Computational study on the effectiveness of flavonoids from Marsilea crenata C. Presl as potent SIRT1 activators and NFκB Inhibitors

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Abstract
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Keywords
Aging; Flavonoid; NFκB; Ovary; SIRT1

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Abstract

Ovarian aging is a natural process in females, and it occurs due to an elevated ROS-induced inflammation caused by oxidative stress. SIRT-1 is a metabolic sensor that tightly regulates oxidative and inflammatory responses. However, this regulative function is antagonized by NFκB. Therefore, the objective of this study was to explore the pathways involved in aging and identify the flavonoid compounds from Marsilea crenata that might be useful as SIRT1 activators and NFκB inhibitors. The screening began with exploring the protein–protein interaction in the experimental process using BioGrid, and the role of the flavonoid was evaluated using STITCH. The interaction between hyperoside, luteolin, naringenin, and quercetin against SIRT1 and NFκB was determined through molecular docking, while their toxicity was also evaluated. The present result demonstrated that seven proteins were similar to the SIRT1 and NFκB pathways. The STITCH result showed six proteins involved in aging, and quercetin was shown to interact with SIRT1. The molecular docking result demonstrated that hyperoside exhibited as a SIRT1 activator for dimeric, heterodimeric, and heterotrimeric chains and NFκB inhibitor for p52-RelB, p50-p65 complex, and IκK, as it had the best binding affinity compared to the other flavonoids. These flavonoids also potentially have at least one toxicity characteristic. In conclusion, flavonoids from M. crenata might be a promising activator of SIRT1 and inhibitors of NFκB.

Keywords: Aging, Flavonoid, NFκB, Ovary, SIRT1

1. Introduction

Ovarian aging is a natural process during the reproductive life of women. Furthermore, the ovary is one organ that begins aging first after approximately 30 years [1,2]. Its function is also inversely correlated with age, as it experiences one of the most devastating regressions compared to other tissues [3,4]. This aging process can cause a steady decrease in follicle numbers and oocyte quality, leading to the ovarian inability to respond to the stimulants adequately. This condition leads to irregular menstruation and follicular function loss [5,6]. However, the detailed mechanism of ovarian aging and female age-related reproductive loss has remained unclear.

Oxidative stress caused by excessive reactive oxygen species (ROS) production is one among many theories that well-described aging process. Interestingly, ROS concentration is higher in older people than in young people [7]. Furthermore, ROS may also activate the nuclear factor kappa beta (NFκB), an inducible transcription factor, which acts as a key modulator of inflammatory responses and is involved in many diseases [8]. Interestingly, the activation of NFκB has antagonized interaction with Sirtuin (SIRT)-1.

SIRT1 is a nicotinamide adenine dinucleotide-dependent histone deacetylase that influences a
A wide range of cellular activities. Therefore, it has long been considered the key to a long life [9,10]. The direct interaction between SIRT1 and NFκB is mediated chiefly through acetylation/deacetylation. Therefore, the targeting of the NFκB/SIRT1 axis is essential for identifying anti-aging compounds, especially in the ovary.

*Marsilea crenata* C. Presl from the family Marsileaceae is commonly known as water clover or *semanggi* in Indonesian. Furthermore, it is frequently found throughout Southeast Asia and is an aquatic perennial fern, widely consumed as a vegetable in Indonesian society. Several studies reported that *M. crenata* contains phytoestrogen, which has anti-osteoporotic activity, elevated estrogen concentration, maintains follicle and oocyte diameter, and alters the uterine histological features [11–16]. In the male, *M. crenata* could restore sperm quality and reproductive hormone [17,18]. Furthermore, the radioimmunoassay methods demonstrated using dried fern specimens showed that it contains phytoestrogen at 1068 pg/g, twice higher than in fresh specimens [19]. Another study reported that *M. crenata* is abundant with bioactive compounds, including naringenin, hyperoside, and quercetin [20], but might have phytotoxic effects [21].

