# Stabilisation Potential of Cell Membrane from Different Polarity Extract of Sirih Bumi (*Peperomia pellucida* [L.] Kunth) as Anti-inflammatory Agent

# (Potensi Stabilisasi Membran Sel dari Ekstrak Berbeda Kepolaran Sirih Bumi (*Peperomia pellucida* [L.] Kunth) sebagai Agen Antiinflamasi)

I GUSTI AGUNG AYU KARTIKA[1]\*, SURYANI[2], DWI HADI SETYA PALUPI[3], I KETUT ADYANA[4]

[1] Yoga and Health Study Program, Faculty of Brahma Widya, Universitas Hindu Negeri I Gusti Bagus Sugriwa Denpasar, Denpasar, 80235, Indonesia
[2] Pharmacology and Toxicology Department, Faculty of Pharmacy, Universitas Jendral Achmad Yani, Cimahi, 40531, Indonesia
[3] College of Pharmacy, Yayasan Pharmasi Semarang, Semarang, 50192, Indonesia
[4] Pharmacology and Clinical Pharmacy Department, School of Pharmacy, Institut Teknologi Bandung, Bandung, 40132, Indonesia

**Abstract:** Sirih bumi (*Peperomia pellucida* [L.] Kunth.) is a medicinal herb. Anti-inflammatory activities are reported. These plant extracts have been shown to reduce inflammation in many studies, but it is unknown which extract works best. The objectives if this study were determine the extract that has the best anti-inflammatory activity from sirih bumi. The activity was assessed from the impact of stabilising red blood cell membranes in vitro. The research was then continued with a literature study on the chemical compounds containing the best extracts. To determine the prediction of the compound responsible for its activity and potency, several in silico tests were carried out, such as PASS online, Pre-ADMET online, and Protox II. The test results showed that the ethyl acetate extract at 200 g/mL concentration showed similar activity to aspirin at the same concentration. The activity of this extract increased with increasing concentration of the extract. Six compounds were reported to be isolated from this type of extract. Dillapiol and its derivative 6-allyl-5-methoxy-1,3-benzodioxol-4-ol are predicted to be compounds that have anti-inflammatory effects by inhibiting the membrane permeability mechanism of ethyl acetate extract. Ethyl acetate extract showed the best anti-inflammatory activity among the other type of extract from sirih bumi. Ethyl acetate extract is a type of extract with the best anti-inflammatory activity from sirih bumi.

**Keywords:** Anti-inflammatory activity, cell membrane stability, dillapiol, *Peperomia pellucida*.

**Abstrak:** Sirih bumi (*Peperomia pellucida* [L.] Kunth.) termasuk tanaman gulma yang kaya akan manfaat farmakologis. Salah satu efek yang dilaporkan yaitu antiinflamasi. Banyak penelitian dilakukan untuk membuktikan aktivitas antiinflamasi dari ekstrak sirih bumi. Namun belum diketahui ekstrak dengan jenis pelarut yang mana memiliki efek terbaik. Penelitian ini bertujuan mengetahui jenis ekstrak yang memiliki aktivitas antiinflamasi terbaik dari sirih bumi. Aktivitas antiinflamasi dinilai dari efek stabilisasi membran sel darah merah secara in vitro. Penelitian kemudian dilanjutkan dengan studi pustaka terkait senyawa kimia kandungan jenis ekstrak terbaik. Untuk mengetahui prediksi senyawa yang bertanggung jawab terhadap aktivitas dan potensinya, dilakukan beberapa uji *in silico* seperti PASS *online*, Pre-ADMET, dan Protox II. Hasil uji menunjukkan ekstrak etil asetat pada konsentrasi 200 µg/mL menunjukkan aktivitas yang sebanding dengan aspirin pada konsentrasi yang sama. Aktivitas dari ekstrak ini semakin meningkat seiring dengan meningkatnya konsentrasi ekstrak. Enam senyawa dilaporkan diisolasi dari jenis ekstrak ini. Apiol, dillapiol, dan derivatnya yaitu 6-allil-5-metoksi-1,3-benzodioksol-4-ol serta β-sitosterol-D-glicopyranoside diprediksi menjadi senyawa yang memiliki efek antiinflamasi dengan mekanisme penghambatan permeabilitas membran dari ekstrak etil asetat. Ekstrak etil asetat merupakan jenis ekstrak dengan aktivitas antiinflamasi terbaik dari sirih bumi.

