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Molecular Dynamics Simulation of Various Bioactive Compounds of Red Betel (*Piper crocatum*) as Anti-Inflammatory Drug-Like Candidates in Rheumatoid Arthritis Treatment

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Abstract

Joint inflammation and damage can be caused by rheumatoid arthritis (RA), a systemic autoimmune disorder. Long-term RA affects the joints, causing pain, edema, and stiffness. Several studies have revealed that the exact origin of RA is still unidentified, but inherited disorder and external factors may contribute to the inflammatory condition. The incidence of RA is mostly caused by uncontrolled-production of pro-inflammatory cytokines under nuclear factor-kappa B (NF-κB) overexpression condition. The nuclear factor-kappa B plays an important role in inflammatory T cell activation, differentiation, and effector function. Therefore, the inhibition of NF-κB can be a strategy to reduce the adverse effects or incidence of RA. Red betel is a perennial herb native to Indonesia that is widely used by conventional healers. Many reports show that red betel leaf extracts contain several critical bioactive compounds such as Apigenin, Luteolin, Hydroxychavicol, Eugenol, and Chavibetol. This present study aims to evaluate and predict the therapeutic potential of those compounds as medication that reduce inflammation against RA by disturbing and inactivating the NF-κB molecular interaction. The LC-MS chromatography was used to evaluate the chemical contents of red betel leaves. Furthermore, *in silico* study, the 3D structure of the active compounds of red betel leaves is obtained from PubChem web server, while NF-κB is obtained from Protein Data Bank (PDB) website. Molecular docking was used to screen the potential compounds compared to aspirin as control. As a result, 2 compounds including Apigenin and Luteolin have smaller bond affinity values, −6.3 and −6.0 kcal/mol, respectively compared to aspirin. Based on molecular dynamics (MD) simulation, the root-mean-square deviation (RMSD) value of protein-ligand complexes have the similar pattern over 1000/ps except the NF-κB-Chavibetol complex which has an unstable pattern. On the other hand, the total energy of the protein-ligand complexes have similar average number of −1.69 kJ/mol. The simulation indicates the ligands have constant binding energy to the protein. Furthermore, numerous pharmacokinetic properties of compounds including absorption, distribution, metabolism, excretion and toxicity are exponentially greater than the control drugs. Finally, red betel active compounds can be used as alternative candidates of RA therapy medication.

Keywords: Apigenin, *In Silico*, Natural compounds, Red betel, Rheumatoid arthritis

Introduction

Rheumatoid arthritis is a chronic condition that causes synovial tissue inflammation and may cause damage to bone and cartilage tissue [1]. Several studies have shown the cause of RA remains unclear, but genetic abnormalities and environmental factors may trigger inflammatory condition [2]. A study conducted by Rudan *et al.* [3], demonstrated the prevalence of RA in Southeast Asia counting 0.40 %, the Eastern Mediterranean 0.37 %, the European area 0.62 %, the American area 1.25 %, and the Western Pacific 0.42 %.

Families with positive RA have a risk to experience RA up to 40 - 60 % [4]. Recently, there are several approved inhibitors for RA such as Tofacitinib and Baricitinib, however it cause side effects including the elevated of cholesterol level. The inflammation caused by RA is due to the overexpression of NF-κB which acts as an inflammatory response regulator, in addition this condition triggers the activation of proinflammatory related genes in innate immune cells [5]. Moreover, the NF-κB has a crucial role in activation, differentiation, and effector function in inflammatory T cells. This underlies the inflammatory

disease as a result of NF-κB activation. Therefore, treatment of inflammatory diseases should have a target to suppress NF-κB activation [6]. Another study conducted by Salminen *et al.* [7], revealed that red betel (*Piper crocatum*) is an alternative plant as treatments to suppress NF-κB expression in order to ameliorate RA incidence.

