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Identification of Chemical Compounds in Black Garlic Extract and Effect on Inhibiting Xanthine Oxidase Enzyme

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Abstract: Xanthine oxidase is an enzyme involved in the catalysis of the oxidation reaction that converts hypoxanthine into xanthine and subsequently into uric acid. Elevated uric acid levels can pose various health risks. Gout treatment can be achieved by inhibiting the activity of the xanthine oxidase enzyme using black garlic. This study aims to identify the chemical compounds in black garlic methanol extract, assess the inhibitory effect of this extract on the xanthine oxidase enzyme using in silico methods, and determine the type of inhibition based on enzyme kinetics. The in silico analysis was conducted to evaluate the binding affinity of flavonoid compounds in black garlic extract with xanthine oxidase. The in vitro analysis tested the inhibition of the xanthine oxidase enzyme by black garlic using the UV-Vis spectrophotometry method at a wavelength of 291.7 nm, based on a decrease in uric acid concentration as an indicator of reduced enzyme activity. The type of inhibition mechanism was determined through enzyme kinetics using the Michaelis-Menten equation, which was transformed into the Lineweaver-Burk equation in a double reciprocal form. Black garlic methanol extract contains 133 chemical compounds, including 22 flavonoid compounds that are thought to inhibit xanthine oxidase. According to in silico studies, quercetin-3-O-malonylglucoside exhibits the lowest binding affinity (-9.2 kcal/mol) with the xanthine oxidase enzyme compared to the xanthine substrate (-5.2 kcal/mol) and allopurinol (-5.3 kcal/mol). Inhibition of the xanthine oxidase enzyme by black garlic demonstrated the highest inhibition of 76.352% at a concentration of 10 ppm of black garlic extract. The inhibition type of the xanthine oxidase enzyme by black garlic methanol extract showed a competitive inhibition mechanism, evidenced by an increase in the K_M value from 0.014 to 0.134 without a significant change in the V_{max} value. Thus, it can be concluded that black garlic extract has the potential to be a natural inhibitor of the enzyme xanthine oxidase that can be used to treat gout or hyperuricemia.

Keywords: Black Garlic, In Silico; Inhibitor; Kinetics; Xanthine Oxidase Enzyme.

Introduction

The xanthine oxidase enzyme is an oxidoreductase enzyme that catalyzes the oxidation reaction converting hypoxanthine into xanthine and subsequently into uric acid. During the process of uric acid formation, the enzyme xanthine oxidase transfers the oxygen contained in its molybdenum to the xanthine substrate, forming H_2O_2 [1]. Gout is the end product of the process of purine metabolism, excreted by the body [2]. The normal limit of uric acid is a maximum of 6.0-7.0 mg/dL [3].

In normal amounts, uric acid has benefits for the body as it can be a natural antioxidant that can prevent cancer and premature ageing, but uric acid levels that exceed normal limits can cause hyperuricemia, gout arthritis, gout nephropathy, hypertension, arteriosclerosis, and even coronary heart disease [3]. The most commonly used gout treatment is to use allopurinol. Allopurinol is a drug to reduce uric acid that has an action mechanism to inhibit the enzyme xanthine oxidase by binding covalently to molybdenum on the active side of the enzyme because it has a similar structure to the xanthine substrate, so that it will compete competitively [4]. Allopurinol has various side effects such as hepatitis, kidney failure, neurological disorders, skin rashes, and allopurinol hypersensitivity [5].

Based on the high side effects produced by the pharmacological drugs for gout, another alternative to an organic ingredient with less risk of side effects is needed. Black garlic is a garlic that has undergone a heating process at high temperatures (60℃-90℃) and certain humidity (70%-90%) until it undergoes a change in color to brown or black [6]. When the process of heating garlic into black garlic, there will be an increase in the total number of polyphenols by 7-11 times, and an increase in the total flavonoid and phenolic content by 1-5 times and 4-8 times. Black garlic contains active compounds such as organosulfur, flavonoids, polyphenols, phenolics, and alkaloids [7].

