In-silico study of *Marselia crenata* compounds as activator Keap1/Nrf2 pathway in ovarian function

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**Abstract** The activity of granulosa cells during folliculogenesis and the presence of follicular atresia causes increased ROS (Reactive Oxygen Spesies) level in the ovaries. ROS accumulation will induce disorders of ovarian physiology, so maintaining ROS levels is necessary for normal ovarian physiology. Nrf2 (nuclear factor erythroid-2-related factor 2) is a protein that plays a role in regulating the expression of genes that induce antioxidant expression. In an inactive, Nrf2 binds to Keap1 in the cytoplasm. Inhibiting the interaction of Keap1-Nrf2 will activate Nrf2 and Nrf2 will move towards the nucleus to regulate some endogenous antioxidants. The research aims to investigate the potential of *Marsilea crenata* compounds as inhibitors of Keap1-Nrf2 interactions. The study used an in-silico approach. Compounds chosen were Naringenin and Hyperoside. Keap1 (ID: 2flu) was obtained from Protein Data Bank (PDB), while Hyperoside (CID: 5281643), and Naringenin (CID: 932) were retrieved from PubChem database. Keap1 was prepared by removing any solvents and other ligands using Discovery Studio v.19 versions. Naringenin and Hyperoside were minimized their affinity using PyRx 0.8 software. Ligand and protein were interacted using autodock vina integrated with PyRx 0.8 tools and visualized by Discovery Studio v.19. Results showed that Naringenin and Hyperoside bound to Keap1 in the same active sites of Keap1-Nrf2 regions. Both of Naringenin and Hyperoside interacted with Keap1 in different areas. The interaction between Keap1 and ligands (Naringenin and Hyperoside) was through the formation of hydrogen bonds and Van der walls forces. The binding energy of Naringenin and Hyperoside with Keap1 was -6.7 and -7.2 Kcal/mol, respectively. Our study predicted that Naringenin and Hyperoside might have a potential activity to inhibit Keap1-Nrf2 interaction and activated Nrf2 to regulate the antioxidant gene.

**Keywords**: Hyperoside, *M. crenata*, Naringenin, Nrf2-Keap1

1. **Introduction**
Mammalian ovaries are primary reproductive organs, which have thousands of ovarian follicles. The ovary is a dynamic organ, which has three main functions, namely folliculogenesis, oogenesis and steroidogenesis [1]. The follicles are a functional unit of the ovary. Ovarian follicles each containing an oocyte, which is surrounded by granulosa and theca cells. During the reproductive cycle, follicles...
will develop from primordial to antral follicles, and the end of follicular development is oocytes ovulated [2]. During the process of follicular development, the level of ROS (Reactive Oxygen Species) increases, which is associated with the metabolism of granulosa cells in follicles that undergo rapid proliferation. Also, the high ROS can also be caused by follicles undergo degenerative process or atresia [3]. Follicular atresia is preceded by the apoptosis of granulosa cells, which is caused by high ROS in the follicle [4,5].

Naturally, ovarian activity (folliculogenesis, oogenesis and steroidogenesis) will generate ROS and a small amount of ROS is needed for ovulation, oocyte maturation, gene regulation control and many others [3,6,7]. However, the accumulation of ROS induces oxidative stress that will disrupt the function of ovarian physiology [8]. Therefore, maintaining a balance between generating and eliminating ROS is very important for normal ovarian function. Antioxidants are indicated as factors that can maintain the ROS level [9].

There are two types of antioxidants, namely endogenous antioxidants and exogenous antioxidants [10]. Exogenous antioxidants are antioxidants obtained from the diet, such as carotenoids, ascorbic acid and some of the flavonoid compounds, and endogenous antioxidants are antioxidants that are synthesized in the body, such as GSH (glutathione), SOD (superoxide dismutase) and CAT (Cctalase) [11]. One that has a role in the synthesis of endogenous antioxidants is Nrf2 (nuclear factor erythroid-2-related factor, a transcription factor that regulates the expression of some endogenous antioxidants [2]. In an inactive, Nrf2 binds to Keap1 in the cytoplasm. Inhibiting the interaction of Keap1-Nrf2 will activate Nrf2 and Nrf2 will move towards the nucleus to regulate some endogenous antioxidants. Flavonoid compounds are thought to have a role in dissociation of the Keap1-Nrf2 interaction [12]. Some flavonoid groups that have potential as Keap1 and Nrf2 dissociation including curcumin [13], genistein [14], routine [12]. Flavonoids can bind to amino acid residues of Keap1 protein, which causes changes in Keap1 conformation. The conformational change of Keap1 causes the dissociation of Keap1-Nrf2 interaction [15].

Indonesia has more than 38,000 species of plants, including water clover. Water clover (Marsilea crenata) is one type of wild plant that used by the community as food, which contains a lot of flavonoids. Phytochemical test results show that M. crenata contains various chemical components of flavonoids, including naringenin, hyperosida, daidzein, genistein [16]. Nowadays, computational applications are widely used to study the role of herbal medicine for health. The research aims to investigate the potential of M. crenata compounds as inhibitors of Keap1-Nrf2 interactions, through the in silico approach.