**Kata kunci:** Anti-inflamasi, dillapiol, membran, *Peperomia pellucida*.

\*Corresponding author
e-mail: ayukartika@uhnsugriwa.ac.id

## INTRODUCTION

PHYSICAL trauma, microbiological agents, or chemicals can cause tissue injury, which induces the body to provide a protective response called inflammation. In this process, chemical mediators were released from damaged tissue, and cell migration occurs[1]. Cell damage due to inflammation in the cell membrane causes leukocytes to release lysosomal enzymes and the cyclooxygenase (COX) pathway in arachidonic acid metabolism to produce prostaglandins. Prostaglandins have various effects on nerve endings, blood vessels, and cells involved in inflammation[2]. The search for inexpensive and effective anti-inflammatory agents leads to an exploration of the potential of natural products. Many natural medicines, especially plants, have been studied for their anti-inflammatory effects. Sirih bumi (*Peperomia pellucida* (L.) Kunth) was the promising plant that was classified as a weed. This plant is rich in pharmacological benefits[3–8].

Several experiments have revealed the anti-inflammatory activity of *Peperomia pellucida (Pp)*. A water extract of *Pp* at a dose of 100 mg/kg showed good anti-inflammatory activity in animal models of carrageenan and arachidonic acid-induced inflammation[9]. This activity is also influenced by harvesting time[10]. In another study using an extract dose of 2500 mg/kg BW rats, *Pp* extract produced almost 100% inhibition of carrageenan-induced oedema in rat models[11]. Using the same method, petroleum ether extract at a dose of 1000 mg/kg BW inhibited oedema significantly compared to controls[12]. In addition, 5 µg/mL of methanol extract was shown to be able to inhibit the release of several inflammatory mediators such as IL-8, IL-6, IL-1α, IL-1β, and TNF-α, although this activity did not reach 30% in an in vitro experiment[13].

Some of the studies above have not concluded which type of extract has the best potential activity. Therefore, the determination of the best type of extract that can be used for further development becomes necessary. This study used in vitro anti-inflammatory activity tests, literature studies, and a series of in silico tests to predict the potential compounds in the best extracts.

## MATERIALS AND METHODS

**MATERIALS.** The materials used in this study were fresh *Pp*, distilled water, methanol, ethyl acetate, 96% ethanol, n-hexane (CV Fadillah, Bandung Kulon, Indonesia), buffer solution (Merck, Darmstadt, Germany), methylprednisolone (Sigma-Aldrich, St. Louis,United States), sodium chloride (Merck, Darmstadt, Germany), and aspirin (Sigma-Aldrich, St. Louis,United States).

**Equipments.** The equipments used in this study were rotary evaporator (Buchi, Tangerang, Indonesia), freeze dryer (Zirbus, Harz, Germany), centrifugator (Drawell, Shanghai,China), incubator (Memmert, Singapore), and UV-Vis spectrophotometer (Beckmann Coulter, Indonesia).

The software used includes Marvin Sketch, ChemDraw, Lipinski Rule of Five (http://www.scfio-iitd-res-in/software/drugdesign/-lipinski.jps), Pre-ADMET Online (https://preadmet. webservice.bmdrc.org/), Protox II (http://tox.charite.de/protox_II/), and PASS Online (http://www.pharmaexpert.ru/-passonline). The database sources used were ChemSpider®, and Pubchem (http://pubchem.ncbi.nlm.nih.gov/).