Red betel is a tropical plant that is widely grown in Indonesia and is widely used as a traditional medicine. Methanol extract 96 % red betel leaf contains flavonoids, saponins, triterpenoids, and tannins. The flavonoid species in red betel are flavonone, isoflavone, auron, catechin, anthocyanidin, and chalcone [8]. Despite its rich-flavonoids content, red betel also contains biofenolics compounds such as hydroxychavicol, eugenol, khavibetol, and piperol which are known to be antimutagenic [9,10]. On the other hand, epigenin compound is the main compound of red betel leaf ethanol extract [11,12]. Thus, this present study aims to evaluate and predict the therapeutic potential of those compounds as anti-inflammatory agents against RA by disturbing NF-κB molecular interaction.

Materials and methods

Red betel leaves extraction

Red betel leaves were obtained from Materia Medica, Batu. About 10 g of dried red betel leaves were grinded to powder, then put into 200 mL of ethanol 95 %. The red betel leaves maceration proceeded for 24 h. After that, the liquid was filtered using minipore filter paper (Sigma Aldrich). The red betel extract was vaporized using evaporator. Thick extract was stored to LC-MS Analysis.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Red betel leaves were extracted using modified methods from previous research [13]. In this present study, Liquid Chromatography Mass Spectrofotometry-8040 LC/MS (Shimadzu) was used to evaluate the chemical constituents of red betel. The LC system was outfitted with a dual solvent transport system, an automated sample injector, and a photodiode array detector. The chromatographic procedure was executed utilizing an Acquity Shim Pack FC-ODS column with dimensions of 2 mm in diameter and 150 mm in length, and a particle size of 3 μm. The mass spectrometer was outfitted with an electrospray ionization source.

Ligand preparation

The 3-dimensional structure of the red betel active compound such as Apigenin (CID 5280443), Luteolin (CID 5280445), Hydroxychavicol (CID 70775), Eugenol (CID 3314), Chavibetol (CID 596375), and Aspirin (CID 2244) were obtained from PubChem database (https://pubchem.ncbi. nlm.nih.gov/). Then, the 3D structure saved in .sdf format for further characterization on its drug likeness properties including molecular mass, lipophilicity, hydrogen bond donors, hydrogen bond acceptors, and molar refractivity.

Protein preparation

The complex 3-dimensional protein structure of NF-kappaB-DNA dimer was taken from Protein Data Bank (GDP Code: 1NFK). The water molecule was removed from protein structure. After that, the hydrogen atoms were added to the structure using PyMOL software.

Docking simulation and molecular dynamics simulations

The identification of red betel bioactive compounds as anti-inflammatory drug candidates was performed using the Autodock Vina/ PyRx 0.8 software [14,15]. The grid on the coordinate box is set on the active side of NF-κB (x = −1.195 Å; y = 9.0149 Å; z = 19.759 Å) which has hydrogen bond residues in Agr54, Agr56, Tyr57, Cys59, Lys241, Gln306 and Thr143 of hydrogen bond with deoxyribonucleic acid (DNA). The results were obtained based on the docking process of the affinity value of binding of compounds or ligands with NF-κB protein. Docking process in addition to using red betel active compounds used Aspirin as a control drug. Binding affinity values are obtained from ligand-protein complex values at the lowest free-bond energy (ΔG). Hence, the total energy and the root mean square deviation (RMSD) of protein-ligand complexes were evaluated through YASARA (www.yasara.org) software [16,17]. Furthermore, the physicochemical properties of red betel active compounds were predicted using the Molinspiration Property Calculator webserver. Data for the canonical smile were gathered from PubChem and analyzed using the Molinspiration Property Calculator webserver. After the data were processed, several variables such as logP, TSPA ($Å^2$), molecular weight, aceptor H bond, and donor H bond were evaluated. Moreover, the pharmacokinetics properties including absorption, distribution, metabolism,

excretion, and toxicity were assessed through pkCSM webserver. The pkCSM webserver was used to process the canonical smile data that were retrieved from PubChem. Ultimately, the assessment of potency activity (PA) was executed in order to ascertain the potential efficacy of the compounds in relation to RA. The canonical smile data were taken from PubChem and processed with pass server.