2. Material And Methods

2.1. Protein and Ligands Preparation

The 3D protein structure of the Keap1 protein (ID: 2flu) was obtained from RCSB Protein Data Bank (PDB). Keap1 prepared by removing any solvents and other ligands using Discovery Studio v.19 versions (http://3dsbiovia.com/products). The 3D structure of hyperoside (CID: 5281643), and naringenin (CID: 932) retrieved from PubChem NCBI database. Hyperoside and Naringenin were minimized their binding energy using PyRx 0.8 software and converted the SDF format into pdb format.

2.2. Molecular Docking between protein-ligand and visualization

Ligand (Hyperoside and naringenin) and Keap1 protein were interacted using autodock vina integrated with PyRx 0.8 tools. The docking result was visualized by Discovery Studio v.19 program (http://3dsbiovia.com/products).

3. Results and Discussion

In order to investigate interactions with these ligands and proteins, the molecular docking for both Hyperoside and Naringenin to Keap1 was performed. The results are shown in Figures 1 and 2.
Interaction of ligand and protein demonstrated by the difference of chemical bond and the binding site of the residues of amino acids (Table 1). In addition, based on the 2D structure there were alkyl bonds and van der Waals forces. Hydrophobic bonds, as well as hydrogen bonds and van der Waals force contributed to the formation of binding affinity or energy binding in the ligand-protein complex. The Visualization result of the Hyperoside and Naringenin with Keap1 from the front view looked the same (Figure 1a and 2a). However, the side view showed that Hyperoside bound to Keap1 on the front surface of Keap1, while Naringenin interacted with Keap1 to the inside (Figure 1b and 2b).

**Figure 1.** Interaction between Hyperoside-Keap1. (a-b) overview of the interaction; (c) the 3D structure; (d) the 2D structure of Hyperoside-Keap1

**Figure 2.** Interaction between Naringenin-Keap1. (a-b) overview of the interaction; (c) the 3D structure; (d) the 2D structure of Naringenin-Keap1

**Table 1.** Interaction result of Keap1 and *M. crenata* compounds (Hyperoside dan Naringenin)

| Protein-Ligand | Energy (Kcal/mol) | Name | Chemistry bond | Types                      |
|----------------|-------------------|------|----------------|---------------------------|
| Keap1-Hyperoside | -6.7              | X:GLY371:HN - :LIG1:O | Hydrogen Bond | Conventional Hydrogen Bond |
|                 |                   | X:VAL467:HN - :LIG1:O | Hydrogen Bond | Conventional Hydrogen Bond |
|                 |                   | :LIG1:H - X:VAL467:O | Hydrogen Bond | Conventional Hydrogen Bond |
|                 |                   | :LIG1:H - :LIG1:O   | Hydrogen Bond | Conventional Hydrogen Bond |
|                 |                   | X:GLY423:CA - :LIG1:O | Hydrogen Bond | Carbon Hydrogen Bond       |
|                 |                   | :LIG1:H - X:ASP422:OD1 | Hydrogen Bond | Carbon Hydrogen Bond       |
|                 |                   | X:VAL420:HN - :LIG1 | Hydrogen Bond | Pi-Donor Hydrogen Bond     |
|                 |                   | X:VAL370:CA - :LIG1 | Hydrophobic   | Pi-Sigma                  |
|                 |                   | X:VAL420:CB - :LIG1 | Hydrophobic   | Pi-Sigma                  |
| Protein-Ligand | Energy (Kcal/mol) | Name | Chemistry bond | Types          |
|----------------|-------------------|------|----------------|----------------|
| :LIG1 - X:VAL420 | Hydrophobic       | Pi-Alkyl |
| :LIG1 - X:VAL467 | Hydrophobic       | Pi-Alkyl |
| :LIG1 - X:VAL420 | Hydrophobic       | Pi-Alkyl |
| :LIG1:H - X:VAL420:O | Hydrogen Bond  | Conventional Hydrogen Bond |
| :LIG1:H - X:VAL604:O | Hydrogen Bond  | Conventional Hydrogen Bond |
| X:ALA366:CA - :LIG1:O | Hydrogen Bond  | Carbon Hydrogen Bond |
| X:CY5S13:CA - :LIG1:O | Hydrogen Bond  | Carbon Hydrogen Bond |
| :LIG1:H - X:VAL418:O | Hydrogen Bond  | Carbon Hydrogen Bond |
| X:GLY419:CA - :LIG1 | Hydrophobic       | Pi-Sigma |
| :LIG1 - X:ALA366 | Hydrophobic       | Pi-Alkyl |
| :LIG1 - X:VAL418 | Hydrophobic       | Pi-Alkyl |
| :LIG1 - X:ALA466 | Hydrophobic       | Pi-Alkyl |
| :LIG1 - X:VAL467 | Hydrophobic       | Pi-Alkyl |
| X:VAL420:HN - :LIG1:O | Unfavorable     | Unfavorable Bump |
| X:VAL606:HN - :LIG1:H | Unfavorable     | Unfavorable Donor-Donor |

