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VIRTUAL SCREENING OF SAPONIN DERIVATIVES TARGETING ENZYMES ENDOTHELIAL NITRIC OXIDE SYNTHASE AND CYTOCHROME P450 2E1

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ABSTRACT: Saponin derivatives from Vietnamese ginseng are proven for their efficacies in modulating oxidative stress, but there had been no reports about the interaction between them and two enzymes, endothelial nitric oxide synthase (eNOS) and cytochrome P450 2E1 (CYP2E1). eNOS and CYP2E1 are proposed to be attractive targets for the development of inhibitors against oxidative stress, a contributing factor in aging, cancer, cardiovascular diseases, diabetes mellitus type 2, and neurodegenerative diseases. Therefore, this study aimed to evaluate the binding abilities of the saponin derivatives on both enzymes eNOS and CYP2E1 using structure-based approaches. An in-house library of 50 saponin derivatives from Vietnamese ginseng was computationally analyzed for their binding affinities and interactions with eNOS and CYP2E1 using Autodock Vina 1.5.6. The results showed that ginsenoside Rc, ginsenoside R3, vina-ginsenoside R20, ginsenoside Re, notoginsenoside R1 and 20(R)-ginsenoside Rh1 established the favorable interactions and exhibited high binding affinities with eNOS and CYP2E1. These compounds are potential candidates for *in-vitro* and *in-vivo* assays to assess their promising application in inhibition of these enzymes. This study also contributed to the understanding of saponin derivatives interactions with eNOS and CYP2E1 in antioxidative stress process.

INTRODUCTION: Ginseng saponins are generally called ginsenosides which are the main active principals of ginseng [1]. Ginsenosides have similar steroid backbone, but their different numbers and sites of hydroxyl groups/sugar moieties exhibit diverse activities [1]. They are classified into the four types, protopanaxadiol, protopanaxatriol, oleanolic acid, and ocotillol, based on the steroidal skeleton and number of hydroxyl groups/sugar moieties attached in the structures [1]. Previous *in-vitro* and *in-vivo* studies revealed that saponin derivatives such as majonoside - R1, majonoside - R2, ginsenoside Rg_1, ginsenoside-Rb_1, and vina - ginsenoside - R2 from Vietnamese ginseng (*Panax vietnamensis*) have biological activity in antioxidative stress [2, 3]. The question is that how these compounds can work and on which proteins, they target in the process of oxidative stress.

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Nowadays, inhibition of enzymes that are involved in oxidative damage such as CYP2E1, eNOS, NADPH oxidase, xanthine oxidase and enzymes of the mitochondrial respiratory chain [4], is a promising antioxidative stress strategy [5]. Consequently, this study investigated two enzymes, CYP2E1 and eNOS, and evaluated binding abilities of 50 saponin derivatives on these enzymes.

CYP2E1 and eNOS are main sources of free radicals which lead to the imbalance between free radicals production and antioxidant defenses in pathological situations.

Cytochrome P450 2E1 (CYP2E1): Cytochrome P450 is a superfamily of enzymes involved in monooxygenation of both endogenous and exogenous substrates including endogenous fatty acids and acetaminophen, halothane, industrial and halogenated solvents, alcohols, bicyclic heterocycles [6]. Among cytochrome P450 enzymes, CYP2E1 is particularly notable for the toxic - produced ability because it is considerably more prone to reactive oxygen species (ROS) and acetaldehyde production from ethanol metabolism than other cytochrome P450 enzymes [6, 7, 8]. Some *in-vitro* experiments proved that inhibition of CYP2E1 was effective in decreasing ROS leading to prevent associated diseases [7]. Although, diallyl sulfide (DAS) is a selective inhibitor of CYP2E1 [9], it is also known to cause toxicity such as DNA fragmentation at high concentration and when being used for longer time. Therefore, it is necessary to discover a new inhibitor of CYP2E1[10].

Endothelial Nitric Oxide Synthase (eNOS): eNOS plays an important role in endothelial cells, and it can produce both of ROS and RNS resulting in vascular dysfunction and associated pathology [11]. There are two forms of eNOS, namely coupled and uncoupled one, respectively. When sufficient substrate L-arginine and cofactor BH4 are present, intact NOS dimers couple their heme and oxygen reduction to the synthesis of NO [12]. According to several experiments, under pathological conditions associated with oxidative stress, coupled eNOS may become dysfunctional or uncoupled eNOS, in which oxygen reduction is uncoupled from NO synthesis [12]. When NOS is uncoupled, electrons flowing from the reductase domain to the heme are diverted to molecular oxygen instead of to L-arginine, resulting in the formation of superoxide [13]. Superoxide rapidly reacts with NO in the formation of peroxynitrite ($ONOO^-$) leading to a decrease of NO bioavailability for physiological needs and production of other free radicals [12]. $ONOO^-$ has been shown to oxidize tetrahydrobiopterin (BH4) to biologically inactive products such as trihydrobiopterin (BH3·) radical or 6, 7- [8H]- H_2- biopterin (BH_2) causing dysfunction of eNOS and increasingly serious pathology [12]. There are no effective inhibitors which have found in the previous studies.

Our research was conducted by a useful technique, namely molecular docking, to identify potential inhibitors of eNOS and CYP2E1 and orientated the design of their structures in drug discovery for treatment of oxidative stress - associated diseases. Molecular docking is a method which predicts the preferred orientation of a ligand to a receptor when they bond to each other to form a stable complex in three dimensional (3D) space [14]. This approach allows obtaining the best geometry of ligand-receptor complex and calculating the energy of interaction for different ligands [14]. Molecular docking can help to identify hit compounds, so that reduce the timeline drug discovery, increase the number of candidate drugs to clinical development, and also decrease the failure rate (currently 90%) of candidate drugs in the clinical stages [15].

MATERIALS AND METHODS:

Protein Structure Preparation: The crystal structure of eNOS (PDB ID: 3NLE) with a resolution of 1.95 Å[16] and CYP2E1 (PDB ID: 3E6I) with a resolution of 2.2 Å[6] were retrieved from the protein data bank (PDB) (http://www.rcsb.org). CYP2E1 had two polypeptides and was co-crystallized with the inhibitor 1H - indazole. Two polypeptides of eNOS was in complex with the inhibitor 6-{{(3'R,4'R)-3'-[2''-(3''' -fluorophenethylamino) eth-oxy]pyrrolidin-4'-yl}methyl}- 4- methylpyridine -2-amine - (3R, 4R)-3 in the structure 3NLE. These proteins were treated by BIOVIA Discovery Studio 2016 [17] and Autodock Tools in Autodock Vina [18]; polar hydrogen atoms were added, and water was removed from these structures.

Ligand Preparation: This study was performed on 50 saponin derivatives of Vietnamese ginseng

belonging to 3 groups: ocotillol, protopanaxadiol and protopanaxatriol **Fig. 1** and **2**. 22 saponin derivatives from Vietnamese ginseng were downloaded from the PubChem database (http://www.pubchem.ncbi.nlm.nih.gov) and the Drugbank database (http://www.drugbank.ca). The other 23 saponin derivatives drawn in ISIS Draw 2.5 software [19] were converted into 3D structures by OpenBabel GUI program [20].

