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# In Silico Studies: Stigmastan-3,6-dione in the Ethyl Acetate Fraction of *Momordica charantia* L. Fruit Has Immunostimulant and Anti-inflammatory Activity

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**ABSTRACT**: *Momordica charantia* L fruit provides an immunomodulatory effect by stimulating certain components of the body's immune system. Bioactive phytochemicals from *M. charantia L.*. function as anti-inflammatory agents by reducing levels of pro-inflammatory cytokine secretion including IL-1, IL-6, IL-8, TNF-α, NF-kB. This research looks at the in silico mechanism of action of the active ingredient of the EtOAc fraction of *M. charantia L.* fruit as an immunostimulant and anti-inflammatory. The materials and methods used were an Intel Core i7 10 Th Gen laptop, Chem Office Professional 17.1, Chem 2D, Chem 3D software, PDB code (6W9K) and ligand (TUA), Stigmastan-3,6 dione ($C_{29}H_{48}O_2$) from the EtOAc fraction of fruit M. charantia L. was docked using MVD software, RMSD and RMSF values were viewed using Yasara Software, pkCSM online tool to predict compound toxicity, toxicity prediction ($LD_{50}$) was used by Protox online tool. The results of molecular docking of standard compounds, namely Methylprednisolone and Prednisolone and Stigmastan-3,6-dione, have Rerank Score values of -120.62, -121.47, -79.50, respectively. The lower the Rerank score value, the lower the binding energy between the protein and the ligand, causing the protein and ligand bonds to be more stable and it is predicted that the activity of the compound will be greater. The movement of RMSD values between 0.6-1.9 Å for the 3 (three) compounds, is still within stable limits and does not undergo conformation. The RMSF value of the 3 (three) compounds has the same amino acid residue pattern. The insilico toxicity prediction for the 3 (three) compounds is still within safe limits. The EtOAc fraction of M. charantia L. fruit with the active compound Stigmastan-3,6-Dione in its mechanism of action in silico shows activity as an anti-inflammatory and immunostimulant that works on the NF-kB pathway.

**KEYWORDS**: *Momordica charantia* L. fruit; Stigmastan-3,6-dione; In silico; Molegro Virtual Docker.

## 1. INTRODUCTION

*M. charantia L.* is one of the natural ingredients that provides an immunomodulatory effect by stimulating certain components of the body's immune system, thus showing great potential as immunoregulatory, anti-inflammatory, anti-microbial, anti-cancer, anti-oxidant, cardiovascular and neurological protector. [1] Bioactive phytochemicals from *M. charantia L.* function as anti-inflammatory agents by reducing levels of pro-inflammatory cytokine secretion including IL-1, IL-6, IL-8, TNF-α, nuclear factor-kappa B (NF-kB). Ethanol extract of *M. charantia L.* can reduce IL-1β expression in RAW 264.7 cell macrophages stimulated by LPS. Ethanol extract and ethyl acetate extract of *M. charantia L.* fruit can inhibit LPS-induced IL-6 secretion in mouse peritoneal macrophage cells (1) *M. charantia L.* extract shows anti-inflammatory activity, especially by increasing the secretion of the anti-inflammatory cytokine IL-10 [1] In this study, the solvent ethyl acetate (EtOAc , $C_4H_8O_2$; molecular weight 88.11 g/mol) was used to obtain the EtOAc fraction of *M. charantia L.* fruit. EtOAc is a semi-polar solvent, so it is able to attract semi-polar compounds in the fruit *M. charantia L.* The expected compound is from the triterpenoid group which has anti-inflammatory activity [2]. This research looks at the in silico mechanism of action of the active ingredient of the EtOAc fraction of *M. charantia L.* fruit as an immunostimulant and anti-inflammatory.

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## 2. MATERIALS AND METHODS

### 2.1. Materials

#### 2.1.1. EtOAc Fraction of *M. charantia L.* Fruit

The fractionation process of *M. charantia L.* fruit is carried out after the extraction process is complete and a thick extract is obtained. The thick extract was then partitioned using the EtOAc solvent, and then separated between the EtOAc fraction and the water fraction.

#### 2.1.2. Instrument Liquid Chromatography - Mass Spectrometry (LC-MS)

General description: name of the solvent A: 0,1%FA/WA, solvent selection A: A1, gradient start: at injection, low-pressure limit: 0 psi. Name of solvent B: Acetonitrile + 0,1FA, solvent selection B: B1, seal wash period: 5000 min, pre-injector volume: 0 µL, high-pressure limit: 18000 psi. Type of ionization: ESI, Polarity: Positive, Acquisition Start Time: 0.00 min, Acquisition End Time: 16.00 min, Start Mass: 50.00 m/z, End Mass: 1200.00 m/z, Scan Time: 0.100 s, Low CE: 6.00 eV, High CE Ramp Start: 10.00 eV, High CE Ramp End: 40.00 eV, Cone Mode: Method Settings, Cone Voltage: 30 V, Collision Mode: Specific, Collision Energy: 6.00 eV.

#### 2.1.3 In Silico test using Molegro Virtual Docker series 6.0 [2]

The material used for the in silico test was a chemical compound from the EtOAc fraction of *M. charantia L.* fruit which was obtained from the results of analysis using a Liquid Chromatography (LC) instrument with tandem Mass Spectrometry (MS) analysis; PDB ID is a receptor code that can be obtained from the Protein Data Bank which consists of 4 characters (6W9K); The SYBYL MOL 2 (SYBYL 2) file format is a portable representation of SYBYL 2 molecules which is a storage format from ChemDraw 3D used to support the docking process using the Molegro Virtual Docker 6.0 series software.