The three dimensions (1c) and two dimensions (1d) interaction map showed that there were 15 amino acid residues of Keap1 interacted with hyperoside, for instance Asp422, Arg470, Ile421, Gly423, Gly371, Gly372, Val370, Val369, Val420, Cys368, Val418, Val467, Ala466, Val514 and Gly419. Those interactions were facilitated by hydrogen bonds, either carbon-hydrogen or conventional hydrogen bonds, and also van der Walls forces. The binding energy of the Hyperoside-Keap1 complex was –6.7 Kcal/mol.

Interaction between Naringenin and Keap1 showed that Naringenin binds to 19 amino acid residues of Keap1 (Fig 2c and 2d), including Val420, Val418, Val604, Ala366, Cys513, Val465, Val606, Gly419, Ala436, Val467, Val514, Val512, ILE416, Cys638, Gly417, Gly605, Gly367, Leu365, Ile559 (Figure 2d). These interactions were made possible through the formation of hydrogen bonding, either carbon-hydrogen or the usual hydrogen bonding, as well as van der Walls forces. After Naringenin-Keap1 formed a complex, the binding energy was –7.2 Kcal/mol.

Figure 3. Interaction site of Keap1-Nrf2 [17]

Naturally, Nrf2 binds to the amino acid residue of Keap1 at 320 to 610 (Figure 3). Based on the docking results, Hyperoside and naringenin bound to the active site of the Keap1, the same as the Nrf2 position bound to the Keap1. The bond occurred between Naringenin and Hyperoside with Keap1 caused changes in the Keap1 conformation and resulted in inhibiting interactions between Keap1 and

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The 11th International Conference on Global Resource Conservation  
IOP Conf. Series: Earth and Environmental Science 743 (2021) 012056  
doi:10.1088/1755-1315/743/1/012056
Nrf2 [15]. Thus, it might be predicted that Naringenin and Hyperoside could be nrf2 activators. Activated Nrf2 would move to the cell nucleus to regulate endogenous antioxidants [17].

Nrf2 was transcription factor, among others, for the expression of antioxidant SOD, CAT [18], Gluthahotion S transferase (GST) [19] and GSH [20]. In vitro studies of MIN6 cells revealed that the administration of Naringenin could increase Nrf2 activation [21]. The same study was reported by Lou et al. [22] that Naringenin could increase the level of Nrf2 in SH-SY5Y cells. Hyperoside treatment in SH-SY5Y cell culture might also increase Nrf2 activation, which was followed by increased expression of antioxidant heme-oxygenase (HO-1) [23]. Meanwhile Xing et al. [24] found that Hyperoside could increase the activity of glutation peroxidase (GSH-Px), CAT, SOD and HO-1. Antioxidants had a great influence on ovarian activity, including oocyte maturation, ovulation, Corpus Luteum function and steroidogenesis. Some of the antioxidants that moved in the regulation included CAT, SOD, GSH and GSH-Px. Catalase in the ovary, first discovered in 1975 through immunohistochemical analysis [9].

During oogenesis, various antioxidant enzymes, together with antioxidant agents such as vitamin C, β-carotene and glutathione, protected oocytes from oxidative damage in the follicular fluid [25]. Glutathione had also been shown to protect oocytes against oxidative damage during the vitro culture of oocytes [26,27]. CAT was expressed during oocyte maturation and played an important role in the development of follicles in pigs, goats and mice [9]. Inhibition of CAT activity causes an increase in ROS accumulation and would cause chromosomal damage during oocyte maturation. CAT was also needed to maintain genome integrity during oocyte maturation in rats [28]. Besides CAT also played an important role in the estrous cycle and steroidogenesis [9].

SOD was an enzyme found in many tissues, including the ovaries. In ovarian SOD expressed on primordial follicles, primers and secondary follicles [9]. Besides being found in granulosa cells, SOD was also found in follicular fluid. SOD activity was also related to progesterone secretion, and protects Corpus Luteum from damage caused by inflammation [29]. SOD was also involved in the synthesis of estradiol, which correlated with oocyte quality [9].

Based on the description above it could be concluded that the activation of Nrf2 was needed to guarantee the normal physiology of the ovaries. Nrf2 activation might be done by releasing the bonds between Keap1 and Nrf2. Naringenin and Hyperoside were flavonoids members that can functioned as Nrf2 activators.

4. Conclusion
Naringenin and hyperoside can dissociate the Keap 1- Nrf2 interaction, and activate Nrf2.

Acknowledgments
This research was funded by Faculty of Natural Sciences through PNBP Brawijaya University, 2020

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