**METHODS. Preparation and Determination of Test Plants.** Fresh *Pp* were obtained from the Cagak and Ciater areas, Subang, West Java, from February to April 2016. The test plants were determined at the Bandungese Herbarium, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology.

**Preparation of Simplicia and Extraction**. The procedure was conducted according to a previous study[6]. The roots of fresh *Pp* were cut and washed under running water. The clean herbs were then air-dried before being dried in an oven at 40-60°C. When it was dry enough, it was immediately grounded and ready to be used for extraction. Extraction (maceration) was carried out in stages using *n*-hexane, ethyl acetate, and 96% ethanol, as well as extraction with water separately. The solvent used was 20 times the weight of the simplicia for 3 x 24-hour macerations in a dark glass jar. The extract obtained was filtered, and then the filtrate was collected. All extracts were concentrated at 40-60°C using a rotary evaporator until there were no solvent droplets or until the weight of the resulting dry extract remained constant, except for the water extract, which was concentrated using a freeze dryer.

**Anti-inflammatory Activity Test with Stabilisation of Blood Cell Membrane Model.** Blood was collected from a healthy volunteer who had not taken non-steroidal anti-inflammatory drugs (NSAIDs) for two weeks before the experiment. The procedure was conducted with the help of the Indonesian Red Cross. Blood was centrifuged at 3000 rpm for 10 minutes. The cells obtained were washed with an isotonic solution (0.9% NaCl) in the same volume. The mixture is centrifuged twice, or until the liquid at the top is clear. The obtained cells were made into a 10% suspension with isotonic solution (0.9% NaCl)[14,15].

The test solution was prepared by mixing 2 mL of hypotonic solution (0.36% NaCl), 0.5 mL of 10% v/v blood cells in isotonic solution (0.9% NaCl), 1 mL of phosphate buffer solution (pH 7.4), and 1 mL of the evaluated sample. A mixed solution without blood cells was used as the test control. Sample and standard concentrations were 100, 200, 400, and 800 µg/mL. Aspirin is used as a standard medication. This mixture was incubated for 30 minutes at 37°C. After being centrifuged, the supernatant was collected. The absorbance of the collected supernatant was measured at 560 nm using an ultraviolet-visible spectrophotometer. The formula calculates the percentage inhibition of hemolysis:

$$= 100\% - \left( \frac{\text{absorbance of tested sample} - \text{absorbance of control}}{\text{absorbance of control}} \right) \times 100\%$$

**Literature Study.** This procedure is conducted to determine the content of phytochemical compounds in the selected extract types. The literature study was conducted using the Google Scholar and PubMed databases using keywords such as *Pp*, ethyl acetate, extract, isolation, and similar words in English.

**Biological Activity Prediction**. Prediction of biological activity is done using an online application called prediction of activity spectra for substances (PASS). The criteria used to determine whether a compound is active or not is the probable activity (Pa) value. If the Pa value $>0.7$, it is considered that the tested compound has an analogous form and activity with the drug compound. If $0.5< Pa <0.7$, the compound being tested has a different form and is less likely to show activity like a drug compound. Finally, if the Pa value was $<0.5$, it can be said that the compound being tested is unlikely to show activity like a drug compound. However, the test compound is likely to be a new chemical compound if this activity is confirmed through laboratory testing[16].

**Prediction of Phytochemical Compound ADMET Profiles.** This test is carried out by analysing the predicted profile of phytochemical compounds with the help of several online applications. Websites accessed were http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp, https://preadmet.bmdrc.kr/, http://tox.charite.de/protox_II/, and http://lmmd.ecust.edu.cn/admetsar1/predict. The compound profile was then matched with the Lipinski rule. The Lipinski rule states that the molecular mass should be less than 500 Daltons, have high lipophilicity (expressed by a Log P of less than 5), less than ten hydrogen bond acceptors, molar refractivity should be between 40 and 130, and have less than five hydrogen bond donors.