### 2.2. Procedure

#### 2.2.1 LC-MS analysis of EtOAc fraction of M. charantia L. fruit

Determine the m/z ratio of the analyte to be analyzed, create an MS File, create a method inlet, condition the Quaternary Sample Manager (QSM) and FTN Sample Manager, inject EtOAc fraction of M. charantia L. fruit.

#### 2.2.2 In Silico test using Molegro Virtual Docker (MVD) series 6.0 [2]

Prepare a laptop with specifications that meet workmanship standards, namely Intel Core i7 10 Th Gen, install or install the laptop with Molegro Virtual Docker (MVD) series 6 software, also install or install the Chem Office Professional 17.1 (Chem 2D) software and Chem 3D).

#### 2.2.3 Target protein download (target receptor)

During the in silico test, it is ensured that the laptop is connected to a fairly stable internet network. This is used to download certain types of proteins needed during the in silico test at the address http://www.rcsb.org/pdb [3]

#### 2.2.4 Activity prediction (Molecular docking)

The chemical compound from the EtOAc fraction of *M. charantia L.* fruit that will be docked has a 2-D molecular structure drawn using the Chem Office Professional 17.1 (5) program, then copied into the Chem Office Professional 17.1 3-D program to create a 3-D structure. After measuring the minimum energy, it is stored in the form of mol 2 {SYBYL2(mol2)}. After saving, the docking process is carried out against the target receptor with the PDB code (6W9K).

#### 2.2.5 *Molecular Docking*

At the end of the in silico test, docking is carried out to be able to predict interactions between molecules and assessment functions such as Rerank Score, Root Mean Standard Deviation (RMSD) and H Bond can be seen. The docking results are said to be valid if the RMSD value, namely the average distance between the reference and the docked ligand, is less than 2. The Rerank Score describes the energy required in the process of ligand interaction with the receptor and from this value the anti-inflammatory and immunomodulatory activity of M. charantia L. can be predicted. The smaller the Rerank Score energy means the more stable the bond. If the ligand bond with the receptor is more stable, it can be stated that the activity is also greater, while

the pharmacophore group shows which groups in the drug structure bind to the receptor and the type of drug bond with the receptor.

### 2.2.6 Molecular Dynamics (MD) Simulation

Simulation of molecular dynamics of compounds from docking results that have rerank score values using YASARA software. [4]

Stage of Molecular Dynamic (MD) Simulation using YASARA software

a. Prepare files for molecular dynamics in one folder.
b. Prepared the script md_run.mcr to carry out molecular dynamic simulations. Several variables were adjusted, namely temperature of 310 K, physiological pH of 7.4. The ion concentration is also regulated as a mass fraction, namely 0.9% NaCl. Then set the duration of the molecular dynamic simulation, namely 1000 ps (1 ns). This molecular dynamic simulation uses the AMBER13 forcefield and snapshot storage every 10 ps.
c. Clicked "File", then selected "Save as". The script file md_run.mcr is saved in the same folder as the protein to be simulated.
d. Open the YASARA Dynamics application.
e. Click the "Options" tab, then click "Macro & Movie" then select "Set Target".
f. Select the protein to be simulated, then click "OK".
g. Click the "Options:" tab, then click "Macro & Movie" then select "Play macro".
h. The running process is carried out with the prepared md_run mcr command script. Then click "OK".
i. Wait until the running process is complete, namely until the simulation time reaches 1000 ps or 1 ns.
j. After running is complete, then click the "File" and "New" tabs, then click "Yes".
k. Click the options tab, then click “Macro & Movie” then select “Set target”. The same target protein as simulated is selected.
l. Next, click the "Options" tab, then click "Macro & Movie" then select "Play Macro".
m. Next, a potential energy analysis is carried out by running the macro md_analyze.mcr and the output is obtained in the form of potential energy. And an RMSF analysis was carried out by running the md_analyzeres.mcr macro and the output was obtained in the form of RMSF.

### 2.2.7 Prediction of Physicochemical Properties and Toxicity of Compounds

Prediction of physicochemical properties such as: molecular weight (MW), logarithm of partition coefficient (Log P), number of bonds between atoms that can rotate (Torsion); Hydrogen Bond Acceptors (HBA), Hydrogen Bond Donors (HBD), and Polar Surface Activity (PSA) are carried out using the pkCSM online tool; Prediction of the toxicity of chemical compounds from the EtOAc fraction of M. charantia L. fruit was carried out using the pkCSM online tool; before docking, chemical compounds from the EtOAc fraction of M. charantia L. fruit as well as comparison compounds (anti-inflammatory drugs and immunomodulators) were drawn in 2-D molecular structures using the Chem Office Professional 17.1 program, then copied into the Chem Office Professional 17.1 program to create a 3-D structure, then saved in the form of a *.sdf or .pdb file; Next, the structure is translated into SMILES format using the online SMILES Translator (https://cactus.nci.nih.gov/translate/). It is in the SMILES format that compounds are processed using the pkCSM online tool (http://biosig.unimelb.edu.au/pkcsm/prediction) [5] to predict the toxicity of compounds; To predict oral toxicity (LD50) in rodents and classify compound toxicity based on the Globally Harmonized System (GHS) the Protox online tool (https://bio.tools/protox) is used. [6]