Although *M. crenata* shows a promising effect, the studies of its beneficial effect were mainly focused on its phytoestrogen compounds (Table 1). Also, there is still a lack of evidence on the contribution of its phytochemicals to the SIRT1/NFκB signaling pathway for delaying ovarian aging. Moreover, this aging condition and related disorders could be delayed or prevented using effective natural antioxidants [22]. In addition, some studies showed that natural compounds might act as SIRT1 activators [23,24], which may further help slow down the aging process of the ovaries. Therefore, in this study, molecular docking was carried out to evaluate the potential of bioactive compounds from *M. crenata* to interfere with NFκB and SIRT1. Furthermore, the toxicity of the compounds was also evaluated.

2. Material and methods

2.1. Ligands and protein preparation

The molecular docking studies were carried out using two extracts of medicinal plants, as presented in Table 2. Furthermore, the database of each natural compound selected from *M. crenata* was based on the laboratory’s previous work.

Six compounds were used as the test molecules, and MG132 was selected as a reference molecule due to its role in blocking nuclear translocation and DNA binding of NFκB complexes [25]. Meanwhile, nicotinamide adenine dinucleotide (NAD) was selected as a reference molecule to activate SIRT1 [26].

All molecules used as the test set in the docking simulation were built in the pdb format for their 3D structure, using PyMol software. Furthermore, these molecules were minimized using Open Babel in PyRx 0.8. The three-dimensional structure of NFκB and SIRT1 were downloaded from the Protein Data Bank (https://www.rcsb.org/). Finally, the cocrystallized NF-κB and SIRT1 structures were removed for their water molecules, and the hydrogen atoms were added to the protein using PyMol as the standard procedure.

2.2. Molecular docking simulation

Molecular docking of each molecule from *M. crenata*, as listed in Table 2, was carried out using PyRx. The results were then visualized in Pymol, and the amino acid interaction was evaluated using Discovery Studio v20. The protein target and its target regions are shown in Table 3.

2.3. Protein interaction and pathway analysis

The BioGRID database (https://thebiogrid.org/) was used to analyze protein interactions between NFκB and SIRT1, with minimum evidence and was set at 17. Furthermore, STITCH (http://stitch.embl.de/) was used to study ligands’ interaction with protein networks.

2.4. Toxicity prediction

The four compounds which were tested for molecular docking were also analyzed to ascertain their toxicity characteristic, such as LD50, class, hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity using the ProTox II database (https://tox-new.charite.de/protox_II/index.php?site=compound_input).

3. Results

3.1. NFκB and SIRT1 protein interaction

The results from the BioGrid database showed that the protein–protein interaction (PPI) involved several proteins that directly interact with NFκB (Fig. 1A) and SIRT1 (Fig. 1B). Furthermore, seven proteins were similarly found in the two signaling pathways marked by red squares. The PPI from the BioGrid database was then inputted on STITCH to...
ascertain each ligand interaction type with NFkB and SIRT1. Further analysis using STITCH observed six proteins involved in the aging process in the SIRT/NFkB axis, particularly Akt1, TP53, CDKN1A, ATM, SIRT1, and RelA (Fig. 1C).

Quercetin regulates the transcription of Akt1 and SIRT1, while luteolin regulates the transcription of Akt1. Therefore, based on the network analysis, quercetin was predicted to be a potent activator of SIRT1.