**Data Processing and Analysis.** Data processing, especially for the results of activity testing, wouldbe carried out qualitatively and quantitatively. Data in the form of numeric data is expressed as the average value ± standard deviation. Quantitative data processing would be done through statistical analysis using SPSS to determine whether there were differences and significant activity magnitudes between treatment groups. The quantitative data obtained were analysed using IBM SPSS Statistics version 2®. The significance level used was 0.05 or 0.01. The one-way ANOVA methode is used if the data is normally distributed and homogeneous. Otherwise, the Kruskal-Wallis test is used.

## RESULTS AND DISCUSSION

**In vitro Anti-inflammatory Activity of Extracts.** Anti-inflammatory activity of *Pp* had been investigated in many studies[17]. Various types of extracts have been examined, including petroleum ether, chloroform and methanol extracts, aqueous or water extracts, ethanol extract, and ethyl acetate extract[8,9,12,18]. The recent study provided a comparison of anti-inflammatory activity among several types of extracts. This study used stabilising the red blood cell membrane in vitro to evaluate anti-inflammatory activity. Even though research is conducted outside the body, it has been made in such a way as to resemble the situation inside the body. Human red blood cells have long been used as a model to study interactions between drugs and membranes.

Red blood cells were known to have a membrane that encloses haemoglobin. When the membrane breaks, the haemoglobin inside would come out. Something similar happens in the lysosomal membrane. When there is an injury to the lysosomal membrane, the substance inside would come out. The level of stability of the red blood cell membrane against disturbances such as induced by hypotonic solutions, can be used as a parameter to determine the stability of the lysosomal membrane[19].

Lysosomes play a role in the inflammatory process because the enzyme they release, namely the phospholipase enzyme, plays a role in converting phospholipids into arachidonic acid, which would produce prostaglandins. Prostaglandins would provide an inflammatory effect through vasodilation and increased permeability of the synovial membrane walls and blood vessel membranes, pain receptors were desensitised so that the effects of other mediators were strengthened.

Lysosomal enzymes released during the inflammatory process also cause various tissue disorders, macromolecular damage, and lipid peroxidation, which were thought to be responsible for

specific pathological conditions such as septic shock, heart attack, and rheumatoid arthritis. In addition, the extracellular activity of this enzyme was thought to be related to acute and chronic inflammation[20]. Therefore, compounds with membrane stabilising activity were expected to significantly protect the cell membrane and limit the inflammatory response.

Hypotonic solutions were one of the solutions that can disrupt the membrane stability of red blood cells. This solution causes hypotonic stress that can lead to the oxidation of lipids and proteins, which can further damage cell membranes. Cell membrane damage was characterised by hemolysis, or damage to red blood cells, so that haemoglobin was released. The amount of hemolysis can be used to assess a test sample's anti-inflammatory activity.

The results of the percentage inhibition of membrane lysis were shown in Figure 1. The ethyl acetate extract had the best inhibitory activity compared to other extracts. The activity of this extract increases with increasing concentrations of the extract. The ethyl acetate extract at a concentration of 200 μg/mL showed similar activity to aspirin at the same concentration. It was demonstrated that the extract has the capability to stabilise the membrane by preventing stress-related plasma membrane breakdown. A Aspirin was known to inhibit the activity of cyclooxygenase (COX), which causes fever, pain, swelling, and inflammation through the formation of prostaglandins (PGs)[21].

The superior activity of ethyl acetate extract compared with n-hexane and water extract in membrane stabilising was also found in another study[22]. In the previous study comparing the similar polarity of *Pp* extracts, ethyl acetate extract had the highest flavonoid content[23]. This might correlate with the highest protective activity of the extract. Compounds that can stabilise membranes can inhibit the initial process of the inflammatory phase. Several studies have reported that the presence of flavonoids plays a role in this activity[20,24,25]. However, other groups of compounds could also make a good contribution.