## 3. RESULTS

**Table 1.** LC-MS analysis of 5 (five) chemical compound components from the EtOAc fraction of M. charantia L. fruit.

| No | Component Name | Observed m/z | Neutral mass (Da) | Observed RT (min) | Detector counts | Response | Adducts | Formula | Mass error (mDa) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Stigmastan-3,6-dione | 429.3729 | 428.36543 | 10.31 | 265799 | 180344 | + H | $C_{29}H_{48}O_2$ | 0.2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2 | Candidate Mass $C_{36}H_{44}O_9$ | 621.3071 | 620.29853 | 9.64 | 2827950 | + H | $C_{36}H_{44}O_9$ | 1.3 |
| 3 | Candidate Mass $C_{34}H_{40}O_9$ | 593.2751 | 592.26723 | 9.51 | 1766435 | + H | $C_{34}H_{40}O_9$ | 0.6 |
| 4 | Candidate Mass $C_{18}H_{33}NO$ | 280.2635 | 279.25621 | 8.77 | 191792 | + H | $C_{18}H_{33}NO$ | 0.0 |
| 5 | Candidate Mass $C_{34}H_{38}O_{10}$ | 607.2545 | 606.24650 | 9.38 | 1721965 | + H | $C_{34}H_{38}O_{10}$ | 0.7 |

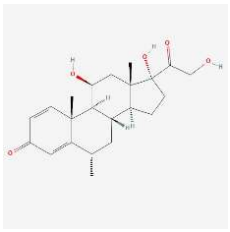
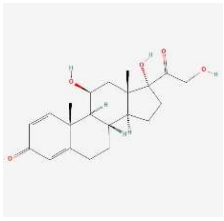
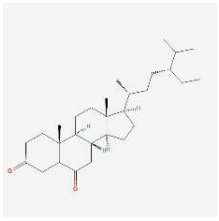
| Ligand | Chemical structure | Moldock Score (Kcal/Mol) | Rerank Score | Hydrogen Bonding | Steric interactions |
|---|---|---|---|---|---|
| Methylprednisolone |  | -135,14 | -120,62 | Asn33, Gln39, Arg80, Gln111, Thr208 | Asn33, Gln39, Ala74, Arg80, Phe92, Gln111, Phe204, Cys205, Thr208, Phe218 |
| Prednisolone |  | -132,84 | -121,47 | Asn33, Arg80, Gln 39, Gln111, Thr208 | Asn33, Arg80, Gln111, Phe204, Cys205, Thr208, Phe218 |
| Stigmastane-3,6-dione |  | -134,85 | -79.50 | Thr208 | Met29, Leu32, Asn33, Leu77, Met73, Gln111, Met115,Phe204, Cys205, Thr208 |

**Table 2.** Molddock values, rerank scores, hydrogen bonds and steric interactions for 3 (three) compounds, namely Methylprednisolone, Prednisolone and Stigmastan-3,6-dione.

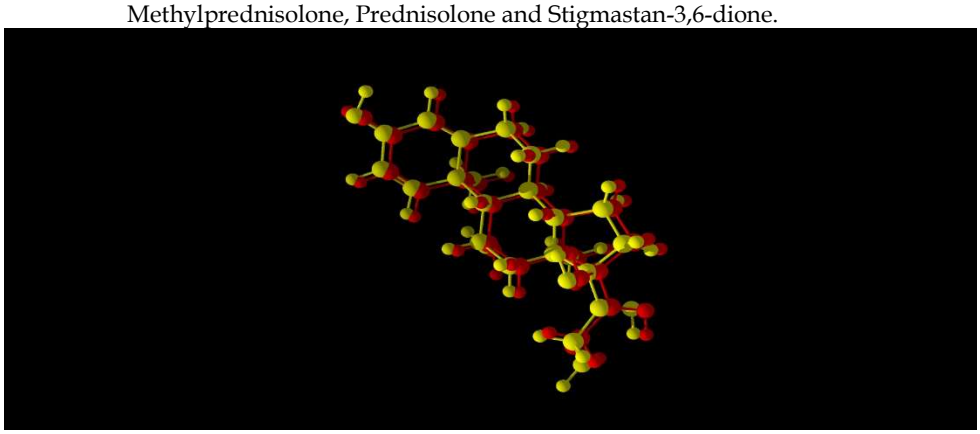


**Figure 1.** Visualization of 6W9K protein redocking validation, native ligand (TUA) pose before docking (yellow) and after docking (red) with RMSD (0.6-1,9 Å).