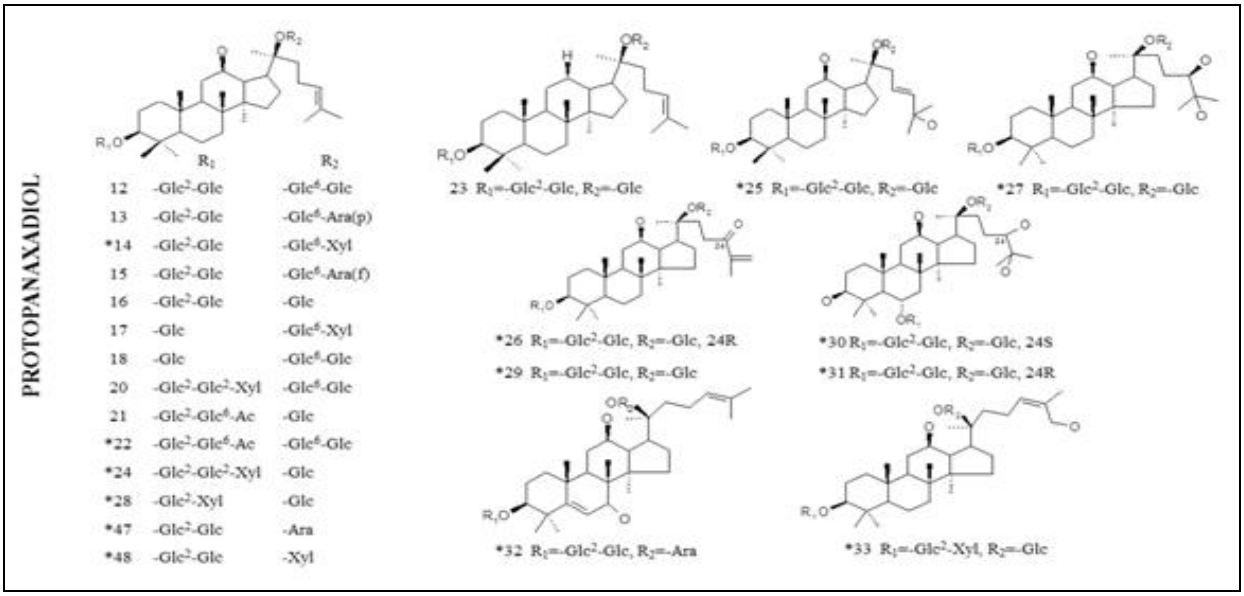

FIG. 1: STRUCTURES OF PROTOPANAXADIOL DERIVATIVES

(12) Ginsenoside Rb_1, (13) Ginsenoside Rb_2, (14) Ginsenoside Rb_3, (15) Ginsenoside Rc, (16) Ginsenoside Rd, (17) Gypenoside IX, (18) Gypenoside XVII, (19) Majoroside-F1, (20) Notoginsenoside Fa, (21) Pseudo-ginsenoside Rc_1, (22) Quinquenoside R_1, (23) Vina-ginsenoside R3, (24) Vina-ginsenoside R7, (25) Vina-ginsenoside R8, (26) Vina-ginsenoside R9, (27) Vina-ginsenoside R13, (28) Vina-ginsenoside R16, (29) Vina-ginsenoside R20, (30) Vina-ginsenoside R21, (31) Vina-ginsenoside R22, (32) Vina-ginsenoside R23, (33) Vina-ginsenoside R24, (47) Vina-ginsenoside R17, (48) Vina-ginsenoside R18. *Compounds were drawn in ISIS Draw 2.5 Software. Ara: α-L- arabinofuranosyl, Glc: β-D-glucopyranosyl, Xyl: β-D-xylopyranosyl.

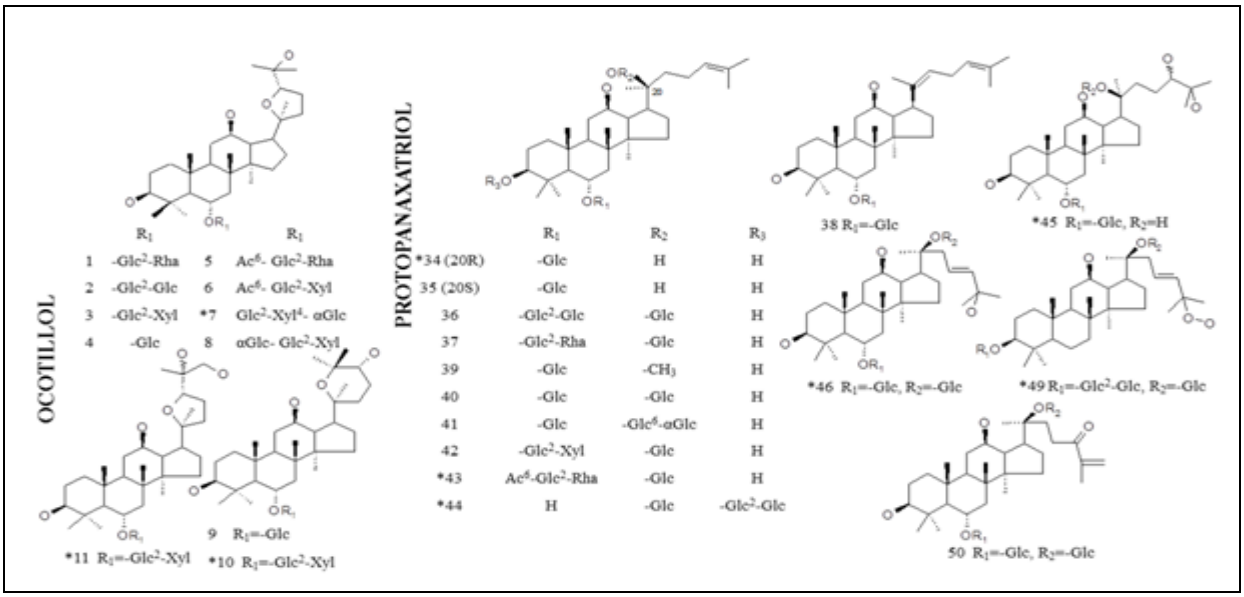

FIG. 2: STRUCTURES OF OCOTILLOL AND PROTOPANAXATRIOL DERIVATIVES

(1) 24*(S)*- Pseudo-ginsenoside F_{11}, (2) Majonoside R1, (3) Majonoside R2, (4) Pseudo-ginsenoside RT_4, (5) Vina-ginsenoside R1, (6) Vina-ginsenoside R2, (7) Vina-ginsenoside R5, (8) Vina-ginsenoside R6, (9) Vina-ginsenoside R10, (10) Vina-ginsenoside R11, (11) Vina-ginsenoside R14, (34) *20(R)*-Ginsenoside Rh_1, (35) *20(S)*-Ginsenoside Rh_1, (36) 20-Glucoginsenoside Rf, (37) Ginsenoside Re, (38) Ginsenoside Rh_4, (39) Ginsenoside Rh_5, (40) Ginsenoside Rg_1, (41) Notoginsenoside R6, (42) Notoginsenoside R1, (43) Pseudo-ginsenoside Rs_1, (44) Vina-ginsenoside R4, (45) Vina-ginsenoside R12, (46) Vina-ginsenoside R15, (49) Vina-ginsenoside R19, (50) Vina-ginsenoside R25. Ac: Acetyl, Rha: α-L-rhamnopyranosyl.