Flavonoid has been widely known for its anti-inflammatory activity[26-29]. The compounds can inhibit nuclear factor-κB (NF-κB) activations, signal activator, and transducer of transcription 1 (STAT-1). Also, they have an inhibitory effect on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in activated macrophages[30]. In addition, they inhibit the secretions of enzymes such as lysozymes and β-glucuronidase and inhibit the secretion of arachidonic acid, which reduces inflammatory reactions[27].

**Literature Study.** In this study, we focus on identifying the phytochemical compounds isolated from the ethyl acetate extract of *Pp* as extract with the highest anti-inflammatory activity. The compound structures were useful for the prediction of biological activity in silico. Several isolated compounds were apiol, dillapiol, a derivate of dillapiol (6-allyl-5-methoxy-1,3-benzodioksol-4-ol), pachypostaudin B, and pellucidin A[4]. Other studies mention the findings of β-sitosterol-DI-glucopyranoside in the ethyl acetate fraction[31]. Among those compounds, only dillapiole has been examined for its anti-inflammatory activity[32]. Dillapiol inhibited oedema formation significantly ($p < 0.05$) in a carrageenan-induced rat paw edema model.

**Biological Activity Prediction**. To our knowledge, this is the *first report* in silico study of the anti-inflammatory activity, especially for membrane stabilising properties of these compounds. The PASS Online web server was used to predict the biological activity of compounds that have been successfully analysed from *Pp*. Predictive data were presented in Table 1.
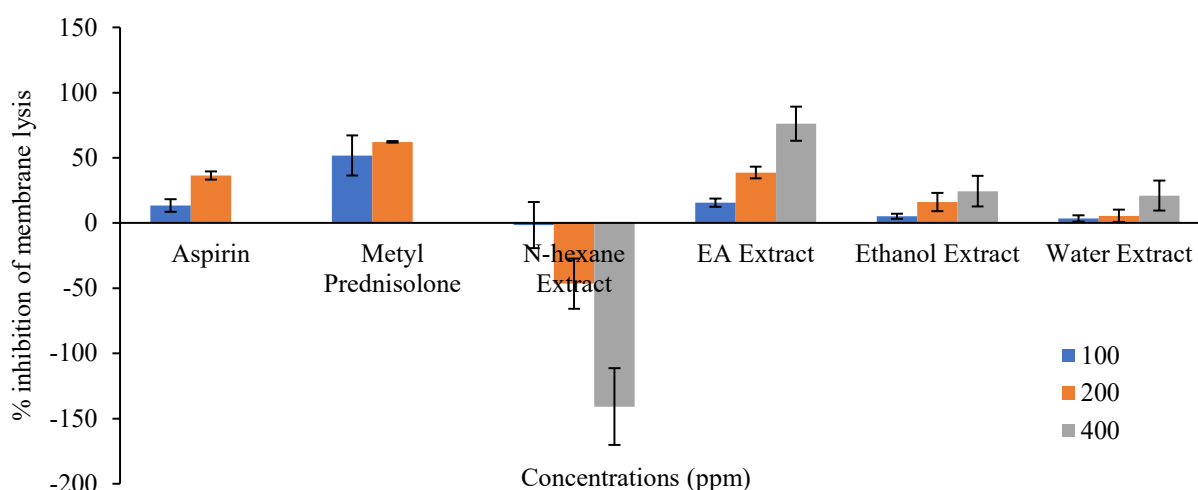


