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In Silico Screening of Ellagic Acid Derivatives and Their Metabolites as Inhibitors for NF-Kb and Tnfα

Sullim Lee
Department of Life Science
College of Bio-Nano Technology, Gachon University
Seongnam-si, Gyeonggi-do, Republic of Korea
sullimlee@gachon.ac.kr

Changho Jhin*
Department of Research and Development
1778 LivingTech. Co., Ltd.
Sejong-si, Republic of Korea
cto@1778tech.com
*Corresponding Author

Abstract— The aim of this study was to evaluate inhibitory effects of ellagic acid derivatives and their metabolites on tumor necrosis factor α (TNFα) and nuclear factor-κB (NF-κB). *In silico* molecular docking was performed. 3-O-methyl-3', 4' -methylenedioxy ellagic acid (MMDEA) and urolithin D were to be potent inhibitors of TNFα among tested molecules. For NF-κB (p50 and p65 subunits), MMDEA was found to be a potent inhibitor comparable with tanshinone IIA, a known NF-κB inhibitor. Ellagic acid derivatives seemed to be more effective than urolithins, their metabolites. All of the ellagic acid derivatives and their metabolites were predicted to have good pharmacokinetical properties according to Lipinski's rule of five. These results suggest that ellagic acid derivatives may potentially down-regulate inflammatory responses..

Keywords- Ellagic acid, Molecular docking, TNFα, NF-κB

I. INTRODUCTION

Ellagic acid (EA) is a polyphenolic compound rich in various fruits and greens such as pomegranates, blackberries and grapes [1]. The structure of EA and its derivatives are presented on Figure 1 (A) - (D). These compounds are generated by hydrolysis of ellgitannins [2], [3]. As metabolites of ellagic acid derivatives, urolithins were produced by intestinal microbes [4]–[6].

Tumor necrosis factor alpha (TNFα) is a cytokine involving activation of a transcription factor of nuclear factor kappa B (NF-κB). TNFα and NF-κB play key role in regulating inflammatory diseases such as inflammatory bowel disease, Crohn's disease and cancer [7]–[9]. Therefore, regulation of TNFα and NF-κB is important for anti-inflammatory responses. TNFα and NF-κB have been focused as target for drug development. He et al. [10] reported the interaction between TNFα and an inhibitory small molecule. Binding site interactions of NF-κB, as a drug target, were also analysed by previous studies [11]–[13].

In this study, as potent TNFα and NF-κB inhibitors, efficacies of ellagic acid derivatives were evaluated by molecular docking. Since not only ellagic acid derivatives, but also their metabolites could have effects on target proteins, ellagic acid metabolites were also evaluated. 3'-O-methyl ellagic acid (MEA), ellagic acid (EA), 3,3'-di-O-methyl ellagic acid (DMEA) and 3-O-methyl-3',4'-methylenedioxy ellagic acid (MMDEA) were evaluated as ellagic acid derivatives, and urolithin A (UA), urolithin B (UB), urolithin C (UC) and urolithin D (UD) were evaluated as metabolites. The structures of the tested molecules were illustrated on Figure 1.

II. MATERIALS AND METHODS

A. Calculation of molecular properties

For evaluating ADME properties of ligands, molecular properties of logP, molecular weight, hydrogen bond acceptors, hydrogen bond donors, number of atoms (natoms), number of rotatable bonds (nrotb) and total polar surface area (TPSA) were calculated by Molinspiration (http://www.molinspiration.com/).

B. Ligands and Macromolecules preparation

The structures of EA and tanshinone IIA were downloaded as mol2 format from ZINC database [14]. The structures of ellagic acid derivatives and their metabolites were generated by modifying structure of EA using Gabedit 2.4.7 [15]. As a known inhibitor of TNFα, ligand307 (ligand ID: 307) was separated from X-ray crystal structure of TNFα with a small molecule inhibitor (PDB ID: 2AZ5) obtained from RCSB protein data bank (http://rcsb.org). For macromolecules, NF-κB p50 and p65 subunits were obtained by separating chain A from X-ray crystal structures of NF-κB p50 homodimer (PDB ID: 1NFK) and NF-κB p50·p65 heterodimer (PDB ID: 1LE9). For TNFα, chain C and D were separated from X-ray crystal structure of TNFα with a small molecule inhibitor (PDB ID: 2AZ5). All of the solvent molecules were removed from the macromolecule structures. Since all the ligands and macromolecules did not contain hydrogen atoms on their structures, hydrogen atoms were added by Add Hydrogen module in UCSF Chimera [16].