Identification of Binding Site on the Surface of eNOS and CYP2E1: Binding site of 3NLE is the domain containing the heme group, key residues Glu363 and Asn368 [16], the co-crystallized ligand, (3R, 4R)-3, and cavity-lining residues which have the distance to this co-crystallized ligand generally 5 Å **Fig. 3A**. Similarly, the binding pocket region of 3E6I consists of heme group, key residues Ala299 and Thr303 [6], 1H - indazole and cavity-lining residues around 1H - indazole 5 Å **Fig. 3B**.

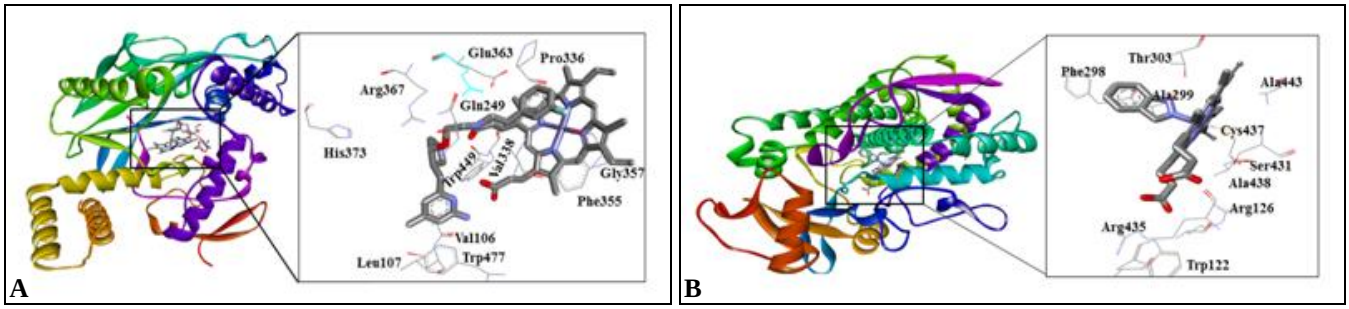

FIG. 3A-B: STRUCTURES AND BINDING SITE OF PROTEINS

(A) eNOS (PDB ID: 3NLE) are showed in complex with 6- {{(3'R, 4'R)- 3'- [2"-(3"' -fluorophenethyl amino)ethoxy] pyrrolidin- 4'- yl} methyl}-4-methylpyridin-2-amin and heme group. (B) CYP2E1 (PDB ID: 3E6I) are shown in complex with 1H – indazole and heme group.

The key docking parameters consisting of the location of the docking site (center x, y, z) and the size of a grid box were identified by AutodockTools in Autodock Vina 1.5.6 **Table 1**.

TABLE 1: COORDINATES AND SIZES OF BINDING SITES ON eNOS (PDB ID: 3NLE) AND CYP2E1 (PDB ID: 3E6I) IN 3D SPACE

Parameter	eNOS	CYP2E1
Center_x	1.040	5.910
Center_y	3.880	-3.609
Center_z	66.029	-8.375
Size_x (Å)	20	26
Size_y (Å)	40	38
Size_z (Å)	26	32

Molecular Docking: Re-docking procedure was performed by docking co-crystallized ligands into the binding site of their holoproteins. The success of re-docking served as a validation of the docking algorithm and the scoring function used in this study if the results were within 2Å of the root-mean-squared-deviation (RMSD) value of ligand after re-docking in comparison with initial co-crystallized ligands.

50 saponin derivatives mentioned above were docked into eNOS and CYP2E1 proteins using Autodock Vina 1.5.6 [18]. The protein was kept rigid while the ligands were fully flexible. The Lamarckian Genetic Algorithm was used to search space for docking, and the binding affinity of a complex was calculated in empirical free energy function [21]. The binding ability of protein-ligand complex was evaluated by docking score or binding affinity (kcal.mol^{-1}). The lower docking score ligand gets, the more potential antioxidative stress ligand is because binding of the ligand into protein will lead to inhibition of enzyme activity [22]. Besides, analysis of the interaction between ligand and protein regarding the hydrogen bond, hydrophobic interaction, π-stacking interaction, Van der Waals force, and electrostatic interaction was conducted using BIOVIA Discovery Studio 2016.

RESULTS AND DISCUSSION:

Re-Docking Results: Re-docking (3R, 4R)-3 and 1H – indazole into their respective binding sites of eNOS and CYP2E1 were successfully performed (RMSD=1.6817 Å and 0.4942 Å respectively). We found that the binding conformations of re-docked ligands reproduced the binding modes of the co-crystallized ligands **Fig. 4a** and **Fig. 5a** with binding affinities of -8.0 kcal.mol^{-1} and -6.1 kcal.mol^{-1} for (3R, 4R)-3 and 1H - indazole, respectively. The hydrogen bonds and hydrophobic contacts of (3R, 4R)-3 with heme propionate D, Pro336 and Gly357 of eNOS after re-docking were showed in **Fig. 4b**.

In comparison with published data revealing that (3R, 4R)-3 forms hydrogen bonds with heme propionate D, Asn340 and Glu363 in the active site Pro336-Asp371 [23], there was a good agreement in the key interactions. Thus, the size and center of the coordinates of the grid box are validated, which

ensures that ligands bind to the binding pocket in the correct conformation. Similarly, when 1H – indazole were re-docked into CYP2E1, it created interactions with the identical amino acids such as Ala299 and Thr303 **Fig. 5b** that the previous studies reported[6]. As a result, this docking protocol was able to reproduce the correct pose.