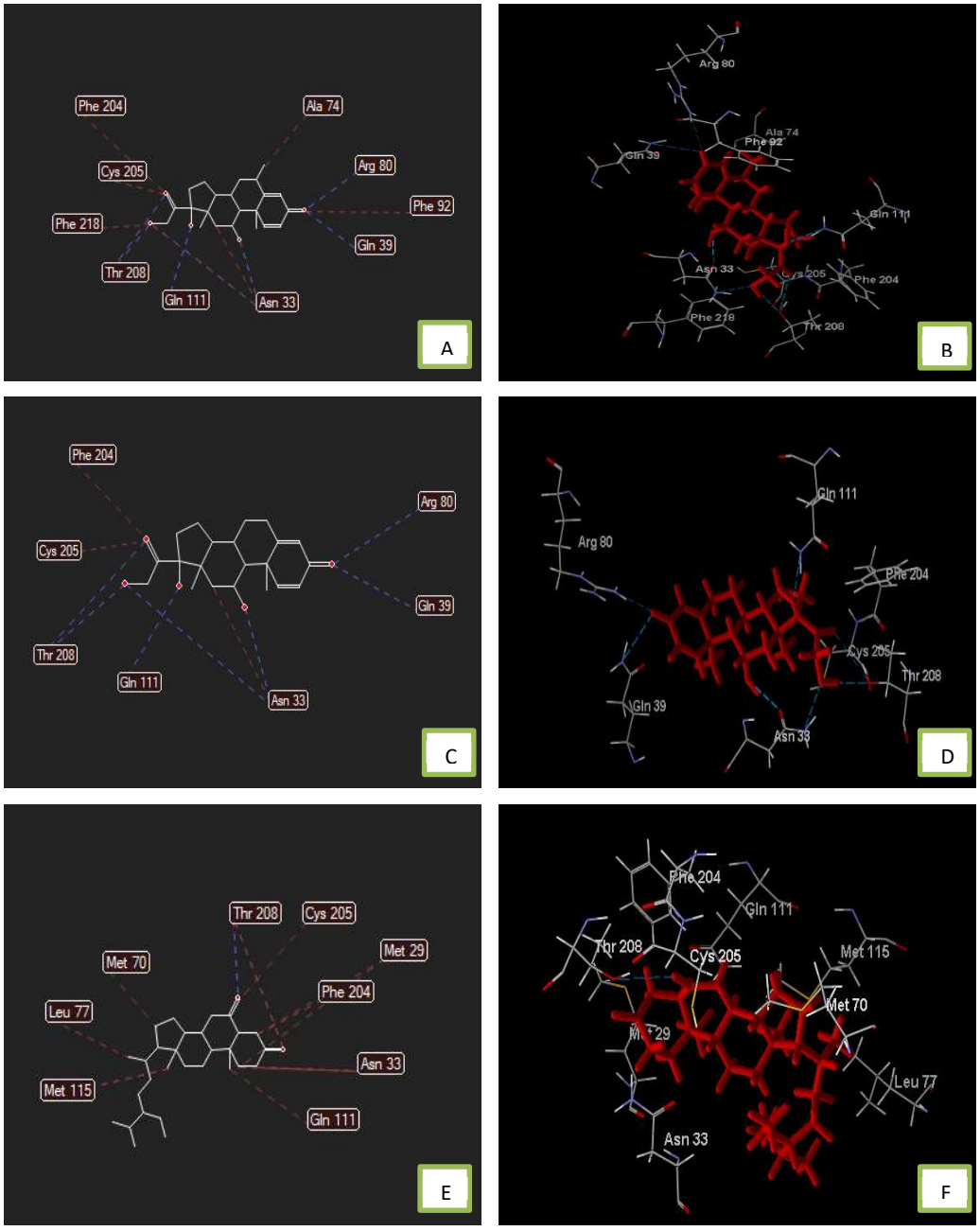



**Figure 2.** Hydrogen bond interactions and steric interactions of amino acid residues with docked compounds. (A: Hydrogen bond interactions in Methyl prednisolone); (B: Steric bond interactions in methyl prednisolone); (C: Hydrogen bond interactions in Prednisolone); (D: Steric bond interactions in Prednisolone); (E: Hydrogen bond interactions in Stigmastan-3,6-dione); (F: Steric bond interactions in Stigmastan-3,6-dione).

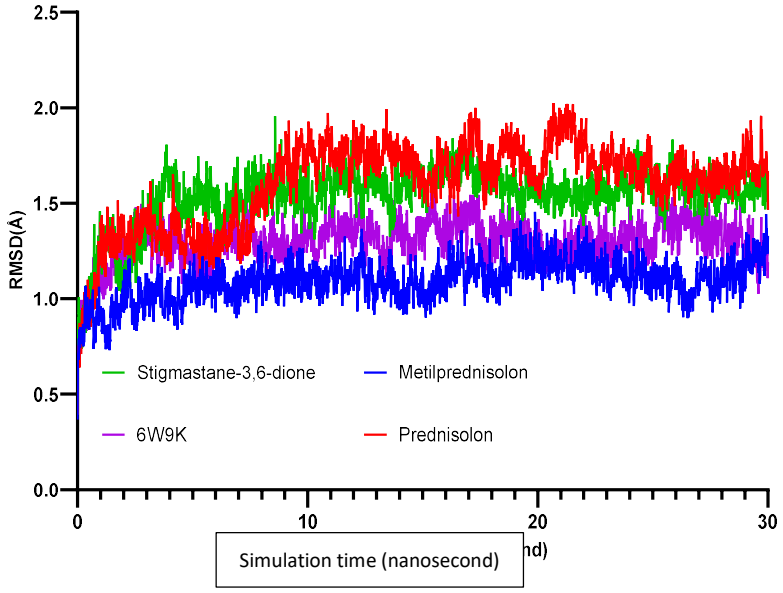



**Figure 3.** Movement of RMSD values (Å) versus simulation time (0-30 nano seconds) of molecular dynamics for the compounds Prednisolone, Methyl prednisolone, Stigmastan-3,6-dione and protein 6W9K.