Results and discussion

Secondary metabolites of red betel extract were obtained from LC-MS analysis. The result demonstrated that the highest peak of red betel compound was Kaempferitrin with retention time 33.483 and molecular weight reach to 578.523 (m/z). Further, the molecular formula prediction for this compound was $C_{27}H_{30}O_{14}$. The second compound was Piperbetol with retention time 12.998 with and molecular weight reach to 386.444 (m/z), and the molecular formula assumption was $C_{22}H_{26}O_6$. The third was Eugenol with retention time 2.694 with a molecular weight of 164.0837 (m/z), and the molecular formula calculation was $C_{10}H_{12}O_2$. The 4 was Chavibetol with retention time 1.831 with a molecular weight of 164.0837 (m/z), and the molecular formula estimation was $C_{10}H_{12}O_2$ (**Figure 1**).

The prediction of inhibition activities of red betel bioactive compound against the NF-κB complex are shown in **Table 1**. The *in silico* prediction showed Apigenin binds to 1NFK with ΔG reach to −6.3 kcal/mol and has a hydrogen bond with a side chain Lys-144, Tyr-57, and Arg-54 (**Figure 2(A)**). Chavibetol binds to 1NFK with ΔG of −4.7 kcal/mol and has a hydrogen bond with a Glu-60 and Arg-54 side chain (**Figure 2(B)**). Eugenol binds to 1NFK with ΔG of −4.7 kcal/mol and has a hydrogen bond with a Glu-60 and Arg-54 side chain (**Figure 2(C)**). Hydroxychavicol binds to 1NFK with ΔG of −4.8 kcal/mol and has a hydrogen bond with the Arg-54 and His-141 side chains (**Figure 2(D)**). Luteolin binds to 1NFK with ΔG of −4.7 kcal/mol and has a hydrogen bond with Asp-239, His-141, Arg-54, and Glu-60 side chains (**Figure 2(E)**), while aspirin (control) binds to 1NFK with ΔG of −4.7 kcal/mol and has a hydrogen bond with a Thr-143, Cys-59, and Val-58 side chain (**Figure 2(F)**). The red betel active compound forms most of the polar bonds with Lys-144, Tyr-57, Arg-54, Thr-143, Cys-59, Val-58, Glu-60, Lys-241, His-141, Asp-239, which form a hydrogen bond with the DNA consensus sequence on the kB side of NF-κB. The red betel active compound may interfere with the NF-κB bond on the kB side with Lys-144 and Lys-241 on the NF-κB complex [18]. Among the 5 active compounds of red betel, the results showed that the lowest free-bonded energy values are Apigenin, Luteolin, Hydroxychavicol, Chavibeto, and Eugenol.

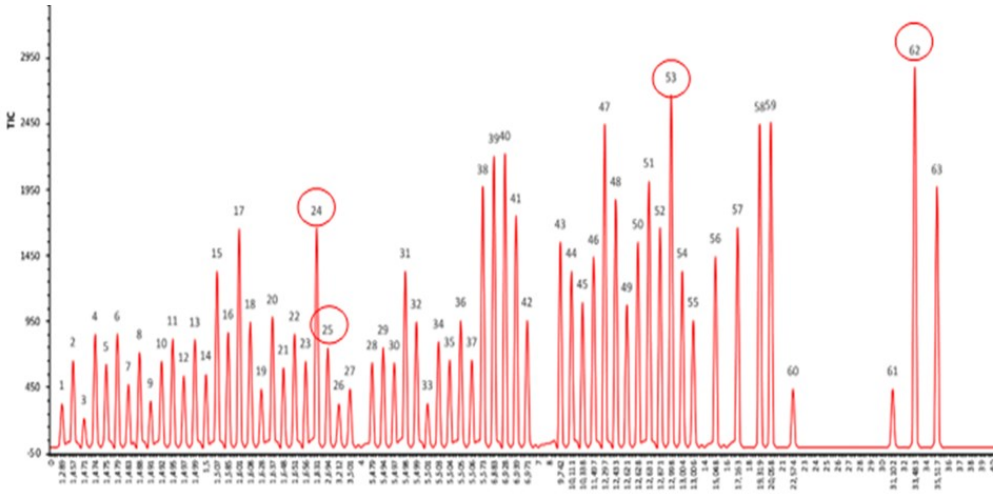

Figure 1 LC-MS analysis of red betel leaves. The results showed there were 4 chemical compounds found in red betel leaves namely Kaempferitrin, Piperbetol, Eugenol, and Chavibetol.