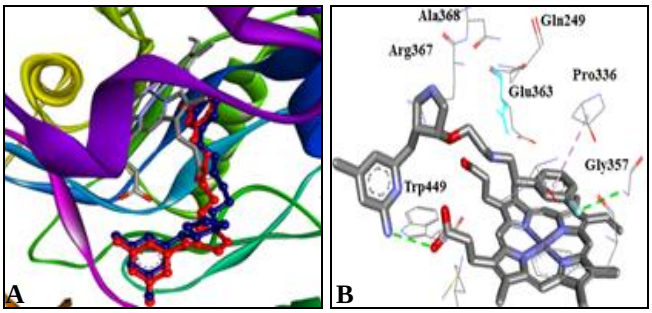

FIG. 4A-B: RE-DOCKING (3R, 4R)-3 INTO THE ACTIVE SITE OF eNOS

(A) Superimposition of (3R, 4R)-3 after re-docking (in red, the best pose) on its structure in the co-crystal structure (in blue) at the active site of eNOS. (B) The interactions between this pose and eNOS residues. (Pink lines: Hydrophobic interactions, green lines: Hydrogen bonds)

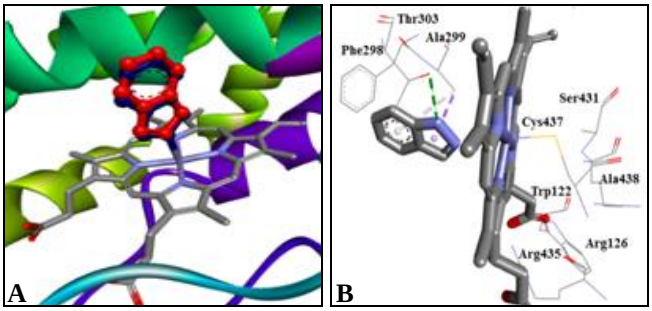

FIG. 5A-B: RE-DOCKING 1H – INDAZOLE INTO THE ACTIVE SITE OF CYP2E1

(A) Superimposition of 1H - indazole after re-docking (in red, the best pose) on its structure in the co-crystal structure (in blue) at the active site of CYP2E1. (B) The interactions between this pose and CYP2E1 residues. (Pink lines: Hydrophobic interactions, green lines: Hydrogen bonds)

Docking Scores: The docking scores of 50 saponin derivatives with eNOS and CYP2E1 range from -7.2 kcal.mol^{-1} to -9.1 kcal.mol^{-1} (**Table S2**) and from -6.2 kcal.mol^{-1} to -8.8 kcal.mol^{-1} (**Table S1**), respectively. Thus, these compounds bonded well with the binding sites of these enzymes and had better binding abilities with eNOS than CYP2E1. The compounds which have lower binding affinities (lower than -8.0 kcal.mol^{-1}) than the other saponin derivatives are the most potent inhibitors of both enzymes eNOS and CYP2E1. From a total of 50 saponin derivatives, the top six compounds, namely ginsenoside Rc, vina-ginsenoside R3, vina-ginsenoside R20, ginsenoside Re, notoginsenoside R1 and 20(R)-ginsenoside Rh1, were selected based on docking results. Among these compounds, ginsenoside Rc got the best binding affinity with eNOS (-9.1 kcal.mol^{-1}) and CYP2E1 (-8.6 kcal.mol^{-1}).

Interactions of Saponin Derivatives with Proteins: Most of the hydrogen bond donors came from protein residues, with the corresponding acceptors deriving from the saponin derivatives. Besides, hydrophobic contacts were formed between carbon atoms of these compounds and non-polar parts of amino acids. All of these hydrogen bonds and hydrophobic contacts were analyzed thoughtfully with the following criteria. First, regarding hydrogen bonds, there is a distance between proton donor (D) and acceptor (A) atoms of < 3.5 Å and an angle D-H…A of > 120^{o}[24]. Second, a limited range of 3.0-4.0 Å in the distance is utilized to evaluate hydrophobic interactions [25].

Saponin Derivatives with eNOS: The docked poses of 50 saponin derivatives fitted well into the binding site of 3NLE through forming hydrogen bonds and hydrophobic contacts. The majority of these saponin derivatives created number of interactions with the key interaction residues consisting of Gln249, Arg252, Ala268, Arg367, Trp449 and Tyr477 than the other amino acids. Analysis of interactions of 50 compounds with the residues in the binding site of eNOS was displayed in **Table S2**.

We found that saponin compounds, which created strong hydrogen bonds with Arg252, got better binding affinities than the others. Therefore, although there is a strong hydrogen bond with Arg252, vina-ginsenoside R12 gain a quite high docking score, -8.9 kcal.mol^{-1}.

Saponin Derivatives with CYP2E1: 50 saponin derivatives were docked well into in the binding pocket region of CYP2E1 and created hydrogen bonds and hydrophobic interaction with this site. Binding of CYP2E1 with these compounds showed

that the immediate 190 $Å^3$ - 470 $Å^3$ active site measured with different subjects bound is not large enough to accommodate a bulky steroid backbone and sugars moieties of saponin derivatives [26-27]. However, their interactions with the major residues Arg126, Arg134, Asn143, Arg344, and Arg444 revealed that they could be potential inhibitors of CYP2E1. **Table S1** illustrated the interactions of 50 compounds with CYP2E1.

TABLE S1: DOCKING RESULTS OF 50 SAPOIN DERIVATIVES WITH CYP2E1

	Compounds	Binding affinity (kJ/mol)	Hydrogen bond			Hydrophobic interaction	
			Interactions	Distance (Å)	Angle D-H…A	Residues	Distance (Å)
OCOTILLOL	Vina-ginsenoside R5	-8.3	Arg126 N-H…O	3.38	144.3	Arg134	3.80
			Arg127 N-H…O	3.06	126.0	Arg444	3.85
			Asn143 N-H…O	2.97	176.8		
			Arg344 N-H…O	3.09	166.1		
			Lys434 O…H-O	2.58	155.8		
	Vina-ginsenoside R6	-7.9	Asn143 N-H…O	3.16	153.7	Arg134	3.35
			Tyr423O-H…O	3.06	144.2		3.82
			Glu440 O…H-O	2.19	161.7		
			Gly441 O…H-O	1.93	143.0		
			Arg444 N-H…O	3.06	130.2		
	Vina-ginsenoside R2	-7.9	Gly139 N-H…O	3.03	120.9	Leu130	3.59
			Asn143 N-H…O	3.19	151.8	Arg134	3.91
			Tyr423 O…H-O	2.82	143.5		
	Majonoside R1	-7.8	Arg126 O…H-O	2.95	141.8	Leu130	3.94
			Asn143 N-H…O	2.96	176.0	Arg134	3.59
			Gly441 N-H…O	2.30	121.3		
	Vina-ginsenoside R11	-7.8	Asn143 N-H…O	2.87	126.5		
			Ala438 O…H-O	2.20	159.8		
	Vina-ginsenoside R14	-7.8	Leu133 N-H…O	2.53	129.7	Ile341	3.81
			Asn143 O…H-O	2.80	129.7		
			Ile341 O…H-O	2.61	128.8		
	Vina-ginsenoside R1	-7.6	Asn143 N-H…O	3.19	144.3	Val436	3.51
			Glu440 O…H-O	2.28	139.8		
	Majonoside R2	-7.5	Leu130 O…H-O	2.53	137.4	Leu130	3.78
			Gly433 O…H-O	2.14	144.0		
	24*(S)*- Pseudo-ginsenoside F_{11}	-7.5	Gly139 N…H-O	2.47	121.4		
	Pseudo-ginsenoside RT_4	-7.5	Asn143 N-H…O	3.07	132.6	Leu130	3.89
				3.29	145.4		
	Vina-ginsenoside R10	-6.9	Asn143 N-H…O	2.81	144.7		
				2.92	159.1		
			Gly433 O…H-O	2.59	125.1		
	Ginsenoside Rc	-8.6	Arg126 N-H…O	2.20	175.7	Leu133	3.99
			Thr131 O…H-O	2.75	151.1		
			Arg134 N-H…O	2.28	173.2		
	Vina-ginsenoside R20	-8.5	Asn143 N-H…O	2.88	148.3		
				2.90	157.6		
			Asp351 O…H-O	2.11	132.2		
			Cys437 O…H-O	2.17	134.4		
				2.22	131.5		
PROTOPANAXADIOL	Vina-ginsenoside R3	-8.1	Arg126 N-H…O	2.34	136.8	Arg134	3.68
	Vina-ginsenoside R21	-7.8	Asn143 N-H…O	2.85	154.7	Val436	3.6
				2.86	141.2		3.77
			Tyr423 O-H…O	2.76	158.9		
			Glu440 O…H-O	2.82	134.8		
			Arg444 N-H…O	3.20	146.3		
	Vina-ginsenoside R22	-7.7	Asn143 N-H…O	3.30	138.3		
	Vina-ginsenoside R16	-7.6	Arg344 N-H…O	2.93	142.4	Arg134	3.56
			Asp351 O…H-O	2.41	124.5		
			Glu440 O…H-O	2.55	122.0		
	Pseudo-ginsenoside Rc_1	-7.5	Arg127 N-H…O	3.27	150.1	Val436	3.65
			Leu130 O…H-O	2.56	128.9		
			Thr131 O-H…O	2.83	154.3		
			Lys434 O…H-O	2.29	147.1		
	Ginsenoside Rd	-7.4	Thr131 O…H-O	3.02	128.3		
			Lys434 O…H-O	2.83	129.0		