Flavonoids can inhibit xanthine oxidase by the mechanism of action, occupying the active site of the xanthine oxidase and binding amino acid residues through hydrogen bonding and hydrophobic interactions. In addition, the hydroxyl group in flavonoids easily captures electrons from the active side of xanthine oxidase, so that it can inhibit the enzyme xanthine oxidase [8]. Black garlics containing flavonoids are thought to competitively inhibit the enzyme xanthine oxidase or mixtures that depend on the structure of the flavonoids [9]. The determination of this type of inhibition can be determined based on enzyme kinetics, namely through the Michaelis-Menten equation, which is transformed into the Lineweaver-Burk equation in a double

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inverse manner. The determination of the kinetics of the enzyme involves the values of V_{max} and K_M

In competitive inhibition, there was no change in the V_{max} value, but the K_M value increased because the affinity of the enzyme to the substrate decreased. In non-competitive inhibition, the V_{max} value decreases, but the K_M value remains unchanged, as this inhibition results in a slower enzymatic reaction [10]. This study examined the effects of black garlic extract as an inhibitor of the xanthine oxidase enzyme by combining in vitro and in silico approaches. In addition, chemical compound identification will be carried out using LC-MS to determine the presence of flavonoid compounds that are suspected to act as inhibitors of the xanthine oxidase enzyme and kinetic analysis of the xanthine oxidase enzyme to determine the type of inhibition of black garlic against the enzyme xanthine oxidase.

Research Methods

Tools and Materials

The tools used are a UV-Vis Spectrophotometer (Shimadzu UV-1800), an LC-MS Spectrophotometer (Shimadzu LC-MS-8040 LC/MS), a vacuum pump, a rotary evaporator, filter paper, laboratory glass tools, an incubator, and a vortex mixer. The ingredients used are black garlic (Malang City), goat milk, xanthin substrate (Merck), methanol (Merck), allopurinol (Hexapharm), Tris-HCl buffer (Kimia Jawa Labora), HCl (Chemindo), aquades, NaOH (Chemindo)

Black Garlic Extraction

100 grams of mashed black garlic are extracted by the maceration method using 1000 mL of methanol for 24 hours. Then filtered using filter paper with a vacuum pump, and then dried using a rotary evaporator. After that, the black garlic methanol extract is stored at a cold temperature until it is used [11], [12].

Identification of Flavonoid Compounds

Concentrated extract of black garlic methanol was identified for its flavonoid compound content using the LC-MS instrument [13].

Molecular Docking and Visualization

Preparation of Receptors and Ligands

The xanthine oxidase enzyme receptor 3NRZ was downloaded in PDB (Protein Data Bank) format and then prepared using the Discovery Studio application. The flavonoid compounds analyzed by LC-MS and allopurinol ligands were downloaded in SDF (Structure Data Format) format from PubChem and then minimized in energy using the PyRx application [14], [15].

Docking Method Validation

The validation method involved redocking native ligands to the enzyme xanthine oxidase as a receptor and then examining the RMSD value. RMSD (Root Mean Square Deviation) is a parameter of the magnitude of the orientation change or deviation of the position of the native ligand after redocking. An RMSD value of ≤ 2Å indicates that the method used is valid [14], [15].

Docking Ligand Test against Receptors

Docking is done using Autodock Vina on PyRx. Before docking, the positions of the x, y, and z coordinate grids are preset to determine the receptor region to be docked with the test ligand and to identify the need for the lowest binding affinity. Then, the interaction of the ligand with the enzyme was analyzed using Discovery Studio [14], [15].

Xanthine Oxidase Enzyme Inhibition Activity Test by Black Garlic Extract

Standard solutions of uric acid with concentrations of 1, 3, 5, 7, and 9 ppm are added with an optimal pH tris-HCl buffer. Then its absorbance was measured at a wavelength of 291.7 nm on the UV-Vis spectrophotometer. After that, a standard curve is made, and a regression equation is obtained [16], [17].