### Table 1. Summary of studies using *M. crenata*.

<table>
<thead>
<tr>
<th>Publication year</th>
<th>Title</th>
<th>Contribution</th>
<th>Advantages/Inconvenient</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016</td>
<td>The effects of water clover (<em>Mar-silea crenata</em>) extract against estrogen, progesterone, and uterine histology on rat (<em>Rattus norvegicus</em>)</td>
<td>Increased estrogen, decreased progesterone, and thickened the endometrium of rats.</td>
<td>The administration of n-hexane extract of <em>M. crenata</em> did not differ statistically on every groups.</td>
<td>[13]</td>
</tr>
<tr>
<td>2017</td>
<td>Isolation and identification of two potential phytotoxic substances from the aquatic fern <em>Mar-silea crenata</em></td>
<td>Two phytotoxic substances of aqueous methanolic extract of <em>M. crenata</em> identified as loliolide and isololiolide.</td>
<td>Both substances may contribute to the phytotoxicity of <em>M. crenata</em>.</td>
<td>[21]</td>
</tr>
<tr>
<td>2018</td>
<td>Alkaline phosphatase activity of <em>Mar-silea crenata</em> Presl. extract and fractions as marker of MC3T3-E1 osteoblast cell differentiation</td>
<td>Increase ALP activity in MC3T3-E1 osteoblast cell.</td>
<td>The fraction 2 and 4 were rich in palmitic acid compounds.</td>
<td>[12]</td>
</tr>
<tr>
<td>2020</td>
<td>Anti-neuroinflammation activity of n-butanol fraction of <em>Mar-silea crenata</em> Presl. in microglia HMC3 cell line</td>
<td>Inhibited the MHC II expression in the microglia HMC3 cell line.</td>
<td>n-butanol fraction of <em>M. crenata</em> at a dose 250 ppm was the best dosage</td>
<td>[16]</td>
</tr>
<tr>
<td>2020</td>
<td>The enhancement of Arg1 and activated ERβ expression in microglia HMC3 by induction of 96% ethanol extract of <em>Mar-silea crenata</em> Presl. leaves</td>
<td>Decreased the activated ERβ and induced of Arg1 in the microglia HMC3 cell line.</td>
<td>The 96% ethanol extract of <em>M. crenata</em> leaves at a dose 250 ppm was the best dosage</td>
<td>[15]</td>
</tr>
<tr>
<td>2020</td>
<td>Effect of water clover (<em>Mar-silea crenata</em>) ethanol extracts on follicle and oocyte diameter of goat: in vitro study</td>
<td>Increased the diameter of antral follicle and oocyte.</td>
<td>Aqueous ethanolic extract of <em>M. crenata</em> at a dose 43.2 μg/mL increased the follicle diameter at day 3 and 6 and oocyte diameter.</td>
<td>[14]</td>
</tr>
<tr>
<td>2020</td>
<td>In vitro and in silico analysis on the bone formation activity of n-hexane fraction of semanggi (<em>Mar-silea crenata</em> Presl.)</td>
<td>n-hexane fraction of <em>M. crenata</em> Presl. Leaves increased bone formation activity in hFOB 1.19 cells.</td>
<td>Ten identified compounds were showed have an ER-β agonists activity.</td>
<td>[11]</td>
</tr>
<tr>
<td>2021</td>
<td><em>Mar-silea crenata</em> ethanol extract prevents monosodium glutamate adverse effects on the serum levels of reproductive hormones, sperm quality, and testis histology in male rats</td>
<td>Increased LH, testosterone, spermatogonia, spermatocytes, spermatids, Leydig cells number, seminiferous tubular diameter, and germinal epithelium thickness and decreased MDA in rat testes.</td>
<td>Aqueous ethanolic extract of <em>M. crenata</em> at doses 0.216, 0.432, and 0.648 mg/g BW improved hormonal and sperm quality in rats.</td>
<td>[17]</td>
</tr>
<tr>
<td>2022</td>
<td>Potential of combination <em>Mar-silea crenata</em> and <em>Curcuma xanthorrhiza</em> to improve sperm quality of male mice exposed by monosodium glutamate</td>
<td>The combination of <em>M. crenata</em> and <em>Curcuma</em> improved the sperm quality in mice exposed by MSG.</td>
<td>Aqueous ethanolic extract of <em>M. crenata</em> at a dose 0.045 mg/g BW combine with 0.1 mg/g BW of <em>C. xanthorrhiza</em> restored sperm quality of mice.</td>
<td>[18]</td>
</tr>
</tbody>
</table>

ascertain each ligand interaction type with NFkB and SIRT1. Further analysis using STITCH observed six proteins involved in the aging process in the SIRT/NFkB axis, particularly Akt1, TP53, CDKN1A, ATM, SIRT1, and RelA (Fig. 1C).

Quercetin regulates the transcription of Akt1 and SIRT1, while luteolin regulates the transcription of Akt1. Therefore, based on the network analysis, quercetin was predicted to be a potent activator of SIRT1.