	Vina-ginsenoside R9	-7.3	Arg344 N-H…O	2.80	152.1	Arg134	3.27
				2.91	138.9		
			Gly441 O…H-O	2.21	150.2		
	Gypenoside XVII	-7.2	Thr131 O-H…O	2.97	167.1	Val436	3.79
			Gly139 N-H…O	3.13	136.6		
	Ginsenoside Rb_3	-7.2	Ala438 O-H…O	2.30	141.6	Ile341	3.75
	Vina-ginsenoside R7	-7.2	Arg126 N-H…O	2.94	151.7		
			Arg344 N-H…O	3.14	120.1		
			Asp351 O-H…O	2.32	140.5		
			Ala438 O-H…O	2.74	120.1		
	Vina-ginsenoside R8	-7.2	His355 N…H-O	2.24	155.9	Leu130	3.88
			Thr432 O…H-O	2.66	123.1		
			Glu440 O…H-O	2.01	152.3		
			Arg444 N-H…O	2.93	129.2		
				3.19	139.6		
				3.07	155.6		
	Majoroside-F1	-7.1	Asn143 N-H…O	3.10	151.6		
			Arg344 N-H…O	3.15	154.4		
			Arg444 N-H…O	2.96	158.4		
	Vina-ginsenoside R24	-7.1	Glu440 O…H-O	2.16	154.9	Ile341	3.78
			Arg444 N-H…O	2.89	150.8		
				2.91	147.5		
	Vina-ginsenoside R23	-7.0	Arg344 N-H…O	3.13	138.2	Val436	3.71
			Glu440 O…H-O	2.38	168.2		
	Ginsenoside Rb_1	-6.9	Gly139 N-H…O	2.83	125.9	Leu130	3.96
				2.97	171.5		
			Arg344 N-H…O	2.78	148.4		
			Asp351 O…H-O	2.28	126.6		
			Ala438 O…H-O	2.88	131.1		
			Arg444 N-H…O	3.05	141.1		
	Vina-ginsenoside R13	-6.9	Leu133 O…H-O	2.39	120.4	Arg444	3.88
			Gly139 N-H…O	2.56	137.4		
			Asn143 N-H…O	2.88	130.7		
			Arg444 N-H…O	3.09	156.0		
	Notoginsenoside Fa	-6.8	Arg126 N-H…O	2.81	136.9	Arg134	4.00
			Tyr423 O…H-O	1.87	171.1		
			Tyr423 O-H…O	2.84	129.3		
			Glu440 O…H-O	1.91	149.3		
			Arg444 N-H…O	2.84	153.9		
				2.98	144.5		
	Ginsenoside Rb_2	-6.8	Arg126 N-H…O	3.15	155.5		
			Gly139 O…H-O	2.40	142.3		
			Ile341 O…H-O	2.40	141.2		
			Arg344 N-H…O	2.84	136.1		
			Cys437 O…H-O	2.88	125.0		
	Gypenoside IX	-6.4	Arg444 N-H…O	3.0	163.7		
	Quinquenoside R_1	-6.2	Arg126 N-H…O	2.82	127.6	Met445	3.84
				1.87	152.7		
			Lys434 O…H-O	2.22	154.2		
	Ginsenoside Re	-8.8	Asn143 N-H…O	3.15	173.8	Arg134	3.71
			Arg344 N-H…O	3.02	156.6		3.91
			Gly441 O…H-O	2.25	123.5		
	Notoginsenoside R1	-8.2	Asn143 N-H…O	3.17	135.5		
			Asp351 O…H-O	2.37	121.6		
				2.88	135.2		
			Ala438 O…H-O	2.11	133.4		
			Arg444 N-H…O	3.18	132.3		
	20(R)-Ginsenoside Rh_1	-8.0	Ser431 O…H-O	2.07	131.2	Leu133	3.48
			Glu440 O…H-O	1.88	165.0	Val436	4.00
			Arg444 N-H…O	3.14	134.0	Leu442	3.75
				3.37	165.6		
	Ginsenoside Rh_4	-7.9	Asn143 N-H…O	3.14	147.7	Ile341	3.63
				3.20	152.7		
	Ginsenoside Rg_1	-7.8	Asn143 N-H…O	2.82	129.0	Arg444	3.89
			Asp351 O…H-O	2.76	145.0		
			Arg444 N-H…O	3.17	122.2		
	Pseudo-ginsenoside Rs_1	-7.7	Arg127 N-H…O	3.05	124.0		