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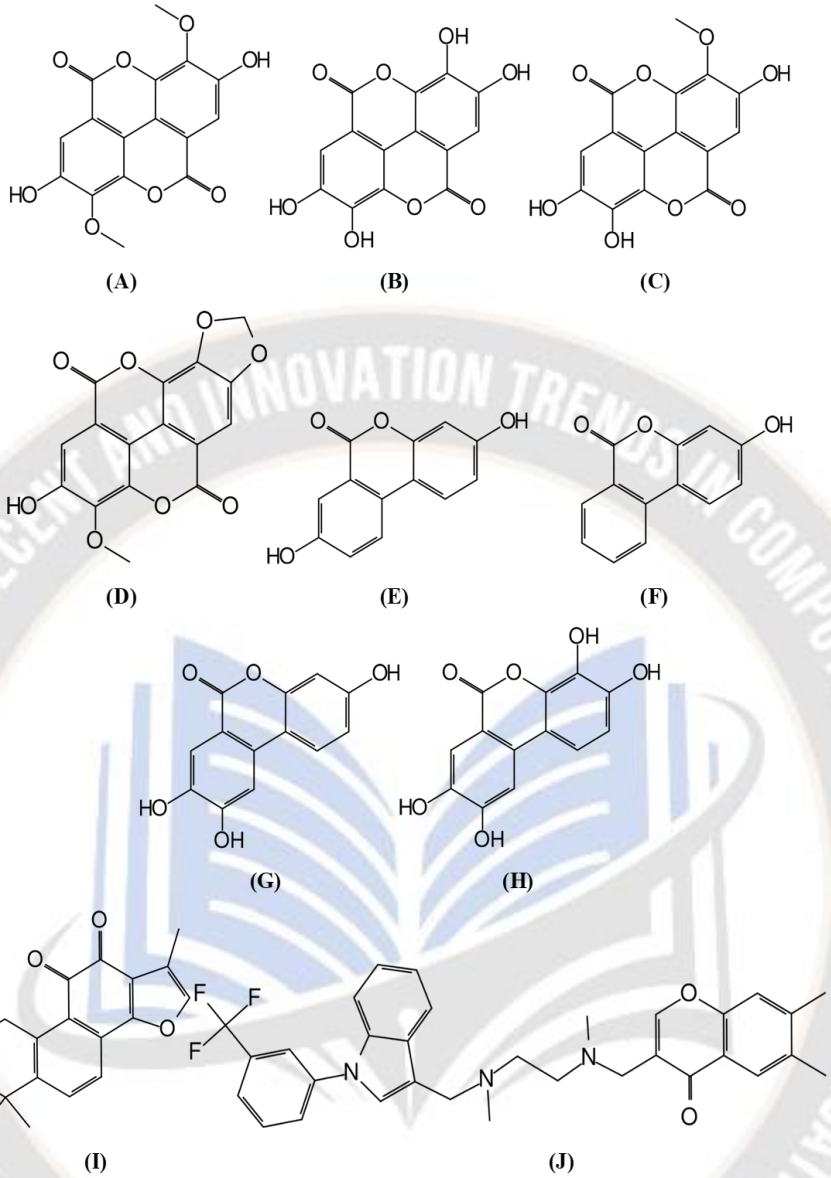

Figure 1. Structures of (A) 3'-O-methyl ellagic acid, (B) ellagic acid, (C) 3,3'-di-O-methyl ellagic acid, (D) 3-O-methyl-3',4'-methylenedioxy ellagic acid, (E) urolithin A, (F) urolithin B, (G) urolithin C, (H)