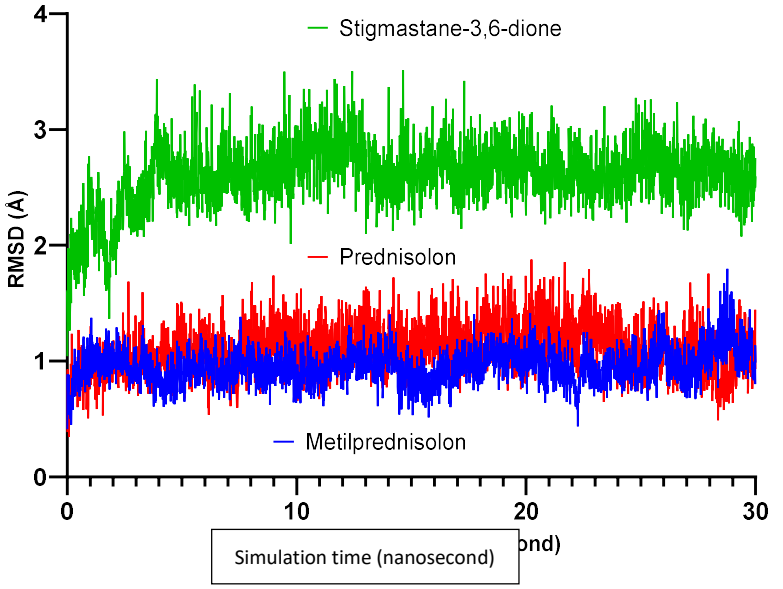



**Figure 4.** Movement RMSD value (Å) versus simulation time (0-30 nano seconds) of average molecular dynamics for the ligand movement Prednisolone, Methyl prednisolone, Stigmastan-3,6-dione.

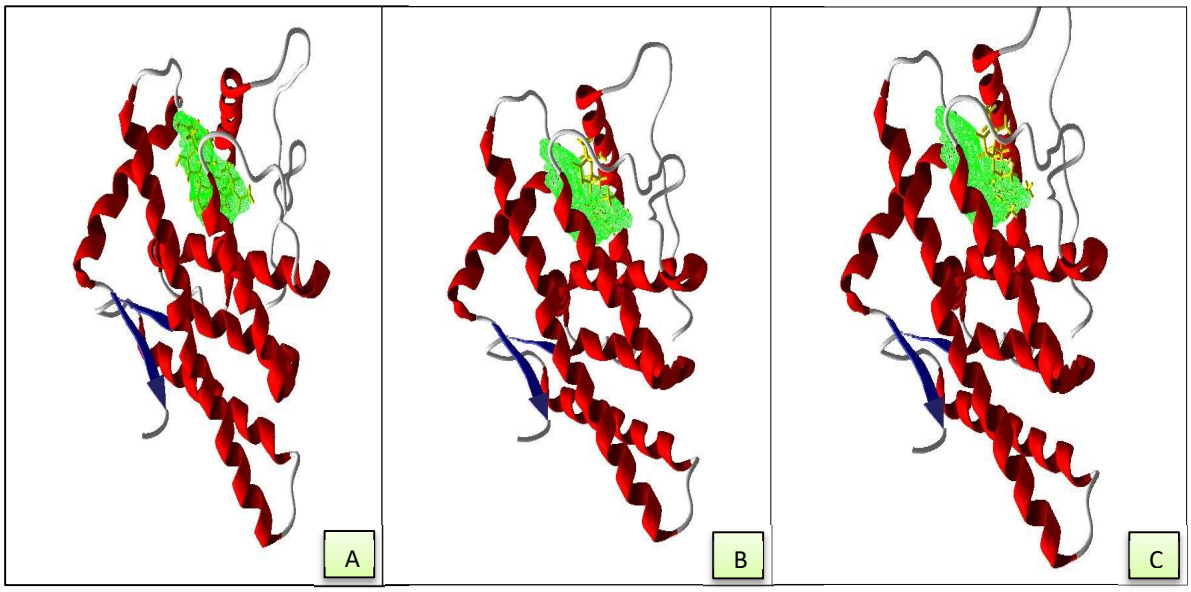



**Figure 5.** Ligand position in the cavity at 10 ns (A); 20 ns (B) and 30 ns (C)

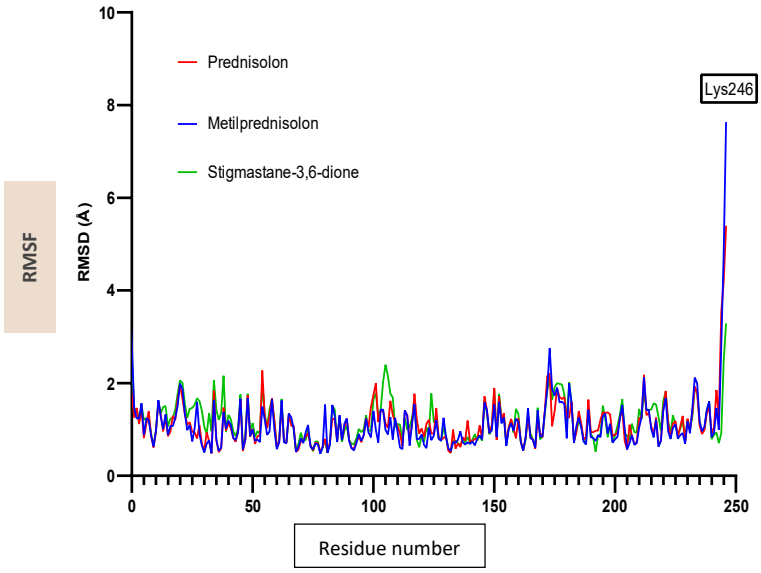



**Figure 6.** Movement of the RMSF value of the protein-inhibitor complex at the binding site

**Table 3.** Prediction of Oral Toxicity of Stigmastan-3,6-Dione, Prednisolone and Methyl Prednisolone Compounds in silico (7,8)

| No | Target | Stigmastan-3,6-Dione | | Prednisolone | | Methyl Prednisolone | |
|---|---|---|---|---|---|---|---|
| | | Prediction | Probability | Prediction | Probability | Prediction | Probability |
| 1 | Hepatotoxicity | Inactive | 0,78 | Inactive | 0,99 | Inactive | 0,98 |
| 2 | Carcinogenicity | Inactive | 0,62 | Inactive | 0,94 | Inactive | 0,80 |
| 3 | Immunotoxicity | Active | 0,99 | Active | 0,99 | Active | 0,99 |
| 4 | Mutagenicity | Inactive | 0,93 | Inactive | 0,53 | Inactive | 0,64 |
| 5 | Cytotoxicity | Active | 0,58 | Inactive | 0,76 | Inactive | 0,74 |
| 6 | $LD_{50}$ (mg/Kg) | 775 | | 1680 | | 1680 | |
| 7 | Predicted Toxicity Class | IV | | IV | | IV | |