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			Asn143 N-H…O	3.28	150.8		
	Vina-ginsenoside R17	-7.7	Arg126 N-H…O	2.80	154.4		
			Asn143 N-H…O	2.90	123.9		
			Ile341 O…H-O	2.30	135.7		
			Arg344 N-H…O	3.10	137.7		
				3.34	123.3		
	Vina-ginsenoside R19	-7.6	Arg126 N-H…O	3.02	146.4		
			Tyr423 O-H…O	3.03	140.7		
			Glu440 O…H-O	2.51	143.3		
			Arg444 N-H…O	3.19	152.7		
				3.31	127.3		
	20-Glucoginsenoside Rf	-7.5	Asn143 N-H…O	3.11	169.4		
			Tyr423 O-H…O	2.70	131.3		
				2.83	120.4		
			Cys437 O…H-O	2.08	120.7		
			Glu440 O…H-O	1.83	173.4		
			Arg444 N-H…O	2.88	148.5		
	Vina-ginsenoside R12	-7.5				Leu130	3.67
	Vina-ginsenoside R15	-7.4	Asn143 N-H…O	2.87		Arg444	3.96
			Ile341 O…H-O	2.42			
			Arg344 N-H…O	3.17			
	20(S)-Ginsenoside Rh_1	-7.3	Arg126 N-H…O	3.14	144.5	Met445	3.73
			Asn143 N-H…O	3.14	160.5		
	Notoginsenoside R6	-7.3	Asn143 N-H…O	2.81	144.7		
				2.93	159.1		
			Gly433 O…H-O	2.59	125.1		
	Vina-ginsenoside R25	-7.3	Asn143 N-H…O	2.96	134.2		
			Ile341 O…H-O	2.03	142.7		
			Ala438 O…H-O	2.34	152.8		
	Vina-ginsenoside R18	-7.1	Arg126 N-H…O	2.87	136.4		
			Gly139 N-H…O	3.10	171.8		
			Asn143 N-H…O	2.87	124.8		
			Arg344 N-H…O	3.20	135.4		
				3.16	130.3		
			Val436 O…H-O	3.06	132.1		
	Vina-ginsenoside R4	-6.8	Ala438 O…H-O	2.69	137.0	Arg134	3.95
						Ile341	3.73
	Ginsenoside Rh_5	-6.5	Asn143 N-H…O	3.06	154.0		
				3.14	153.8		

TABLE S2: DOCKING RESULTS OF 50 SAPONIN DERIVATIVES WITH eNOS

	Compounds	**Binding affinity (kJ/mol)**	**Hydrogen bond**			**Hydrophobic interaction**	
			Interactions	**Distance (Å)**	**Angle D-H…A**	**Residues**	**Distance (Å)**
OCOTILLOL	Vina-ginsenoside R6	-8.9	Ser248 O-H…O	2.93	122.4	Val106	3.8
			Gln249 N-H…O	3.13	128.5		
			Arg374 N-H…O	2.93	120.3		
	24*(S)*- Pseudo-ginsenoside F_{11}	-8.6	Arg252 N-H…O	3.23	143.5	Ala268	3.72
			Tyr477 O-H…O	2.81	164.8		3.64
	Majonoside R1	-8.6	Arg367 N-H…O	2.74	124.8	Val106	3.77
			Arg374 N-H…O	3.05	133.2		
				3.08	159.6		
	Majonoside R2	-8.6	Ser248 O…H-O	2.21	137.3	Val106	3.74
			Arg252 N-H…O	3.20	132.9	Trp449	3.83
			Arg367 N-H…O	2.75	120.5		
			Ala448 O…H-O	2.33	123.0		
	Vina-ginsenoside R2	-8.4	Trp246 O…H-O	2.56	152.8	Val106	3.53
			Arg252 N-H…O	3.32	133.1	Trp449	3.92
			Ala448 O…H-O	2.29	138.1		
	Pseudo-ginsenoside RT_4	-8.1	Ser248 O…H-O	2.02	158.6		
			Arg252 N-H…O	2.80	142.0		
			Arg367 N-H…O	3.14	125.0		
			Tyr477 O…H-O	3.16	140.3		
	Vina-ginsenoside R11	-8.1	Ala268 O…H-O	2.09	153.2		
			Glu363 O…H-O	2.50	124.7		
			Arg367 N-H…O	3.16	126.1		
	Vina-ginsenoside R5	-7.8	Ala268 O…H-O	2.07	145.3		

			Tyr477 O-H…O	2.70	139.2		
			Tyr477 O…H-O	1.89	156.5		
				1.91	136.5		
	Vina-ginsenoside R10	-7.8	Asn340 O…H-O	1.82	158.5	Trp449	3.68
	Vina-ginsenoside R14	-7.8	Gln249 N-H…O	2.80	132.4	Trp449	3.87
			Tyr477 O…H-O	2.22	124.2		
			Tyr477 O-H…O	2.88	172.6		
			Asp480 O…H-O	2.01	145.0		
				2.27	153.4		
	Vina-ginsenoside R1	-7.6	Arg252 N-H…O	3.14	128.9	Trp449	3.8
			Ala448 O…H-O	2.49	170.5		
	Ginsenoside Rc	-9.1	Arg252 N-H…O	2.36	140.9		
			Glu271 N-H…O	2.74	166.3		
			Arg367 N-H…O	2.95	155.9		
				2.99	143.3		
				3.26	126.0		
			Arg374 N-H…O	3.21	152.4		
	Vina-ginsenoside R3	-8.9	Gln249 N-H…O	3.10	128.4	Ala268	3.67
			Ala268 O…H-O	2.35	144.5	Trp449	3.76
			Glu363 O…H-O	2.55	125.5		3.86
			Tyr477 O-H…O	2.79	135.6		
			Asp480 O…H-O	2.18	164.5		
	Vina-ginsenoside R23	-8.9	Gln249 N-H…O	3.14	120.3	Leu107	3.96
			Arg252 N-H…O	3.07	170.0		
			Tyr477 O-H…O	2.88	141.5		
			Asp480 O…H-O	1.97	140.9		
				2.36	145.8		
	Majoroside-F1	-8.8	Ser248 O…H-O	2.37	136.9		
			Arg252 N-H…O	2.80	143.4		
				2.96	161.2		
	Pseudo-ginsenoside Rc_1	-8.8	Arg252 N-H…O	2.85	129.1	Val106	3.53
			Glu271 O…H-O	2.31	162.7	Ala268	3.65
			Asn340 O…H-O	2.16	127.3		3.88
			Arg374 N-H…O	2.73	155.9	Trp449	3.66
	Quinquenoside R_1	-8.6	Arg252 N-H…O	3.05	132.5	Val106	3.43
				3.09	166.9		
	Vina-ginsenoside R20	-8.4	Gln249 N-H…O	3.13	132.6		
			Arg252 N-H…O	3.39	155.5		
			Ala268 O…H-O	2.36	146.6		
			Asn269 O…H-O	1.90	126.5		
			Glu363 O…H-O	2.09	129.0		
	Gypenoside XVII	-8.3	Gln249 N-H…O	3.07	133.8	Val106	3.81
			Arg252 N-H…O	3.01	139.2	Trp449	3.66
			Ala268 O…H-O	2.01	157.2		3.93
			Asp371 O…H-O	2.49	137.4		
			Arg374 N-H…O	2.85	147.9		
	Vina-ginsenoside R8	-8.3	Arg252 N-H…O	2.80	137.6		
				3.13	142.8		
			Ala268 O…H-O	2.24	142.8		
			Asn368 O…H-O	2.05	152.7		
	Vina-ginsenoside R21	-8.1	Gln249 N-H…O	3.14	167.7		
			Glu271 O…H-O	2.29	121.4		
			Asp371 O…H-O	2.11	134.3		
			Arg374 N-H…O	3.15	136.6		
			Tyr477 O-H…O	3.31	135.0		
	Gypenoside IX	-8.0	Gln249 N-H…O	3.26	155.2		
			Arg252 N-H…O	2.93	128.4		
			Tyr333 O-H…O	2.70	146.3		
			Arg374 N-H…O	3.26	147.6		
	Vina-ginsenoside R16	-8.0	Arg252 N-H…O	2.91	174.3	Trp449	3.75
				3.18	128.8		3.92
			Asn368 O…H-O	2.51	133.9		
			Asp480 O…H-O	2.16	154		
	Vina-ginsenoside R22	-8.0	Gln249 N-H…O	2.78	124.9	Ala268	3.88
			Ala268 O…H-O	1.98	166.6		
			Arg252 N-H…O	2.60	147.1		
				3.09	124.6		