C. Molecular docking

Molecular docking simulation was performed by Autodock Vina [17] (version 1.1.2), Autodock4 [18] (version 4.2.5.1) and DOCK6 [19] (version 6.6) on AMD FX 8-core system with Linux OS (kernel version 3.5.0). Before performing molecular docking by Autodock Vina and Autodock4, non-polar hydrogen atoms were abstracted, Gasteiger partial charges were assigned to ligands and macromolecules, and ligands and macromolecules were converted into pdbqt format using Auto Dock Tools 1.5.6 [18]. Lamarckian genetic algorithm was applied to docking experiments by Autodock4, with maximum 2.5 million energy evaluations, gene mutation rate of 0.02 and crossover rate of 0.8. For Autodock Vina, the exhaustiveness parameter was increased by 100. Before performing molecular docking by DOCK6, AM1-BCC partial charges were assigned to the ligands and macromolecules by UCSF Chimera [16]. Two different docking methods were applied for docking by DOCK6; rigid ligand docking method (RGD) and flexible ligand docking method (FLX). Simulation boxes for NF-κB p50 and p65 subunits were constructed large enough to cover the DNA binding regions and adjacent amino acids. For TNFα, the simulation boxes were constructed to cover the binding site of ligand307. UCSF Chimera was used to analyse hydrogen bonds and contacts between ligands and macromolecules. Besides default scoring functions of Autodock4, Autodock Vina and DOCK6, a scoring function from X-score [20] software was also adapted to obtain the best docked conformation and affinity. The output structure files generated from Autodock4, Autodock Vina and DOCK6 were converted into mol2 format by Openbabel [21] (version 2.3.2) and used as input files for evaluating binding affinities by X-score.

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III. Results and Discussion

The molecular physicochemical properties were predicted by Molinspiration for evaluating ADME (absorption, distribution, metabolism, and excretion) properties. The predicted properties of ligands were presented on Table I. According to Lipinski's rule of five [22], drug-like molecules which have no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, a molecular weight of less than 500 daltons, and logP below 5 were estimated to have good ADME properties. Also, improved drug-likeness was observed on molecular with TPSA of less than 140 $Å^2$, number of total atoms from 20 to 70 and less than 10 rotatable bonds [23], [24]. DMEA, MEA and MMDEA met these criteria, and the TPSA of EA was predicted as 141.3 $Å^2$, slightly over 140 $Å^2$. Urolithins also predicted to have good ADME properties according to Lipinski's rule of five, but the number of atoms were lesser than 20. Tanshinone IIA, known NF-κB inhibitor, also well met the criteria. However, ligand307 had two violations in Lipinski's rule of five, logP and molecular weight, thus orally intake of ligand307 seemed to be ineffective. Since TNFα inhibitory effect of ligand307 was revealed via *in vitro* assay [10], drug delivery and ADME for oral intake was not considered. As a result, ellagic acid and their derivatives seemed to be less effective TNFα inhibitors than ligand307, but the molecular properties were more adequate than ligand307 for ADME.

The free energies between ligands and TNFα are presented in Table II. The hierarchical orders of free energies resulted from Autodock Vina and Autodock 4 had similar tendencies. From the results by Autodock Vina and Autodock 4, the docking free energies of MMDEA and UD were the lowest among the ellagic acid derivatives and ellagic acid metabolites, respectively. However, in case of DOCK6, the lowest docking free energies were observed on MEA by both of RGD and FLX methods. This difference in the hierarchical order of free energy may have resulted from differences in scoring functions. The scoring functions of Autodock Vina and Autodock4 imply solvent effect by implicit solvent method, meanwhile the standard scoring function of DOCK6 neglects solvent effect. It could be explained by comparing the TPSA of the tested molecules. The TPSA of MMDEA (108.4 $Å^2$) is smaller than that of other ellagic acid derivatives (119.3 – 141.3 $Å^2$). Therefore, rather than other ellagic acid derivatives, MMDEA supposed to be little affected by solvent interaction that lowering protein-ligand affinity and increasing free energy. Also, neglected solvent interaction on DOCK6 scoring function could be a cause of large free energy differences compared with free energies calculated by Autodock Vina and Autodock4.

