	-	-	-
1H-Indazole	-	-5.93	-	-
α-Naphthoflavone	-	-	-11.63	-
Quinidine	-	-	-	-8.87
Putative AMCLE compounds				
Galangin	-11.17	-9.23	-11.37	-9.49
Methyl Gallate	-8.43	-8.91	-8.03	-6.39
Gallic Acid	-7.94	-3.70	-8.51	-3.75
Protocatechuic Acid	-7.74	-4.43	-7.83	-4.20
Caffeic Acid	-7.46	-9.05	-8.35	-7.08

Aside from secondary plant metabolites, it was also determined that AMCLE contained crude proteins with significant antioxidative properties.[27] Their study, however, focused on vortexing and centrifugation rather than boiling and steeping the extract. This difference may explain the absence of proteins in AMCLE as they may have been denatured due to heat. AMCLE did not contain any detectable amount of carbohydrates, and no study has reported the presence of carbohydrates in AMCLE.

As for the putative identities of the compounds, the researchers consulted multiple online journal articles related to *M. calabura* L.[7,28,29] However, these studies were limited to isolating specific phenols, flavonoids, and triterpenes. The qualitative phytochemical test results showed no triterpenes in the extract; hence, particular triterpenes were excluded in the succeeding experiment. The list was further narrowed down to compounds isolated through aqueous extraction. The putative compounds were composed of four phenols: gallic acid (PubChem CID: 370), caffeic acid (PubChem CID: 689043), methyl gallate (PubChem CID: 7428), protocatechuic acid (PubChem CID: 72), and one flavonoid: galangin (PubChem CID: 5281616).

The Lipinski Rule of Five (Ro5) determines the bioavailability of orally administered drugs in humans.[25] The Ro5 contains four physicochemical parameters for a chemical compound to be an orally active drug. These conditions are less than or equal to five hydrogen bond donors and less than or equal to ten hydrogen bond acceptors, a molar mass of less than or equal to 500 g/mol, and a partition coefficient (log P) of less than or equal to five.[30] Through the rule of five, potential compounds exhibiting a predicted poor absorption and bioavailability may be avoided to save on drug development resources. The phytochemicals have shown no violations with Ro5.

The enzymes chosen in the study are all part of the CYP family of enzymes. They are mainly responsible for converting the common over-the-counter drug acetaminophen into its toxic metabolite, NAPQI, a common cause of drug-induced hepatotoxicity. Drug-induced hepatotoxicity caused by NAPQI accounts for more than 50% of acute liver failure and 20% of liver transplant cases alone.[31] The conversion of acetaminophen into NAPQI occurs during phase 1 of drug metabolism through an oxidation reaction with the CYP system.[32] Such conversion is facilitated by the isozymes CYP1A2, CYP2D6, CYP2E1, and CYP3A4 at varying percentage levels, with CYP2E1 having the most significant influence at both therapeutic and toxic doses.[13]

Ritonavir, a CYP3A4 inhibitor, incurred two violations, specifically with its molecular mass and log *P* value. This result implies that the drug ritonavir falls under another drug group called Beyond the Rule of Five drugs (bRo5). Common to drugs under this classification is a molecular mass higher than 500 daltons brought about by either the natural or peptidic nature of the compound. In ritonavir, the presence of two thiazole rings serves as the leading cause of inhibition. One of the ritonavir's inhibition mechanisms includes type II ligand binding through a water molecule displacement and coordination of the heme iron with the ligand's nitrogen atom.[21] Alongside a high molecular weight, ritonavir also displays a high log *P* value which indicates high lipophilicity. This property predicts that ritonavir has poor epithelial permeability and solubility. Its absorbance is primarily regulated by many transporters such as Pgp, BCRP, and OATPs.[33] A high log P has also been favorable in ritonavir's hERG ion channel, modulating overall toxicity.[34]

The docking score reported represents the Gibbs free energy, which provides information about the stability of the crystal docked structure.[25] The more negative docking score denotes low free energy, implying a high binding affinity between the protein and the ligand.[25] The crystal docked structures of the different putative AMCLE compounds with CYP3A4 are shown in Figure 1. The *in silico* screening results show a notable finding that all compounds had a higher binding affinity when docked to CYP3A4, except for caffeic acid. The inhibitory characteristics of gallic acid on CYP3A4 remain unclear despite demonstrating time-dependent weak inhibition of CYP3A4 through its oxidative products.[35] Similarly, literature was scarce for the inhibitory effects of protocatechuic acid and methyl gallate on CYP3A4, but the results of this study suggest their potential inhibitory property to the CYP3A4 enzyme. Meanwhile, galangin, which has hydrophobic interaction, hydrogen bonding, and pi-stacking with several CYP3A4 amino acid residues, may explain its low free energy and apparent inhibitory effect. A study supports that galangin inhibited the CYP3A4-mediated metabolism of xenobiotics.[36] Ritonavir displayed multiple interactions with the protein's active site, such as hydrophobic and van der Waals interactions. Additionally, ritonavir was anchored to the active site via a direct hydrogen bond with ser119 supported by water bridges between the terminal isopropyl group of the inhibitor's thiazole group, as shown in Figure 1A.[21] This instance may explain why ritonavir had the lowest free energy when docked to CYP3A4.