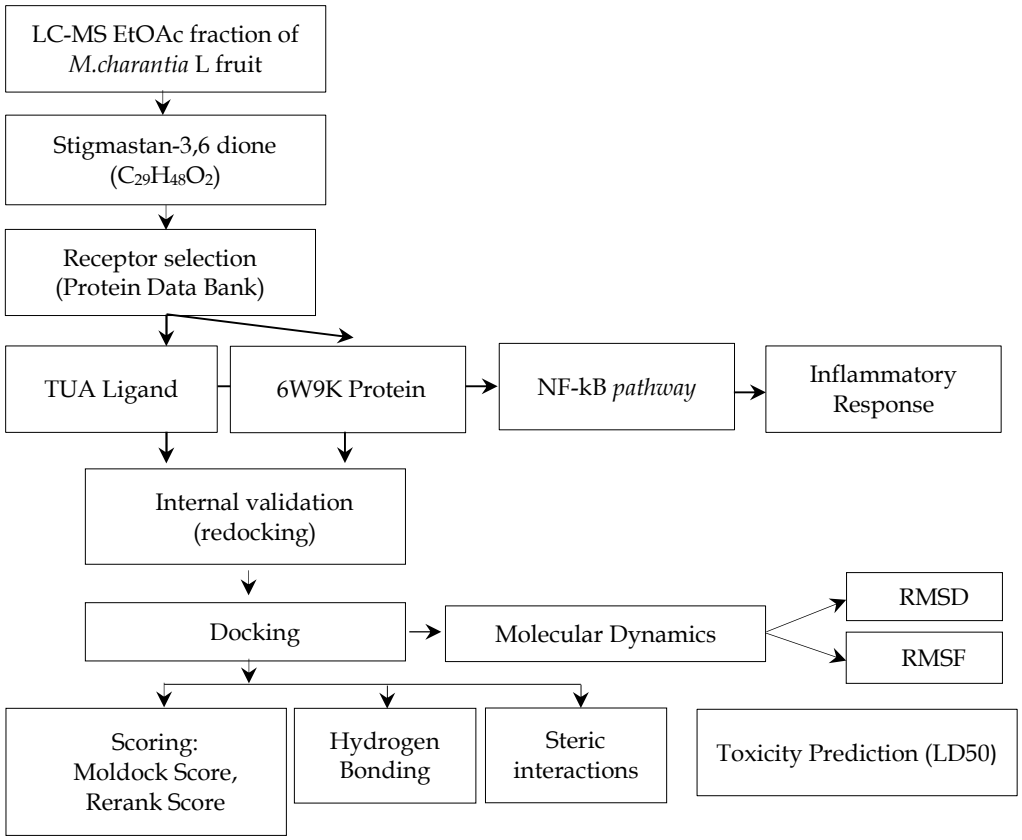



**Figure 7.** In silico mechanism of action diagram of Stigmastan-3,6 dione ($C_{29}H_{48}O_2$) on the EtOAc fraction of *M.charantia* L fruit.

## 4. DISCUSSION

### 4.1 LC-MS analysis of EtOAc fraction of M charantia L fruit

The EtOAc fraction of *M. charantia L.* fruit was analyzed using LC-MS. Raw data is obtained from the mass-charge ratio range between 100 to 1200. In this analysis, one of the parameters is using XEVO QToF (Quadrupole Time of Flight)- ESI (Electrospray ionization). The results of LC-MS analysis of the EtOAc fraction of *M.charantia* L fruit showed that there were 5 (five) chemical compound components, namely Stigmastan-3,6 dione ($C_{29}H_{48}O_2$); mass candidate $C_{36}H_{44}O_9$ ; $C_{34}H_{40}O_9$; $C_{18}H_{33}NO$; dan $C_{34}H_{38}O_{10}$ with the mass/charge weight (m/z) of each being 429,3729; 621,3071; 593,2751; 280,2635 dan 607, 2545. Can be seen in Table 1.

Stigmastan-3,6 dione ($C_{29}H_{48}O_2$) is found in *M.charantia* L plants (9) is a class of steroids (10)triterpenoid (11) and has anti-inflammatory activity (12). Solvents for removing the Stigmastan-3,6 dione compound are chloroform, dichloromethane, ethyl acetate, DMSO, acetone and others (10). Terpenoids are a natural compound formed from isoprene which is difficult to obtain through total chemical synthesis. Based on their chemical framework, terpenoids can be divided into monoterpenoids ($C_{10}$), sesquiterpenoids ($C_{15}$), diterpenoids ($C_{20}$), sesterterpenes ($C_{25}$), triterpenoids ($C_{30}$), and polyterpenoids (longer $C_5$ chains). As one of the most abundant and diverse groups of natural products, terpenoids have attracted widespread attention in recent years due to their important and diverse biological activities, including potent anticancer, anti-oxidation, anti-viral and anti-inflammatory activities(13). In other research, it was reported that *M. charantia L.* was identified as containing the compounds sitosterol ($C_{29}H_{52}O$) and stigmastadienol ($C_{29}H_{48}O$) which are aglycones of charantin [14,15].