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				3.29	131.9		
			Asn340 O…H-O	1.99	172.8		
	Ginsenoside Rb_1	-7.9	Gln249 N-H…O	2.80	138.5	Leu107	3.68
			Arg252 N-H…O	2.93	164.4	Trp449	3.62
			Ala268 O…H-O	2.24	131.6		3.92
			Arg367 N-H…O	3.00	170.4		
			Asp480 O…H-O	1.77	160.1		
	Ginsenoside Rd	-7.9	Gln249 N-H…O	3.07	122.4	Trp449	3.66
			Arg252 N-H…O	3.05	147.5		
			Ala268 O…H-O	2.07	132.7		
			Asn340 O…H-O	2.43	145.3		
			Arg367 N-H…O	3.19	128.5		
			His373 N…H-O	2.20	154.6		
			Tyr477 O-H…O	2.80	145.0		
	Vina-ginsenoside R24	-7.8	Arg252 N-H…O	2.79	137.7		
			Ala268 O…H-O	1.89	163.6		
			Arg367 N-H…O	2.87	158.6		
				3.30	122.3		
	Notoginsenoside Fa	-7.7	Gln249 N-H…O	2.88	146.3	Ala268	3.87
				2.89	122.1		
			Arg252 N-H…O	2.80	173.0		
				2.97	136.3		
	Vina-ginsenoside R7	-7.6	Arg374 N-H…O	3.15	134.8	Trp449	3.63
			Tyr477 O-H…O	2.69	128.1		
	Vina-ginsenoside R9	-7.4	Glu363 O-H…O	2.51	151.0		
			Arg367 N-H…O	2.93	126.8		
			Asp371 O-H…O	2.09	163.5		
			Asp480 O-H…O	2.15	147.0		
				2.34	134.0		
	Ginsenoside Rb_2	-7.3	Ser248 O…H-O	2.44	146.8		
			Arg367 N-H…O	3.17	128.3		
				3.32	164.1		
			Asp371 O…H-O	2.04	133.2		
			His373 N…H-O	2.52	122.5		
			Arg374 N-H…O	2.97	144.7		
	Ginsenoside Rb_3	-7.3	Asn340 O…H-O	2.36	140.8	Trp449	3.54
			Tyr477 O…H-O	2.01	150.6		3.62
				2.90	176.4		
			Asp480 O-H…O	1.99	175.0		
	Vina-ginsenoside R13	-7.2	Gln249 N-H…O	3.02	132.2	Tyr477	3.66
			Arg367 N-H…O	3.27	152.1		
			Tyr477 O…H-O	1.94	157.9		
	20(R)-Ginsenoside Rh_1	-9.1	Arg252 N-H…O	3.12	152.0	Ala268	3.84
			Tyr477 O-H…O	3.01	151.4		
			Tyr477 O…H-O	2.18	143.4		
			Asp480 O…H-O	2.32	120.9		
				2.49	148.1		
	Ginsenoside Rh_4	-9.1	Arg252 N-H…O	2.79	137.4	Ala268	3.85
			Tyr477 O-H…O	3.11	149.1	Val338	3.65
			Asp480 O…H-O	2.16	128.0		
				2.29	140.2		
PROTOPANAXATRIOL	Notoginsenoside R6	-8.9	Gln249 N-H…O	3.10	164.5		
			Arg252 N-H…O	3.04	147.3		
			Asp480 O…H-O	2.44	126.3		
	Vina-ginsenoside R12	-8.9	Arg252 N-H…O	2.31	138.4		
			Glu363 O…H-O	2.00	149.2		
			Arg374 N-H…O	2.25	124.6		
			Tyr477 O-H…O	2.96	134.2		
	Notoginsenoside R1	-8.8	Ser248 O…H-O	2.41	130.8		
			Ser248 O-H…O	3.14	120.4		
			Arg252 N-H…O	2.69	151.8		
				3.20	126.7		
			Glu271 O…H-O	2.34	130.3		
	Vina-ginsenoside R4	-8.7	Ser248 O…H-O	2.44	123.8	Val106	3.50
			Gln249 N-H…O	3.04	127.9	Trp449	3.62
			Arg252 N-H…O	2.93	138.4		3.99
				2.98	147.2		

Vina-ginsenoside R15	-8.6	Gln249 N-H…O	2.96	123.8		
		Arg252 N-H…O	2.88	131.4		
			3.04	142.8		
Ginsenoside Re	-8.5	Ser248 O-H…O	3.12	120.8	Trp449	3.68
		Gln249 N-H…O	2.83	125.3		3.89
			3.05	173.0		3.97
		Arg252 N-H…O	2.84	165.8		
			3.05	129.6		
		Asn269 O…H-O	1.99	129.7		
		Glu271 O…H-O	2.12	145.1		
Ginsenoside Rh_5	-8.4	Gln249 N-H…O	3.17	122.4	Ala268	3.62
		Arg367 N-H…O	3.14	152.3	Trp449	3.82
Pseudo-ginsenoside Rs_1	-8.4	Arg252 N-H…O	3.03	124.1	Val106	3.74
		Arg367 N-H…O	3.13	154	Arg367	3.90
		Tyr477 O-H…O	3.20	173	Trp449	3.68
						3.99
Ginsenoside Rg_1	-8.3	Arg252 N-H…O	2.92	156.3	Val106	3.74
			3.02	144.2	Ala268	3.71
		Ala268 O…H-O	2.04	171.3		
20(S)-Ginsenoside Rh_1	-8.3	Arg252 N-H…O	3.09	150.1		
		Asn340 O…H-O	2.48	137.1		
		Tyr477 O-H…O	2.90	122.1		
Vina-ginsenoside R17	-8.3	Glu363 O…H-O	1.60	151.4	Ala268	3.7
			2.22	151.5	Ile272	3.61
			2.35	128.2		
		Asn368 O…H-O	1.90	154.8		
		Arg374 N-H…O	2.40	144.3		
		Asp480 O…H-O	2.16	150.7		
20-Glucoginsenoside Rf	-8.0	Gln249 N-H…O	3.05	126.7		
		Tyr477 O…H-O	2.18	150.1		
		Tyr477 O-H…O	3.06	122.6		
Vina-ginsenoside R19	-8.0	Trp246 O…H-O	2.38	123.7		
		Asn340 O…H-O	2.37	162.6		
		Arg374 N-H…O	3.19	144.0		
		Tyr477 O-H…O	3.10	129.7		
		Asp480 O…H-O	2.10	156.9		
Vina-ginsenoside R25	-7.8	Ser248 O…H-O	2.61	141.0		
		Ala268 O…H-O	1.98	147.0		
			2.24	154.9		
Vina-ginsenoside R18	-7.6	Glu271 O…H-O	1.85	166.3	Tyr477	3.96