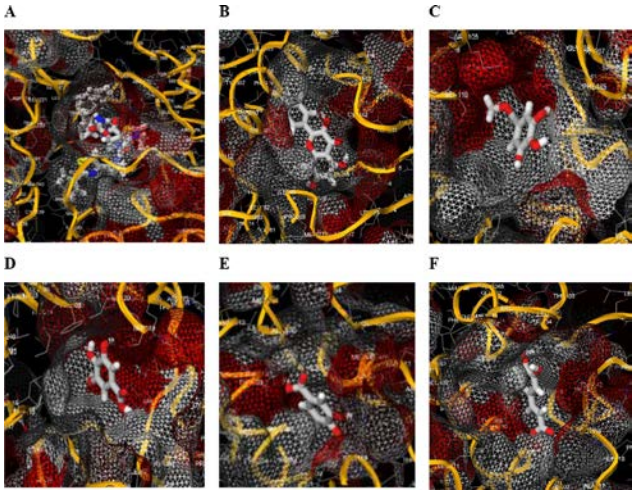

Figure 1: Protein surface view of CYP3A4 crystal-docked structure with the different putative AMCLE compounds. A: ritonavir, B: galangin, C: methyl gallate, D: gallic acid, E: protocatechuic acid, F: caffeic acid.

For CYP2E1, the results have shown that galangin and caffeic acid demonstrated low free energy when bound to CYP2E1. Their binding interactions with CYP2E1 and the other compounds are shown in Figure 2. Galangin's has the lowest free energy, while gallic acid has the highest. The low free energy can be attributed to the high number of hydrogen bonding and hydrophobic interaction with CYP2E1 amino acid residues. Previous studies show that galangin effectively modulated CYP2E1 microsomal activity in paracetamol-treated mice, proving that it decreases hepatic oxidative stress.[37] Caffeic acid's low free energy, when docked with CYP2E1, has not been fully elucidated by existing literature. However, the phenethyl ester of caffeic acid has been shown to decrease CYP2E1 activity by depleting the hydroxylation of aniline, which is a CYP2E1 dependent reaction.[38] In a study using Epilobium hirsutum, methyl gallate displayed an 80% decrease in aniline 4-hydroxylase enzyme activity modulating CYP2E1 activity.

The inhibitor for CYP1A2, α-naphthoflavone (ANF), exhibited the lowest free energy, which may be due to the hydrophobic effect, the aromatic interactions between ANF and CYP1A2 amino acid residues, and pi-stacking with phe226, as shown in Figure 3, contributing to favorable binding energy.[19] Consistent with galangin high number of hydrophobic interactions with CYP1A2 amino acid residues and pi-stacking with phe226 may attribute to high binding affinity with the protein. Galangin appears to be a potent inhibitor of CYP1A2, as previous studies have reported.[24]

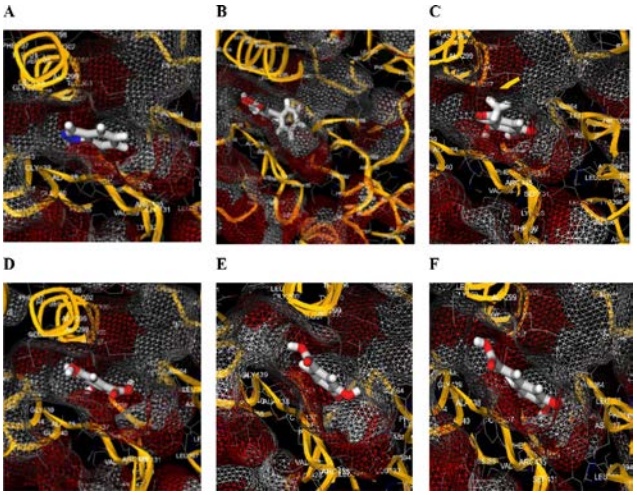

Figure 2: Protein surface view of CYP2E1 crystal-docked structure with the different putative AMCLE compounds. A: 1H-indazole, B: galangin, C: methyl gallate, D: gallic acid, E: protocatechuic Acid, F: caffeic acid.