The candidate mass $C_{36}H_{44}O_9$ has a MWof 620.29853 (Da), but the MWidentified is 621.3071 m/z, this is because the identified molecule is irradiated by 1 $H^+$ proton so that the MWof the analyte is increased by the weight of 1 H atom, namely 1. It is estimated that the compound this is Nimbolinin D (triterpenoid group),

with a MWof 620.7 g/mol as an anti-inflammatory and inhibits Nitric Oxide (NO) [16]. Limonoids type Nimbolini D, inhibit NO production with IC50 24.4 and 7.9 µM. Limonoids are structurally diverse secondary metabolites with potential anti-inflammatory pharmacological properties [17].

The candidate mass $C_{34}H_{40}O_9$ has a MWof 592.26723 (Da). It is estimated that this compound is Moreollic acid (an organic acid compound), with a BM of 592.7 g/mol as an anti-microbial, inhibiting tumor cell proliferation [18,19].

The candidate mass $C_{18}H_{33}NO$ has a MWof 279.25621 (Da). It is estimated that this compound is Linoleamide (Primary fatty acid amide/PFAMs), with a MWof 279.5 g/mol as an important signaling molecule [20]. The 7 (seven) main PFAMs are lauramide, myristamide, linoleamide, palmitamide, oleamide, stearamide and behenamide [21]. Fatty acids are known as self-defense agents in organisms and have various biological activities including anti-inflammatory. As we know that NF-kB activation is involved in the development of inflammation and inhibition of transcriptional factors as a target for inflammation treatment. Research that has been conducted shows that fatty acids, including linoleamide, are natural inhibitors of NO and NF-kB production [22].

The candidate mass $C_{34}H_{38}O_{10}$ has a MWof 606.24650 (Da). It is estimated that this compound is Trigonosine B (diterpenoid group), with a BM of 606.7 g/mol, which has an anti-neuroinflammatory role [23] antimicrobial, antiviral, antitumor [24].

To determine the most compounds contained in the EtOAc fraction of *M. charantia L.* fruit, it can be seen from the number of detector counts which can be seen in Table 1. The detector counts data is the signal intensity calculated by the detector, where the greatest intensity is the most dominant compound in the EtOAc fraction of *M. charantia L.* fruit. From this intensity, it can be concluded that the materials containing the most compounds are candidate mass $C_{36}H_{44}O_9$ > candidate mass $C_{34}H_{40}O_9$ > candidate mass $C_{34}H_{38}O_{10}$ > Stigmastan-3,6 dione ($C_{29}H_{48}O_2$) > candidate mass $C_{18}H_{33}NO$, with each detector count being 2827950; 1766435; 1721965; 265799 and 191792.

**4.2 In Silico test using Molegro Virtual Docker (MVD) series 6.0.**

The receptor selection from the Protein Data Bank (PDB) is 6W9K, where the cell origin is Homo sapiens, there are no mutations, the resolution is 1.60 Å and the native ligand is TUA. 6W9K protein preparation using the Molegro Virtual Docker software application and the preparation results show that there are no error warnings, so it can proceed to the internal validation or redocking process (Figure 1). The 6W9K protein is a structure of the glucocorticoid-derived receptor ligand binding domain with prednisolone and the coregulator fragment PGC1a [25]. The 6W9K protein has a biochemical function as binding to estrogen response elements and a biological process as an intracellular steroid hormone receptor signaling pathway. The 6W9K protein has 2 (two) bound ligands, namely TUA (prednisolone) and GOL (glycerol) [26]. The glucocorticoid receptor is a steroid hormone-activated transcription factor that binds to various elements of the glucocorticoid response to up or down regulate the transcription of thousands of genes involved in metabolism, development, stress and inflammatory responses. [27]

**4.3 *Molecular Docking***

Docking is a simulation method that predicts the structure of a receptor-ligand complex, where the receptor is a protein and the ligand is a small molecule. This simulation is equivalent to the lock-key theory of enzyme specificity, where the lock is the receptor and the key is the ligand. The goal in a protein-ligand docking simulation is to position the key (ligand) in the lock (ligand binding pocket in the protein). From a computational view, we view protein-ligand docking as an optimization problem, where our goal is to find the best solution (the correct position for the ligand) from a set of possible locations. Protein-ligand docking often uses one or more of the following computational methodologies: genetic algorithms, differential evolution, Lamarckian genetic algorithms, fast shape matching, incremental construction, geometric distance, simulated annealing, and others. Protein-ligand docking methodology can produce several positions for the key in the lock. Therefore, a scoring function is needed to evaluate all possible key positions, and then a selection can be made for the best location [28]. Molecular docking or molecular docking is a computational procedure that efficiently predicts noncovalent bonds between macromolecules (target proteins) and small molecules (ligands) with the aim of predicting bond conformations in the form of position, type and affinity based on bond energy. The insilico test produces a bond energy value, where the bond energy shows the amount of energy needed to form a bond between the ligand and the receptor. The lower the bond energy, the

more stable the bond between the ligand and the receptor. If the bond between the ligand and the receptor is more stable, the activity will be greater [29].