For further analysis, conformations of docked ligands and interaction with TNFα were analysed. Resulted conformations of ligand307 by different docking softwares and methods were compared with the X-ray crystallized structure (Figure 2). Only the docked ligand307 structure by Autodock Vina was precisely predicted (RMSD < 2.0 Å). Therefore, the ligand conformations resulted by Autodock Vina were used for further analysis. There were no hydrogen bond found between ligand307 and TNFα. The binding free energies, inhibition constants (Ki) and ligand-contact residues of TNFα are presented on Table III. All of the ligands were in contact with residues of TYR59, TYR119, LEU120 and GLY121 of chain C and D. Amongst the residues that in contact with ligands, LEU57 was suggested as a key residue for TNFα inhibitory effect. Only two ligands, ligand307 and MMDEA, were in contact with LEU57 among tested ligands, and the first and second lowest binding free energies were observed on ligand307 and MMDEA with TNFα, respectively. However, the binding free energy and Ki of ligand307 were lower than those of MMDEA. Same tendency was observed on Ki calculated by X-score. Ki of MMDEA and ligand307 were 2.45 μM and 0.10 μM, respectively. To sum up, MMDEA was the most potent TNFα inhibitor among ellagic acid derivatives and their metabolites, but it did not seem to be

Table I. Physicochemical properties of ligands

	logP	Molecular weight	H-bond acceptors	H-bond donors	Number of atoms	number of rotatable bonds	TPSA($Å^2$)
DMEA	1.50	330.2	8	2	24	2	119.3
EA	0.94	302.2	8	4	22	0	141.3
MEA	1.22	316.2	8	3	23	1	130.3
MMDEA	1.85	328.2	8	1	24	1	108.4
UA	2.12	228.2	4	2	17	0	70.7
UB	2.62	212.2	3	1	16	0	50.4
UC	1.63	244.2	5	3	18	0	90.9
UD	1.37	260.2	6	4	19	0	111.1
Tanshinone IIA	4.16	294.4	3	0	22	0	47.3
Ligand307	6.36	547.6	5	0	40	9	41.6

Table II. Predicted binding free energies (kcal/mol) of ellagic acid derivatives and their metabolites with TNFa by different methods

	Autodock Vina	Autodock4	Dock6 rigid	Dock6 flex
DMEA	-7.5	-6.9	-34.0	-31.1
EA	-7.6	-6.6	-33.9	-34.6
MEA	-7.6	-6.9	-34.6	-34.8
MMDEA	-8.0	-7.5	-32.7	-34.6
UA	-7.2	-6.7	-26.4	-27.3
UB	-7.2	-6.7	-25.4	-26.7
UC	-7.7	-7.0	-27.7	-29.1
UD	-8.0	-7.0	-30.1	-30.4
Ligand307	-8.8	-11.1	-40.2	-50.0