### 3.2. SIRT1 protein interaction with bioactive compounds of *M. crenata*

This study showed that the selected bioactive compounds from *M. crenata* could potentially be SIRT1 activators. Furthermore, hyperoside was
shown to have the strongest binding affinity to SIRT1, compared to other *M. crenata* bioactive compounds. It also had the same binding affinity to NAD as the drug reference in the SIRT1 heterodimeric and heterotrimeric chains, as indicated in Table 4 below.

The molecular docking analysis showed that hyperoside, luteolin, naringenin, and quercetin had the same binding site with NAD as the chemical reference (Fig. 2). Furthermore, from the amino acid interaction, Ser365 was the most frequently appearing compound in all–ligand interaction with SIRT dimeric chains (Table 4).

The analysis also ascertained that Asn226 and Glu230 were the most frequently occurring amino acids on the SIRT1 heterodimeric chains (Table 4).
Thr177 frequently appeared during the all–ligand interaction in the chains, except with quercetin. Meanwhile, Glu230 also appeared in the interaction, except with naringenin (Table 4).

3.3. NFκB protein interaction with bioactive compounds of M. crenata.

In this study, the docking results showed that the selected bioactive compounds from M. crenata might be potential candidates as NFκB inhibitors. Furthermore, similar to the previous results, hyperoside had the strongest binding affinity to p52-RelB, p50-p65 NFκB, and IkB, compared to other ligands (Table 5). It also appeared to have a better binding affinity than MG-132 as a drug reference on p52 and IkB. Finally, xanthorrhizol had the strongest binding affinity than beta-curcumene and curcumin at IkK complexes.

The molecular docking analysis showed that hyperoside, luteolin, naringenin, and quercetin have the same binding site with MG-132, as a drug reference (Fig. 3). Furthermore, from the amino acid interaction, it was ascertained that Asp219 was frequently present in all–ligand interactions with the p52-RelB NFκB complex (Table 5). Furthermore, Arg33 was the most frequently occurring amino acid in the all–ligand interaction with the p50-p65 NFκB complex (Table 5). Finally, Asn150, Asp166, Gly24, and Thr185 amino acids most frequently appeared in the all–ligand interaction with IkK protein (Table 5).

3.4. Oral toxicity prediction of bioactive compounds from M. crenata

The oral toxicity prediction of bioactive compounds from M. crenata showed that quercetin belongs to class 3, naringenin to class 4, Luteolin, and hyperoside to class 5 (Table 6). Surprisingly, the selected M. crenata bioactive compounds were predicted to have one toxicity feature, particularly hyperoside and naringenin (Table 6).

4. Discussion

SIRT1/NFκB axis has become an important topic of study due to its antagonistic effect on maintaining homeostasis and balancing immune and inflammatory responses [27]. In the cytoplasm, NFκB binds with its inhibitory protein, IkB. As soon as a stimulus enters the cell, the phosphorylation of this IkB protein begins, followed by the ubiquitination and degradation of the NFκB inhibitory protein. NFκB is then released and translocated to the nucleus to activate gene transcription [28].

In contrast, SIRT1 counteracts NFκB action through deacetylated RelA/p65 subunit lysine 310, resulting in ubiquitination and degradation of RelA/p65. This condition reduces transcriptional activity and restricts proinflammatory target gene expression [29,30]. Interestingly, the deacetylation of RelA/p65 by SIRT1 promotes the interaction of p65/p50, which is the most ubiquitous NFκB heterodimer with IkB [31]. This interaction causes the NFκB complex to be transported back to the cytoplasm from the nucleus, becoming inactive and delaying many pathophysiological processes, such as oxidative stress, inflammation, and aging [27]. Numerous studies have discovered some SIRT1 activator agents [32,33]. This is because SIRT1 enhancement might increase the quality of life through “healthy” aging.