The results of molecular docking of 3 (three) compounds, namely Methylprednisolone (standard compound), Prednisolone (standard compound) and Stigmastan-3,6-dione have different Rerank Score values, can be seen in Table 2. The lower the Rerank score value, the higher the bond energy. the protein with the ligand will be lower and this will cause the protein and ligand bonds to be more stable. If the bond between the protein and the ligand is stable, it can be predicted that the activity of the compound will be greater. For several studies, we also looked at and compared the value of the Moldock score [30]. The MVD program has 4 (four) scoring functions (MolDock Score, MolDock Score with GRID, Plants Score and Plants Score with GRID) [28].

Hydrogen bonds play an important role in the stability of protein structures, interactions of proteins with ligands due to their participation in secondary structural elements, such as alpha helices and beta sheets (28) Hydrogen bond interactions and steric interactions of amino acid residues with the docked compound can be seen in Figure 2 (2 dimensions).

**4.4 Molecular Dynamics (MD) Simulation**

Molecular dynamics (MD) simulations in molecular biology and drug discovery have expanded dramatically in recent years. These simulations capture the behavior of proteins and other biomolecules in atomic detail and excellent temporal resolution. Vast improvements in simulation speed, accuracy and accessibility, together with developments in experimental structural data, have increased the appeal of biomolecular simulations for experimentalists. Simulations have proven useful in deciphering the functional mechanisms of proteins and other biomolecules, in uncovering the structural basis of disease and in the design and optimization of small molecules, peptides and proteins (31) Molecular dynamics (MD) simulations predict how each atom in a protein or other molecular system will move over time on a general model of physics that governs interactions between atoms [32].

Dynamic molecular simulations were carried out in two aspects, namely measuring the Root Mean Standard Deviation (RMSD) and RMSF (Root Mean Square Fluctation), using Yet Another Scientific Artificial Reality Application (YASARA) software (4)

The results of molecular dynamic simulations on the compounds Prednisolone, Methyl prednisolone, Stigmastan-3,6-dione and protein 6W9K with a simulation time of 30 ns can be seen in Figure 6. The docking results are said to be valid if the RMSD value is the average distance between the reference and the docked ligand less than 2. The movement of Root Mean Standard Deviation (RMSD) values ranges from 0.6 – 1.9 Å for the 4 (four) compounds. Based on this, it can be concluded that these 4 (four) compounds are still within stable limits and do not undergo significant conformation because the average RMSD value is still below 3 Å [33].

RMSD (Root Mean Square Deviation) movement is a measure used to compare shifts or changes in molecular conformation before the simulation and during the simulation for a certain time. RMSD analysis describes changes in protein structure during the simulation so that the stability of the protein and ligand structures can be known. The lowest RMSD value indicates the most stable complex. An RMSD value ≤ 3 (Å) indicates that the enzyme-inhibitor complex is stable and does not experience significant conformational changes and is an ideal value [34].

Figure 5 shows that the position of the ligand in the cavity is also influenced by time in units of nanoseconds (ns). At 10 ns, the presence of ligand in the cavity is still stable within it; at 20 ns, the ligand shifts but is still in the cavity, likewise at 30 ns. The results of molecular dynamic simulations from 0-30 nanoseconds show that the 3 (three) Prednisolone, Methylprednisolone, and Stigmastan-3,6-dione have similar amino acid residue patterns.

For the stability of the ligand complex with the protein, it is necessary to look at other parameters such as RMSF (Root Mean Square Fluctuation) which describe the conformational shift of each amino acid residue during the simulation, especially in the binding site area (Figure 6). The RMSF value was evaluated to determine fluctuations in ligand interactions with amino acids in the protein during the simulation. The RMSF value describes the flexibility of ligand interactions with each amino acid residue. The low flexibility of amino acid residues indicates the stability of interactions in the active site area that binds to the test compound

because the atoms that make up amino acid residues tend not to change many positions during the molecular dynamics simulation [35].

### 4.5 Toxicity

The purpose of carrying out in silico toxicity tests is as a preliminary test before carrying out in-vitro and in vivo tests and to predict the toxicity of a compound. Computational toxicity prediction can apply the 3 R's ethical principles in animals, namely Replacement; Reduction and Refinement. Toxic dose or $LD_{50}$ value in mg/KgBW. $LD_{50}$ is the lethal dose to 50% of test subjects when exposed to a compound. Prediction of oral toxicity ($LD_{50}$) in rodents and classification of compound toxicity based on the Globally Harmonized System (GHS)[36].

a. Class I: Fatal if swallowed ($LD_{50} \leq 5$ mg/kg b.w)

b. Class II: Fatal if swallowed ($5$ mg/kg $< LD_{50} \leq 50$ mg/kg b.w)

c. Class III: Toxic if swallowed ($50$ mg/kg $< LD_{50} \leq 300$ mg/kg b.w)

d. Class IV: Harmful if swallowed ($300$ mg/kg $< LD_{50} \leq 2000$ mg/kg b.w)

e. Class V: May be harmful if swallowed ($2000$ mg/kg $< LD_{50} \leq 5000$ mg/kg b.w)

f. Class VI: Non toxic ($LD_{50} > 5000$ mg/kg b.w)

Table 3 can be concluded that the insilico prediction of the toxicity of the compounds Stigmastan-3,6-Dione, Prednisolone and Methyl Prednisolone is still within safe limits. A summary of the in silico mechanism of action of Stigmastan-3,6 dione ($C_{29}H_{48}O_2$) on the EtOAc fraction of *M.charantia* L fruit can be seen in Figure 7.

## 5. CONCLUSION

The EtOAc fraction of *M. charantia L.* fruit with the active compound Stigmastan-3,6-Dione in its mechanism of action in silico shows activity as an anti-inflammatory and immunostimulant that works on the NF-kB pathway.

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