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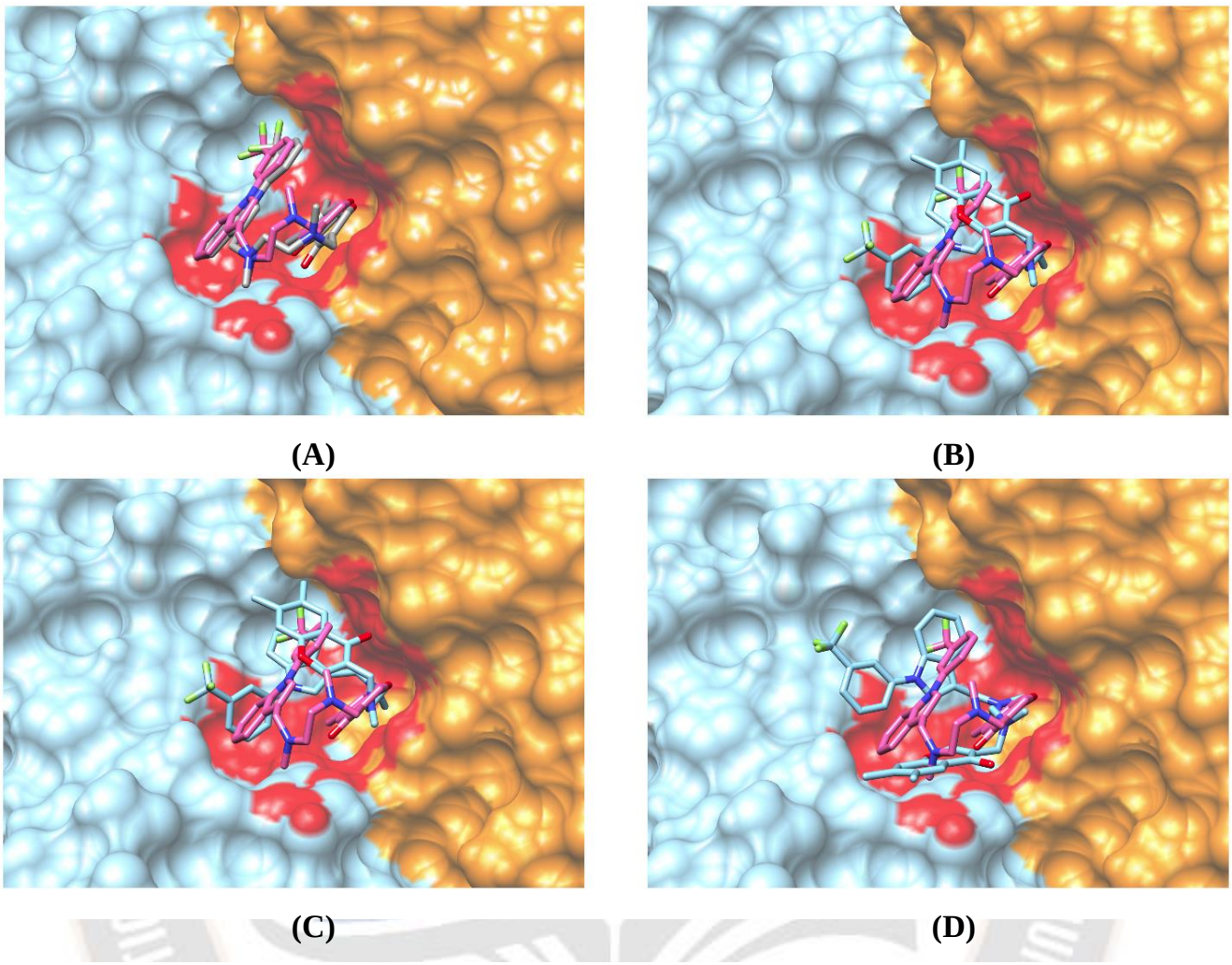

Figure 2. X-ray crystal structure (pink) and predicted structures of ligand307 docked on surface of TNFα (PDB ID: 2AZ5) chain C (sky blue) and D (orange) predicted by (A) Autodock Vina, (B) Autodock4, (C) DOCK6 with rigid ligand method and (D) DOCK6 with flexible ligand method.s

more effective than the known TNFα inhibitor of ligand307 at the molecular level.

We also performed molecular docking analysis with NF-κB, one of TNFα-induced transcription factors. The binding free energies between ligands and NF-κB p50 and p65 subunits are presented on Table IV and Table V. Similar as the docking result of TNFα, inconsistency was observed on hierarchical order of docking free energies calculated by various docking softwares and methods. Generally, the docking free energies of ellagic acid derivatives were lower than those of urolithins. The binding free

TABLE III. BINDING FREE ENERGIES, INHIBITION CONSTANTS AND CONTACT RESIDUES OF ELLAGIC ACID DERIVATIVES AND THEIR METABOLITES WITH TNFA PREDICTED BY AUTODOCK VINA