Interestingly, some chemicals just set up a hydrogen bond or hydrophobic contact with CYP2E1 although they got good binding affinities. For example, vina-ginsenoside R3 have docking score of 8.1 kcal.mol^{-1} but it just formed a hydrophobic contact with Arg134. In contrast, despite the formulation of 3 hydrogen bonds with Arg126, Lys434 and Arg444 and hydrophobic interaction with Met445, Quinquenoside R1 had the highest binding affinity, -6.2 kcal.mol^{-1}.

DISCUSSION: During the last 40 years or so, oxidative stress has been increasingly recognized as a contributing factor in aging and a long list of several other human diseases such as cancer, cardiovascular diseases, diabetes mellitus type 2, and neurodegenerative diseases… because free radicals damage biological macromolecules [28]. Previous studies have concentrated on free radicals - removed strategies using either antioxidants or drugs that enhance endogenous antioxidants [29]. However, many antioxidants have not successfully passed the scrutiny of clinical trials for the prevention and treatment of various diseases [5]. Furthermore, reports of toxicological studies linked some synthetic antioxidants to liver damage, cancer and other diseases [30]. Therefore, inhibition of free radical-produced enzymes is also another seemingly promising antioxidative stress strategy [5].

In this study, we found that a series of saponin derivatives, especially ginsenoside Rc, vina-ginsenoside R3, vina-ginsenoside R20, ginsenoside Re, notoginsenoside R1 and 20(R)-ginsenoside Rh1, could significantly inhibit the activities of eNOS and CYP2E1 because of their low binding affinities (lower than -8.0 kcal.mol^{-1}) in the binding sites of these proteins. These active sites were validated by the re-docking procedure under the criterion of RMSD < 2 Å and being fitted well of

re-docked poses in binding pockets. Among these hit compounds, ginsenoside Rc gained the best binding affinity with eNOS (-9.1 kcal.mol^{-1}) and CYP2E1 (-8.6 kcal.mol^{-1}).

The results of binding modes of these compounds were analyzed to show details of interactions with key amino acids. The important residues involved in forming H-bonds and hydrophobic contacts between ligands and these two enzymes in **Table 2**. For most of the investigated hit compounds, their interactions with the proteins were from H-bonds rather than hydrophobic contacts because the presence of sugar moieties reduces the hydrophobic character of the compounds. Regarding ligands containing methyl, akyl group, and cyclohexane, hydrophobic interactions were often presented with Val106, Leu107, His373, Trp449 and Trp477 of eNOS and Arg126 of CYP2E1. Hydroxyl group of sugar moieties of ligands formed H-bonds with Gln249, Glu363, and Arg367 in the binding site of eNOS and Arg126, Ser431, Cys437, Ala438 in the counterpart of CYP2E1. Analysis of interactions between ginsenoside Rc and both enzymes, eNOS and CYP2E1, as illustrated in **Fig. 6**.

TABLE 2: THE IMPORTANT RESIDUES IN THE BINDING POCKETS OF eNOS AND CYP2E1 AND THE RESIDUES INVOLVED IN FORMING H-BONDS AND HYDROPHOBIC CONTACTS BETWEEN THE PROTEINS AND LIGANDS IN BOLD

Proteins	Residues making up the binding pocket
eNOS	Val106, Leu107, Gln249, Pro336, Val338, Phe355, Gly357, Glu363, Arg367, His373, Trp449, Tyr477
CYP2E1	Trp122, Arg126, Phe298, Ala299, Thr303, Ser431, Arg435, Cys437, Ala438, Ala443

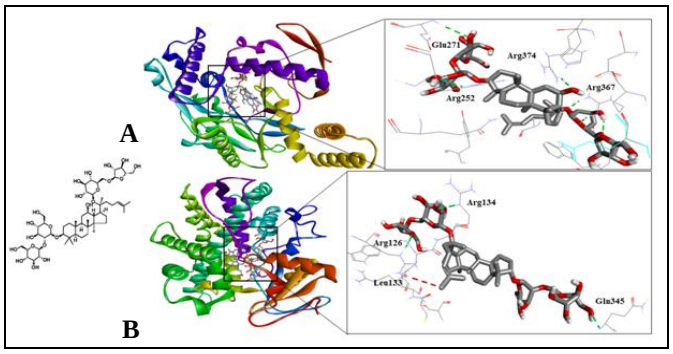

FIG. 6A-B: INTERACTIONS OF GINSENOSIDE Rc WITH PROTEINS

(A) Interactions between ginsenoside Rc and eNOS. (B) Interactions between ginsenoside Rc and CYP2E1. (Red lines: Hydrophobic interactions, green lines: Hydrogen bonds).

According to a large number of previous investigations on ginseng, the antioxidative ability of ginsenoside Rc, ginsenoside Re and 20(R)-Ginsenoside Rh_1 in free radical-induced hemolysis of human erythrocytes are proved [31]. Especially, ginsenoside Re possesses significant antioxidant efficiency in diabetic rat [32] and cardiomyocytes of chink [33].

Besides, the former scientists demonstrated that notoginsenoside R1 is capable of scavenging free radical, abating the lipoxidation and increasing the activity of antioxidases, thus suppressing oxidative stress in *ex-vivo* and *in-vitro* experiments [34]. They also reported that notoginsenoside R1 could decrease the level of oxidative stress and inflammation in atherosclerotic mice [35]. These evidence are contributed factors to the inhibited abilities of top 6 compounds against eNOS and CYP2E1.

CONCLUSION: Development of antioxidative stress agents from phytochemicals is important in modern drug discovery. In the present study, an in-house library of 50 saponin derivatives was screened. This result proves that ginsenoside Rc, vina-ginsenoside R3, vina-ginsenoside R20, ginsenoside Re, notoginsenoside R1 and 20(R)-ginsenoside Rh1 were an efficient therapeutic candidate to treat oxidative stress - associated diseases. Further, studies on these 6 hits could be carried out to validate their antioxidative stress activity and drug design. Furthermore, docking is a very useful computational tool to screen the antioxidant ability of these derivatives targeting another oxidative stress-related enzymes.

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CONFLICT OF INTEREST: The authors declared that there is no conflict of interest.

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