On the other hand, the RMSD of protein-ligand complexes and the total energies were evaluated via MD simulation. In general, MD simulation demonstrates the interaction strength and stability of the complex between the receptor-ligand [19]. Based on the simulation, the RMSD value of protein-ligand complexes have the similar pattern over 1,000/ps except the NF-κB-Chavibetol complex which has unstable pattern (**Figure 3(A)**). Furthermore, the total energy of the protein-ligand complexes have similar average number on −1.69 kJ/mol (**Figure 3(B)**). This simulation indicates the ligands have constant binding energy to the protein.

Table 1 Free energy and physicochemical properties of red betel.

Compound	ΔG (kcal/mol)	LogP	TPSA (Å^2)	Molecular weight	Aceptor H bond	Donor H bond	Violation (Lipinski rule)
Apigenin	−6.3	2.46	90.89	270.24	5	3	0
Eugenol	−4.1	2.10	29.46	164.20	2	1	0
Chavibetol	−4.7	2.10	29.46	164.20	2	1	0
Hydroxychavicol	−4.8	1.79	40.46	150.18	2	2	0
Luteolin	−6.0	1.97	111.12	286.24	6	4	0
Aspirin (Control)	−4.7	1.43	63.60	180.16	4	1	0

The physicochemical properties of red betel active compounds are predicted using the Molinspiration Property Calculator webserver (**Table 1**). The physicochemical prediction aims to estimate the resemblance to the drug in terms of inhibition. According to Kumar and Bora, there are some things to consider in order to estimate the resemblance of the drug, for example the LogP of the compound should be less than 5, the hydrogen bond donor must be less than 5, the H bond acceptor less than 10 and the molecular weight should be less than 500 [20]. Membrane permeability cells in a molecule must have a topological polar surface area (TPSA) of less than 140 Å^2. The inhibitor molecules of red betel all have values less than the maximum value, it can be predicted that they have good cell membrane permeability. The 3-dimensional visualization of the NF-κB complex (1NFK) with the red betel active compound attached to the Hydrogen bond is described in **Figure 2**.

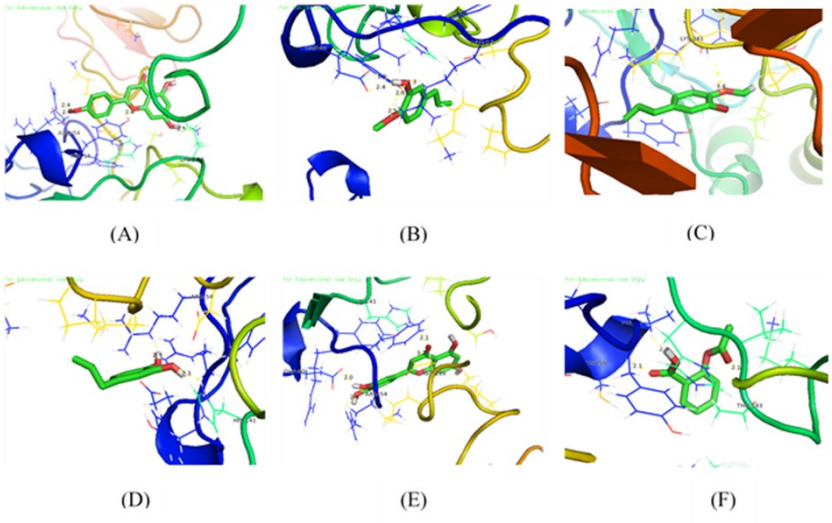

Figure 2 Three-dimensional visualization of NF-κB complex (1NFK) with red betel active compound bonded with hydrogen bond. (A) Apigenin with Lys-144, Tyr-57, Arg-54, (B) Chavibetol with Glu-60 and Arg-54, (C) Eugenol with Lys-241, (D) Hydroxychavicol with Arg-54 and His- 141, (E) Luteolin with Asp-239, His-141, Arg-54, and Glu-60, (F) Aspirin with Thr-143, Cys-59, and Val-58.