	ΔG (kcal/mol)	Ki[a] (μM)	Contact residues	
			Chain C	Chain D
DMEA	-7.5	3.2	TYR59 GLN61 TYR119 LEU120 GLY121 TYR151	TYR59 SER60 TYR119 LEU120
EA	-7.6	2.7	TYR59 GLN61 TYR119 LEU120 GLY121	TYR59 SER60 TYR119 LEU120
MEA	-7.6	2.7	TYR59 GLN61 TYR119 LEU120 GLY121 TYR151	TYR59 SER60 TYR119 LEU120
MMDEA	-8.0	1.4	LEU57 TYR59 GLY121	TYR59 TYR119 TYR151
UA	-7.2	5.3	TYR59 GLN61 TYR119 LEU120 TYR151	TYR59 SER60 TYR119 LEU120
UB	-7.2	5.3	TYR59 TYR119 LEU120	TYR59 SER60 TYR119 LEU120
UC	-7.7	2.3	TYR59 SER60 TYR119 LEU120 GLY121 TYR151	TYR59 SER60 TYR119 LEU120
UD	-8.0	1.4	TYR59 SER60 TYR119 LEU120 GLY121 TYR151	TYR59 SER60 TYR119 LEU120 GLY121
Ligand307	-8.8	0.4	LEU57 TYR59 TYR119 LEU120 GLY121 GLY122 GLY151	LEU57 TYR59 SER60 TYR119 LEU120 GLY121 GLY151

[a]Inhibition constant (Ki) was calculated by following equation; Ki = 1/Kbinding = exp(ΔG/RT); where R is gas constant, 8.31441 J/K/mol, and T is room temperture of 298.15 K

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TABLE IV. PREDICTED BINDING FREE ENERGIES (KCAL/MOL) OF ELLAGIC ACID DERIVATIVES AND THEIR METABOLITES WITH NF-ΚB P50 SUBUNIT BY DIFFERENT METHODS

	Autodock Vina	Autodock4	Dock6 rigid	Dock6 flex
DMEA	-7.3	-6.4	-27.1	-29.8
EA	-7.2	-6.1	-30.2	-31.9
MEA	-7.3	-6.6	-26.8	-31.6
MMDEA	-7.7	-6.5	-28.7	-29.1
UA	-6.4	-5.9	-23.6	-25.3
UB	-6.4	-6.3	-22.7	-23.4
UC	-6.9	-6.3	-26.5	-28.3
UD	-6.8	-5.7	-27.0	-29.4
Tanshinone IIA	-7.6	-7.3	-23.8	-23.6

TABLE V. PREDICTED BINDING FREE ENERGIES (KCAL/MOL) OF ELLAGIC ACID DERIVATIVES AND THEIR METABOLITES WITH NF-ΚB P65 SUBUNIT BY DIFFERENT METHODS

	Autodock Vina	Autodock4	Dock6 rigid	Dock6 flex
DMEA	-7.9	-9.1	-31.8	-32.2
EA	-8.4	-9.1	-30.7	-31.7
MEA	-8.3	-9.2	-30.2	-32.9
MMDEA	-8.2	-9.6	-31.2	-31.7
UA	-7.6	-8.5	-26.0	-26.3
UB	-7.2	-8.2	-23.3	-23.4
UC	-7.7	-8.7	-27.1	-28.3
UD	-7.9	-8.3	-28.0	-30.2
Tanshinone IIA	-7.9	-9.9	-26.4	-25.9

energies of tanshinone IIA, a known inhibitor of NF-κB, was lower than urolithins, but higher than MMDEA for both cases of p50 and p65 NF-κB subunits. Meanwhile, the calculated binding free energies by different softwares and methods were not same each other. For DOCK6, binding free energies of tanshinone IIA against both p50 and p65 subunits were higher than those of UC and UD. For Autodock4, however, the lowest binding free energy was observed on tanshinone IIA. But only little gaps were observed between NF-κB binding free energies of tanshinone IIA and ellagic acid derivatives. From these results, the ellagic acid derivatives could be suggested as NF-κB inhibitors compared with tanshinone IIA. Followed by binding free energy evaluation, binding site analysis was conducted.