This study demonstrated that flavonoids from M. crenata might be promising SIRT1 activators. This is
evident due to the binding of hyperoside, luteolin, naringenin, and quercetin to the allosteric site of the SIRT1 protein. The allosteric site is the point of binding for the active regulator of SIRT1 (AROS) [34]. These sites were identified in the amino acid residues 183–243, and typically interacted with hydrophobic chains at Thr209, Pro211, Pro212, Leu215, Thr218, Ile223, and Ile227. Meanwhile, hydrophilic reactions occurred at Asn226 [23]. The previous study reported that Glu230 and Asp226 are essential for promoting SIRT1 activity by stabilizing the activated conformation of SIRT1 [35,36]. Furthermore, it showed that Asp226 could interact directly on the substrate with SIRT1 [37].

The molecular interaction results in this study are supported by other studies which showed that hyperoside, luteolin, naringenin, and quercetin could enhance the expression of SIRT1. They also revealed that the concentration of this protein was suppressed by nicotinamide [33,38–40].
Quercetin contains five hydroxyl groups, and it is believed that the 4’OH group in its B-ring is responsible for carrying out activities [41]. Furthermore, the 3’OH groups on the B ring are attractive to electrophilic attacks due to more excess negative charges than naringenin [42].

Luteolin is another bioactive compound of *M. crenata*, and it is identical to quercetin in the hydroxyl groups on the A and B phenyl rings. Meanwhile, naringenin does not contain hydroxyl groups at the –3 nor –4 position of its C ring compared to quercetin. On the other hand, hyperoside substitutes the hydroxyl group on C3 with galactoside, which influences its antioxidant capacity compared to quercetin [43]. This is in line with the results of this study, which showed that hyperoside has a stronger binding affinity than quercetin as shown in Table 3. The greatest negative values of hyperoside can be explained based on Gibb’s energy that the interaction between the ligand and protein will eventually achieve an equilibrium state when the binding affinity values are more negative [44–46].

This study showed that flavonoids from *M. crenata* might be promising as NFkB inhibitors on NFkB p52-RelB complex and NFkB p50-p65 heterodimer. Furthermore, the previous study reported that Arg52 and Arg54 are key residues on p52 NFkB [25], and Arg52 on p52 was rotated around 15° to make polar contacts with guanine and cytosine [47].

Arg33, present on NFkB p50-p65 heterodimer, is one of the key residues on p65. In addition, it helps the recognition of gua25’ and gua24’, and establishes a protein on the RelA [48]. Finally, RelA heterodimer is mediated through the transcription genes involved in survival, proliferation, and inflammation.

In line with the STITCH results, RelA was involved in the aging pathway, and its inhibition might provide a better understanding of the action mechanism of the flavonoid of *M. crenata*. On the
other hand, Asn150, Asp166, Gly24, and Thr185 might be important IκK proteins. All Asp residues in the catalytic loop formed a polar contact with ATP-phosphatase directly as well as through the magnesium atoms [49]. Therefore, the bioactive compounds from M. crenata might show anti-inflammatory activity by binding with NFκB. Hyperoside, luteolin, naringenin, and quercetin target the different sites of the NFκB proteins, which may have a beneficial effect to inhibit
inflammation and break the vicious cycle of oxidative stress.

However, the limitation of the current study was the result only describes the PPI of the SIRT1/NFκB axis with four main flavonoids of *M. crenata*. Then, the molecular docking only has a success rate of around 60–75% in identifying the right poses. However, it allows for arranging the cost-benefit ratio, lowering the error rate, and avoiding wasting time and resources before the experimental laboratory begins [50,51]. In this scenario, based on the result, the flavonoid might be predicted as a promising candidate to extend lifespan and possibly maintain the ovary health via the SIRT1/NFκB axis. They may also delay the negative impact of aging.