**Figure 1. Percentage of red blood cell membrane lysis inhibition by *Pp* extract.**

**Table 1. Predictions of the biological activity of detected compounds and compounds isolated from *Pp.***

| Compound | Pa | Pi | Pharmacological activity |
|---|---|---|---|
| Derivate of dillapiol | 0.895 | 0.012 | Membrane integrity agonists |
| | 0.745 | 0.004 | MMP-9 expression inhibitors |
| | 0.595 | 0.091 | Membrane permeability inhibitors |
| | 0.444 | 0.075 | Anti-inflammatory |
| | 0.240 | 0.064 | Treatment of bone disease |
| | 0.168 | 0.101 | Antiosteoporosis |
| | 0.154 | 0.139 | Bone formation stimulant |
| Pachypostaudin B | 0.404 | 0.094 | Anti-inflammatory |
| | 0.163 | 0.106 | Antiosteoporosis |
| | 0.155 | 0.065 | MMP-9 expression inhibitors |
| Pelucidin A | 0.645 | 0.011 | MMP-9 expression inhibitors |
| | 0.328 | 0.137 | Anti-inflammatory |
| | 0.272 | 0.052 | Treatment of bone disease |
| | 0.258 | 0.049 | Antiosteoporosis |
| | 0.191 | 0.175 | Angiogenesis stimulant |
| | 0.168 | 0.111 | Bone formation stimulant |
| Dillapiol | 0.901 | 0.011 | Membrane integrity agonists |
| | 0.696 | 0.007 | MMP-9 expression inhibitors |
| | 0.495 | 0.059 | Anti-inflammatory |
| | 0.418 | 0.195 | Membrane permeability inhibitors |
| | 0.177 | 0.104 | Treatment of bone disease |
| Apiol | 0.822 | 0.031 | Membrane integrity agonists |
| | 0.676 | 0.008 | MMP-9 expression inhibitors |
| | 0.430 | 0.188 | Membrane permeability inhibitors |
| β-sitosterol-D-glicopyranoside | 0.891 | 0.003 | Membrane integrity agonists |
| | 0.848 | 0.005 | Membrane permeability inhibitors |
| | 0.630 | 0.002 | Membrane permeability enhancer |
| | 0.544 | 0.045 | Anti-inflammatory |
| | 0.429 | 0.022 | Treatment of bone disease |
| | 0.402 | 0.023 | Antiosteoporosis |
| | 0.396 | 0.019 | Angiogenesis stimulant |
| | 0.158 | 0.153 | Non-steroidal anti-inflammatory agent |
| | 0.157 | 0.132 | Bone formation stimulant |

Apiol, dillapiol, derivates of dillapiol, and β-sitostrol-D-glicopyranoside were predicted to be involved in anti-inflammatory activity by stabilising cell membranes in ethyl acetate extracts. The prediction showed they have the pharmacological activity of membrane integrity agonists with pa score higher than 0.7. Based on the value of Pa, the possibility of the emergence of cell membrane stabilising activity from these compounds in the experiment was high. In addition, it was considered that the tested compound has an analogous form and activity to the drug compound[16]. The activity of the membrane integrity agonists was important for anti-inflammatory activity of *Pp*. Integrity of the lysosomal membrane ensures the prevention of the release of lysosomal enzymes into the cytoplasm. Membrane integrity was usually used to assess lysosomal dysfunction. Lysosomal disfunction was known to play a role in several diseases including autoimmune and inflammatory disorders, cancer, metabolic disorders, and neurodegenerative diseases[33].

The derivatives of dillapiol were newly discovered compounds, so research on their activity was still very limited. There has been no research into its anti-inflammatory activity. Apiol has also never been tested as an anti-inflammatory agent. However, the anti-inflammatory activity of dillapiol has been previously demonstrated through in vivo studies[32].

Dillapiol could be used as a prototype for a new anti-inflammatory agent due to its moderate anti-phlogistic properties. The presence of alkyl groups on the side chain, the benzodioxole ring, and methoxy groups on the aromatic ring were essential for an anti-inflammatory activity[32].