Docking results from Autodock Vina were used for binding site analysis (Figure 3). The binding free energies, Ki and ligand-contact residues of NF-κB p50 and p65 subunits were shown on Table VI and Table VII. The binding site discovered in this study were coincided with previous molecular docking studies with various ligands [11], [12]. Among ligand-contact residues of NF-κB p50 subunit, 8 out of 9 tested ligands were in contact with GLY65, GLY66, SER72 and LYS77. As GLY65 and LYS77 constituted DNA binding site, ellagic acid derivatives and metabolites could be potent competitive inhibitors of NF-κB p50 subunit against DNA. Especially, the carbonyl oxygen of DMEA, EA, MEA, MMDEA and tanshinone IIA had hydrophilic interaction with GLY66 of NF-κB p50 subunit. Since these ligands had lower binding free energies than the other ligands, the polar interaction might play a key role in ligand-enzyme binding. For NF-κB p65 subunit, all tested ligands were in contact with ARG35, TYR36, GLY92, ASN115 and GLY117. DMEA, EA, MEA and UD had hydrogen bonds with TYR36 of NF-κB p65 subunit. Manuvakhova et al. [13] also suggested same region as a binding site. Among the ligand–contact residues, ARG35 and TYR36 constitute the DNA binding site, therefore, ellagic acid derivatives and metabolites could be potent inhibitor for NF-κB p65 subunit. To sum up, ellagic acid derivatives and metabolites were potent inhibitor for both of NF-κB p50 and p65 subunits. Among them, MMDEA was observed as the most potent inhibitor according to binding free-energy evaluation and binding site analysis. Urolithins, ellagic acids metabolites, observed as inferior inhibitors for NF-κB than ellagic acids derivatives.

IV. CONCLUSION

In this study, for evaluating TNFα and NF-κB inhibitory effects of ellagic acid derivatives and metabolites, *in silico* molecular docking was performed. For TNFα inhibitory effect, ellagic acid derivatives and metabolites seemed to be inferior compared with known TNFα inhibitor of ligand307 in molecular level, but the ADME properties seemed to be better than those of ligand307. For NF-κB, the inhibitory effects of ellagic acid derivatives and metabolites were comparable to known NF-κB inhibitor of tanshinone IIA. The ellagic acid derivatives, especially MMDEA, seemed to be more effective than their metabolites. TNFα and NF-κB are involved with inflammation, thus, ellagic acid including MMDEA can be used as anti-inflammatory substances. Only *in silico* simulation was conducted on this study, further *in vitro* and *in vivo* research is necessary for evaluating MMDEA as anti-inflammatory drugs. Although the anti-inflammatory effects were not confirmed by *in vitro* and *in vivo* on this study, the importance of this study is

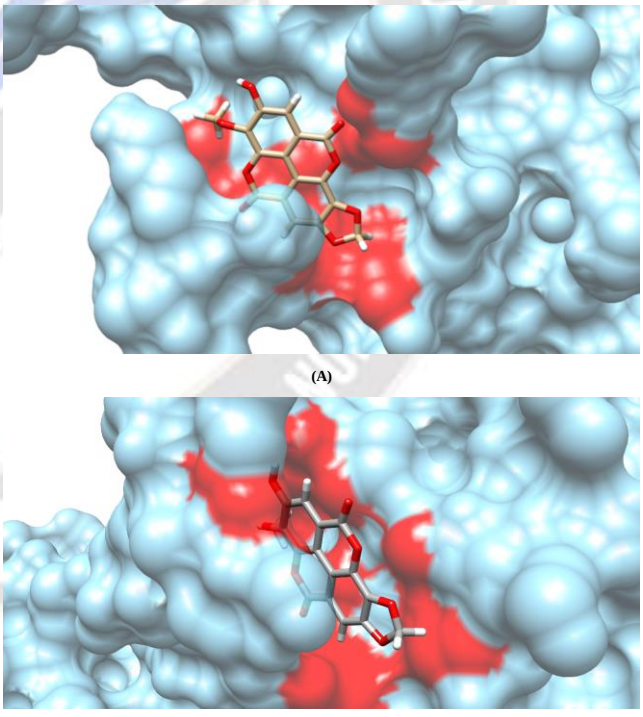

Figure 3. Binding mode of MMDEA with NF-κB (A) p50 and (B) p65 subunits predicted by Autodock Vina.

that not only ellagic acid, but also ellagic acid derivatives and metabolites were evaluated and compared. In conclusion, MMDEA was suggested as a potent inhibitor for TNFα and NF-κB by *in silico* methods.

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