The results indicate that the intestinal absorption, Caco-2 permeability, and water solubility values of 5 red betel compounds were significantly higher than those of the control drugs. According to **Table 2**, compounds exhibit favorable absorption when the absorption value exceeds 80 %, while absorption is deemed inadequate when it falls below 30 %. Compounds with a predictive value greater than 0.90 are regarded as having high Caco-2 permeability. The aqueous solubility of a compound is indicative of its ability to dissolve in water at a temperature of 25 °C. Enteral administration of lipid-soluble drugs results in lower absorption rates compared to water-soluble drugs. This suggests that the solubility of a drug plays a significant role in its absorption [21].

The VDss value distribution of 5 compounds showed high category, while the drug control include in the low category. It was found that all compounds can penetrate the blood brain barrier. The compound can penetrate the blood brain barrier if the Log BB value is > 0.3, and cannot penetrate if log BB is < −1.

Similarly, the 5 red betel compounds also can penetrate to the central nervous system (CNS). According to this findings, the further study related alteration of these compounds on central nervous system and RA are necessary. A study highlighted the crucial points on what condition the drug should pass or not cross the blood-brain barrier, including the influence to the central nervous system [22]. Accordingly, compound with a logPS > −2 are considered to penetrate the central nervous system [21]. The CYP3A4 is an isoenzyme derived from cytochrome p450 which is an important detoxification enzyme found mainly in the liver. The metabolism properties showed that 5 red betel compound are not the member of CYP3A4 substrates which means the red betel compounds have no significant effect on metabolism and non-contraindication mechanism [20,23]. Five red betel compound values for the clearance property known as total clearance were less than 2 mL/min/kg, indicating that it has a long half-life to be cleared by the liver, gall, as well as the kidney [21,24].

The toxicity properties can be determined by maximum tolerated dose for human (log mg/kg/day), and 5 red betel compounds and drugs are in the low category. Maximum Recommended Tolerated Dose (MRTD) less than or equal to 0.477 log (mg/kg/day) is considered low, and high if greater than 0.477 log (mg/kg/day). LD50 values are a widely accepted metric for evaluating the acute toxicity of various substances and comparing their relative toxicity. The LD50 refers to the quantity of a substance administered in a single dose that results in the mortality of half of a cohort of experimental animals. The safety of drug development is a crucial issue due to the potential for drug-induced liver injury, which is a significant cause of drug attrition. Our research findings indicate that 5 compounds present in red betel are safe for liver function.

Table 2 Prediction absorption properties of red betel compound & control drug of pkCSM.

Compound	Intestinal Absorption (Human) (% Absorbed)	Caco-2 Permeability (10^{-6}cm/s)	Water Solubility (log mol/L)	VDss (Human) (log L/kg)	BBB Permeability (log BB)	CNS Permeability (log PS)	CYP3A4 Substrate	CYP3A4 Inhibitor	Total Clearance (log mL/min/kg)	Renal OCT2 Substrate	Max. Tolerated Dose (human) (mg/kg/day)	Oral Rat Acute Toxicity (LD50) (mol/kg)	Hepatotoxicity
Apigenin	93.25	1.007	−3.329	0.822	−0.734	−2.061	No	No	0.57	No	0.328	2.450	No
Eugenol	92.04	1.559	−2.250	0.240	0.374	−2.007	No	No	0.28	No	1.024	2.118	No
Chavibetol	91.84	1.497	−2.260	0.203	0.389	−2.007	No	No	0.28	No	1.011	2.062	No
Hydroxychavicol	92.09	1.676	−1.379	0.477	0.361	−2.048	No	No	0.21	No	0.087	2.307	No
Luteolin	81.13	0.096	−3.094	1.153	−0.907	−2.251	No	No	0.49	No	0.499	2.455	No
Aspirin (Control)	76.94	0.090	−1.868	−1.716	−0.332	−2.489	No	No	0.72	No	1.016	2.286	No

Table 3 Prediction potential of red betel compound & control drug of pass server.