**Table 2. Prediction of ADMET phytochemical compounds from *Pp* in silico.**

| Parameter | Requirement | Derivate of dillapiol | Dillapiol | Apiol | β-sitostrol-D-glicopyranoside |
|---|---|---|---|---|---|
| *Lipinski's rule of 5-drug likeness* | | | | | |
| Massa (Dalton) | ≤ 500 | 208.21 | 222.24 | 222.24 | 592.00 |
| Donor hydrogen bonds | ≤ 5 | 0 | 0 | 0 | 4 |
| Acceptor hydrogen bonds | ≤ 10 | 4 | 4 | 4 | 6 |
| Log P | ≤5 | 1.86 | 2.16 | 2.16 | 6.32 |
| Molar refractivity | 40-130 | 54.68 | 59.57 | 59.57 | 165.49 |
| | | | | | |
| *ADME* | | | | | |
| % Human Digestive Absorption | Normal range Low=0-20% Moderate=20-70% Good=>70% | 94.497932 | 98.88827 | 98.88827 | 89.91 |
| Permeability to Caco-2 cells (nm/s) | Low=<4 Moderate=4-70 High=>70 | 38.6126 | 57.2419 | 57.2419 | 26.30 |
| % Plasma protein binding | >90% (high) | 87.580785 | 89.088539 | 88.847870 | 100 |
| Blood brain barrier | Low=<0.1 Moderate=0.1-2 High=>2 | 0.722516 | 1.72265 | 1.72265 | 6.16872 |
| | | | | | |
| *Toxicity* | | | | | |
| $LD_{50}$ prediction | | 1000 mg/kg | 1000 mg/kg | 1000 mg/kg | 23,000 mg/dL |
| Toxicity class prediction (similarity; accuracy) | | 4 (97.47%;72.9%) | 4 (100%; 100%) | 4 (100%; 100%) | - |
| Hepatotoxicity (possibility) | | Not active (0.76) | Not active (0.75) | Not active (0.75) | - |
| Carcinogenicity (possibility) | | Active (0.58) | Active (0.55) | Active (0.55) | Not active (0.9442) |
| Immunotoxicity (possibility) | | Active (0.88) | Active (0.60) | Active (0.60) | - |
| mutagenicity (possibility) | | Not active (0.64) | Not active (0.64) | Not active (0.64) | - |
| Cytotoxicity (possibility) | | Not active (0.90) | Not active (0.92) | Not active (0.92) | - |
| Acute toxicity to rat ($LD_{50}$, mol/kg) (possibility) | | 2.6986 | 2.4136 | 2.4136 | 2.9608 |
| Acute toxicity to fish ($pLC_{50}$, mg/L) | | 0.4954 | 0.1389 | 0.1389 | 1.5294 |

Considering these three compounds have similar structures, there was a great chance that the apiol and derivate of dillapiol had similar actions.

Besides the mechanism of membrane stabilisation, its capability of COX-2 and cytokine inhibition also contributed to the anti-inflammatory activity of *Pp*. Also, it showed anti-arachidonate 5-lipoxygenase and antihyaluronidase activity[13,34,35].

**Prediction of Phytochemical Compound ADMET Profiles.** The results of the profile prediction of apiol, dillapiol, the derivate of dillapiol, and β-sitostrol-D-glicopyranoside are presented in Table 2. The requirements of the Lipinski Rule of 5 were used in this study to help distinguish between nondrug-like and drug-like molecules. The probability of success or failure was determined using the drug-likeness of molecules to comply with two or more of the rules[36].

Based on these data, the three isolates have potential as candidates for anti-inflammatory drugs. However, more attention needs to be given to their safety. Apiol, dillapiole, and its derivatives have the potential to cause carcinogenicity and immunotoxicity. Close monitoring was required, especially for high-risk individuals.

**CONCLUSION**

Semi-polar and polar extracts were proven to stabilise the membrane. The type of extract with the best activity from *Peperomia pellucida* was ethyl acetate extract. The compounds thought to be responsible for this activity were apiol, dillapiol, derivates of dillapiol, and β-sitosterol-D-glicopyranoside.

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