5. Conclusion

In summary, the results showed that seven proteins were found similarly in the SIRT/NFκB signaling pathways with quercetin, which was predicted as a potent activator of SIRT1 based on BioGrid and STITCH, respectively. Hyperoside has the strongest binding affinity value in NFκB and SIRT1 proteins. The toxicity prediction showed that the flavonoids of *M. crenata* at least have one toxicity characteristic by inducing hepatotoxicity, carcinogenicity, immunotoxicity, mutagenicity, or

### Table 5. The binding affinity calculation and amino acid interaction over molecular docking simulations of selected *M. crenata* bioactive compounds against NFκB.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Compounds</th>
<th>Binding Affinity (kcal/mol)</th>
<th>Hydrogen bond(s) interaction</th>
<th>van der Waals interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NFκB p52-RelB Complex (3DO7)</strong></td>
<td>MG-132</td>
<td>−6.7</td>
<td>Arg52, Lys221, Phe53, Tyr55</td>
<td>Arg52, Lys221</td>
</tr>
<tr>
<td></td>
<td>Hyperoside</td>
<td>−6.9</td>
<td></td>
<td>Arg54, Asp219, Glu58, Lys43, Pro223, Ser222, Tyr285,</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>−6.7</td>
<td>Glu58, His140, Ser188</td>
<td>Arg54, Asp219, Phe53</td>
</tr>
<tr>
<td></td>
<td>Naringenin</td>
<td>−6.4</td>
<td>Arg52, His140, Ser188</td>
<td>Glu58, Asp219</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>−6.5</td>
<td>His140, Ser188</td>
<td>Asp219, Leu187, Lys143, Pro223, Ser222, Tyr55</td>
</tr>
<tr>
<td><strong>NFκB p50-p65 Heterodimer Complexed to kappa B DNA (1VKX)</strong></td>
<td>MG-132</td>
<td>−7.4</td>
<td>Arg33, Lys218, Arg605</td>
<td>Lys572</td>
</tr>
<tr>
<td></td>
<td>Hyperoside</td>
<td>−6.9</td>
<td>Arg33, Lys218, Arg605</td>
<td>Ala192, Arg187, Arg246, Arg35, Asp217, Glu247,</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>−6.7</td>
<td>Arg605</td>
<td>Arg187, Arg246, Arg33, Asn186,</td>
</tr>
<tr>
<td></td>
<td>Naringenin</td>
<td>−6.4</td>
<td></td>
<td>Asp217, Glu247, Glu606, Glu193,</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>−6.4</td>
<td>Arg605, Asp217</td>
<td>Ala192, Arg33, Asn186, Asp217, Glu606, Glu193,</td>
</tr>
<tr>
<td><strong>IκK (3RZF)</strong></td>
<td>MG-132</td>
<td>−6.4</td>
<td></td>
<td>Asp166, Gly149, Gly184, Gly22, Ile165, Lys106,</td>
</tr>
<tr>
<td></td>
<td>Hyperoside</td>
<td>−7.9</td>
<td>Leu21, Thr185</td>
<td>Lys147, Thr23</td>
</tr>
<tr>
<td></td>
<td>Luteolin</td>
<td>−7.7</td>
<td>Leu21, Lys147</td>
<td>Asp150, Asp166, Gly149, Gly184,</td>
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<tr>
<td></td>
<td>Naringenin</td>
<td>−7.1</td>
<td></td>
<td>Gly22, Ile165, Thr23, Val29,</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>−7.8</td>
<td>Asp166</td>
<td>Asp150, Gly149, Gly184, Gly22,</td>
</tr>
</tbody>
</table>

### Table 6. Oral toxicity prediction using ProTox II.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LD50 (mg/kg)</th>
<th>H</th>
<th>Cg</th>
<th>I</th>
<th>M</th>
<th>Cy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperoside</td>
<td>5000</td>
<td>5</td>
<td>(−)</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>Luteolin</td>
<td>3919</td>
<td>5</td>
<td>(−)</td>
<td>(+)</td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Naringenin</td>
<td>2000</td>
<td>4</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(+)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>159</td>
<td>3</td>
<td>(−)</td>
<td>(+)</td>
<td>(−)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Note: H = hepatotoxicity, Cg = carcinogenicity, I = immunotoxicity, M = mutagenicity, and Cy = cytotoxicity.
cytotoxicity. Molecular docking is useful for drug discovery and optimization of bioactive compounds to reduce the error rate before the further examination. However, the computational studies should be integrated with pre-and clinical studies to discover their medicinal effect on ovarian aging.

Conflict of Interest

All authors declare no potential conflicts of interest regarding the present study.

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