Sample testing of black garlic methanol extract (with concentrations of 2, 4, 6, 8, and 10 ppm) and allopurinol 2 ppm was carried out through 1 mL of test solution, adding 2.9 mL of 0.02 M Tris-HCl buffer solution, pH 7.5 and 2 mL of xanthine substrate with a concentration of 0.15mM. Then incubated at a temperature of 33℃ for 10 minutes. Then, 0.1 mL of xanthine oxidase enzyme is added and homogenized with a vortex mixer. Next, the solution is incubated for 25 minutes at a temperature of 33℃. Then, 1 mL of HCl 0.1N was added to the solution, and then its absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 291.7 nm. As for the sample control test, it is done in the same way without adding an enzyme solution [8], [18], [19].

Determination of Xanthine Oxidase Enzyme Kinetics with the Addition of Black Garlic Methanol Extract

1 mL of test solution was added, 2.9 mL of Tris-HCl buffer solution 0.02 M, pH 7.5, and 2 mL of xanthin substrate with a concentration of 0.05, 0.10, 0.15, 0.20, and 0.25 mM. Then incubated at a temperature of 33℃ for 10 minutes. And then 0.1 mL of xanthine oxidase enzyme is added and homogenized with a vortex mixer. Next, the solution is incubated for 30 minutes at the optimal temperature. Then 1 mL of HCl 0.1N was added to the solution to stop the enzymatic reaction, and then the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 291.7 nm.

The determination of enzyme kinetics is carried out by graphing the relationship between substrate concentration [S] and enzyme activity (V) then the data is converted and plotted on a graph between 1/[S] and 1/V then, the V_{max} and K_M values are determined based on the Lineweaver-Burk curve equation through [20]:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

If 1/V is y, and 1/[S] is x, then y = a + bx. So that:

$a = 1/V_{max}$
$b = K_M/V_{max}$

Results and Discussion

The black garlic extraction process is carried out using the maceration method with methanol solvent for 24 hours. The maceration method was chosen in this study because the method has a simple working process and includes cold extraction that does not require heating, so that it can minimize the occurrence of chemical compound damage [21]. Based on the results of the extraction, a yield of 38.38% was obtained and black garlic extract with a black, brownish, and thick color. Black garlic methanol extract was analyzed for its chemical compound content using an LC-MS (Liquid Chromatography-Mass Spectrometry) instrument, and 133 active compounds were obtained, which could be identified as shown in Figure 1.

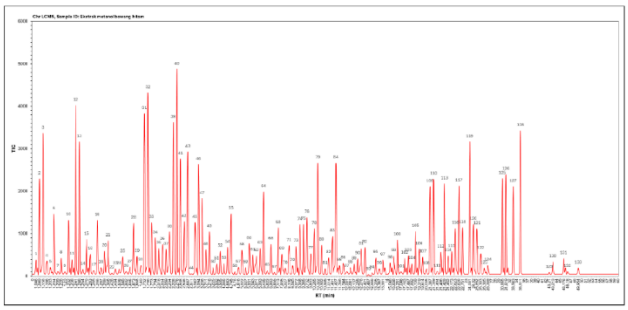

Figure 1. LC-MS Chromatogram Results of Black Garlic Methanol Extract

The results of the chemical compound identification analysis using LC-MS showed that there were 3 dominant compounds contained in black onion methanol extract, namely amino acids (24 compounds), organosulfur (23 compounds), and flavonoids (22 compounds). Based on the results of the analysis 22 flavonoid compounds with 5 flavonoid compounds can be identified that are suspected to inhibit the enzyme xanthine oxidase, namely quercetin, kaempferol, luteolin, catechin, and apigenin. Commercially obtained quercetin can exhibit mixed type [22] and competitive type inhibition [23]. Commercially obtained kaempferol provides competitive inhibition to the commercial xanthine oxidase enzyme from cow's milk by competing with the xanthine substrate during the catalysis process to occupy the active site of the enzyme [24]. Several other studies regarding competitive inhibition of commercial xanthine oxidase enzymes from cow's milk by flavonoid compounds have also been conducted, such as inhibition of xanthine oxidase by commercial luteolin compounds [25], commercial catechin [9], and commercial apigenin [26].