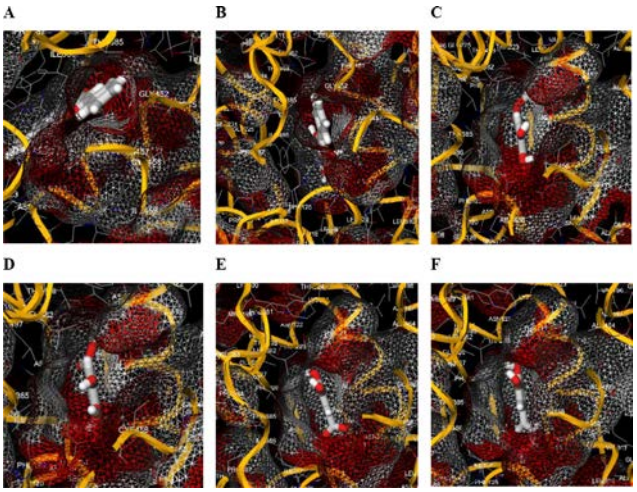

Figure 3: Protein surface view of CYP1A2 crystal-docked structure with the different putative AMCLE compounds. A: α-naphthoflavone, B: galangin, C: methyl gallate, D: gallic acid, E: protocatechuic acid, F: caffeic acid.

CYP2E1 and CYP2D6 appear to have a comparable trend in binding energies of the docked compounds, as shown in Figure 4. The hydrophobic interactions, hydrogen bonds, and pi-stacking of galangin with CYP2E1 may have resulted in low free energy. Galangin's inhibition of CYP2D6 has not been fully elucidated by literature, but it has been shown to inhibit CYP mRNA-expression levels, which were elucidated through real-time quantitative polymerase chain reaction experiments.[39] Quinidine's high binding affinity to CY2D6 is primarily due to its hydrophobic interactions; however other studies suggest potential hydrogen bonds formed between the carboxyl and the hydroxyl group of quinidine to the enzyme.[40]

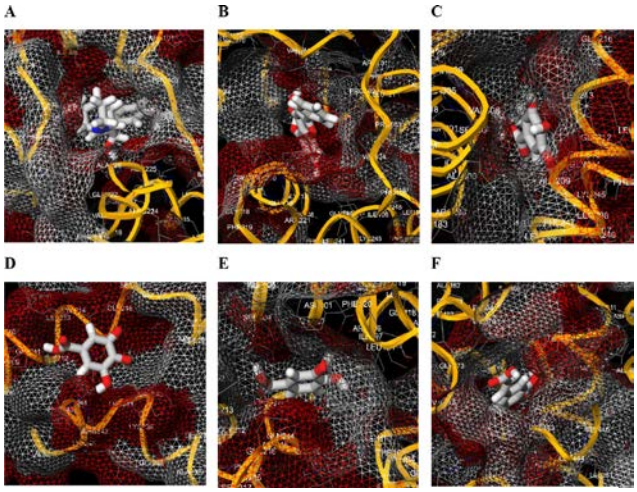

Figure 4: Protein surface view of CYP2D6 crystal-docked structure with the different putative AMCLE compounds. A: quinidine, B: galangin, C: methyl gallate, D: gallic acid, E: protocatechuic acid, F: caffeic acid.

CONCLUSION

Phenols, tannins, saponins, alkaloids, and flavonoids were present in all concentrations of AMCLE. After further evaluations, only gallic acid, protocatechuic acid, caffeic acid, methyl gallate, and galangin were considered the putative identities of the phytochemicals present in AMCLE. All the putative AMCLE compounds abided with Lipinski's Ro5 suggesting high oral bioavailability. The predicted binding affinity of these metabolites to CYP450 show that galangin, a flavonoid, exhibited the highest binding affinity to most CYP450 enzymes. Besides, the binding affinity of galangin is comparable with the binding affinity of some of the enzyme inhibitors, such as ritonavir and α-naphthoflavone, inhibitors of CYP3A4 and CYP1A2, respectively. Overall, the protective effect of AMCLE against drug-induced hepatotoxicity may involve galangin. However, further studies are needed to support this claim.

ACKNOWLEDGEMENT

We would like to acknowledge Dr. Miriam de Vera for the comments and suggestions in the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

AMCLE: Aqueous *M. calabura* L. leaf extracts; **Ro5:** Lipinski's Rule of Five.

SUMMARY

Aqueous *M. calabura* L. leaf extract contains phenols, tannins, saponins, alkaloids, and flavonoids. One flavonoid, galangin, has the highest binding affinity to the CYP450 enzymes compared to all the putative metabolites tested. Besides, it outranked most of the known CYP450 enzyme inhibitors. These data suggest that the CYP450-associated hepatoprotective property of AMCLE may be attributed to galangin.

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Cite this article: Nas JSB, Enriquez JGFB, Villa-Ignacio AJY, Bungay AAC, Salunga TL. Molecular Docking of Putative Compounds in Aqueous *Muntingia calabura* L. Leaf Extracts with Cytochrome P450 Proteins. Asian J Biol Life Sci. 2022;11(1):136-43.