Potential	Pa					
	Apigenin	**Eugenol**	**Chavibetol**	**Hydroxy-chavicol**	**Luteolin**	**Aspirin (Control)**
Antimutagenic	0.926	0.878	0.878	0.878	0.944	0.436
Antioxidant	0.740	0.474	0.474	0.533	0.782	0.252

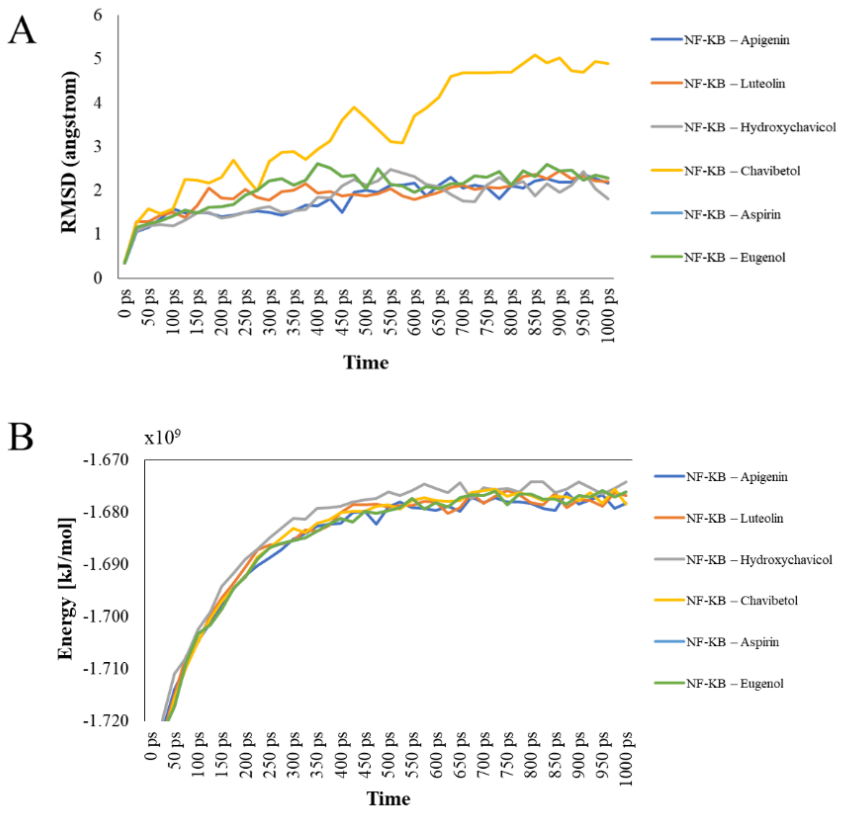

Figure 3 Molecular dynamics simulation. (A) RMSD values of protein-ligand complexes interaction. (B) Total energies simulation of protein-ligand complexes interaction.

Based on **Table 3**, red betel compounds have the potential as high antimutagenic experimentally and *in silico* and have a high likelihood of being candidates for drugs because the Pa score is more than 0.7, while the potential for aspirin is very low. Compounds of apigenin and leutonin have high antioxidant activity experimentally and *in silico* and have a high probability of becoming drug candidates because the Pa value is more than 0.7 [25], while the potential for aspirin is very low. Based on Arulsevan, antioxidant compounds will inhibit free radicals and ROS in cells and will turn them into stable compounds [26]. Reactive oxygen species is a key signal in producing proinflammatory mediators, because ROS can activate Nf-κB and trigger an increase in proinflammatory cytokines [27].

Conclusions

The current *in silico* research provides much insight into the inhibition of NF-κB by red betel active compounds. Red betel active compounds (Apigenin, Chavibetol, Eugenol, Hydroxychavicol, and Luteolin) can be predicted to be NF-κB inhibitors for rheumatoid arthritis treatment therapy. This can be supported by physicochemical properties that show good results for drug similarities. Red betel compounds have the potential as antimutagenic and antioxidants which are quite good compared to control drugs. Further study related regarding *in vivo* experiment need to perform in order to validate the present results of compounds toward the RA.

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