Molecular Docking and Visualization

The in silico method predicts the interaction between two molecules, specifically examining how they bind and the type of interaction occurring between ligands and receptors in stable binding regions [27]. This process comprises three stages: the preparation stage of receptors and ligands, the validation stage of the docking method, and the docking stage of test ligands on receptors. In the preparation stage, water molecules and ligands associated with the xanthine oxidase enzyme are removed to avoid interference with the docking between the receptors and target ligands. The method validation stage is carried out by redocking native ligands. The results of this validation show that the RMSD value obtained is 0.085 Å, indicating that the docking method used is valid and applicable. An RMSD value of ≤ 2Å indicates the method is valid, while a smaller RMSD value reflects a closer similarity between the native ligand's position as a result of the redocking process and its original position on the receptor before the docking process [14], [15] . The docking stage results in an energy affinity value, which is presented in Table 1 to illustrate the interaction bonds.

Table 1. Binding affinity of the test ligand to the xanthine oxidase enzyme receptor

Compound	Binding Affinity (kcal/mol)
Hypoxanthine (native ligand)	-5.2
Xanthine (Substrate)	-5.2
Allopurinol (Positive control)	-5.3
Quercetin	-7.6
Kaempferol	-6.7
5-deoxykaempferol	-7.5
Quercetin-3-O-malonylglucoside	-9.2
Quercetin-3-O-Glucoside	-8.6
Quercetin-3-Glucoside-7-Rhamnoside	-8.6
Naringenin	-7.5
Luteolin	-7.7
Luteolin-7-Glucoside	-8.5
Kaempferol-3-Rhamnoside	-7.3
Kaempferol-3-(6"-Malonylglucoside)	-8.9
Kaempferol-3-O-D-Glucoside	-8.4
Catechin	-7.2
Apigenin	-7.7
Vitexin	-9.1
Apigetrin	-8.1
Astragalin	-8.4
Hyperoside	-8.2
Reynoutrin	-7.3
Rhamnetin	-7.6
Naringin	-7.0
Isorhamnetin-3-O-β-D-galactopyranoside	-6.7

Based on the analysis results, the flavonoid compounds in black garlic methanol extract have the potential to be inhibitors of the enzyme xanthine oxidase because they have a smaller binding affinity than hypoxanthine, xanthine, and allopurinol. Based on Table 1, the flavonoid compounds in black garlic methanol extract have a binding affinity of -6.7 to -9.2 kcal/mol, with the smallest affinity possessed by the compound quercetin-3-O-malonylglucoside (-9.2 kcal/mol). Hypoxanthine and xanthine, which are substrates of the xanthine oxidase enzyme, have a binding affinity of -5.2 kcal/mol, while allopurinol (positive control) has a binding affinity of -5.3

kcal/mol. The smaller the binding affinity value, the more stable, strong, and potentially influential the receptor [28].

Based on molecular visualization, the xanthin, allopurinol substrates, and flavonoid compounds in black garlic methanol extract have different ligand interactions, either hydrophobic, electrostatic, or unfavorable. These bonds affect the size and size of binding affinity between the ligand and the receptor [29]. Xanthine substrates interact with amino acid residues on the xanthine oxidase enzyme through hydrogen (ASN 1287) and hydrophobic bonds (ALA1283, ARG1282, ARG1279), while allopurinol interacts through hydrogen bonds (GLU939, TYR977, LEU998), hydrophobic bonds (ALA938, CYS999), and electrostatic (ASP1276). The compound quercetin-3-O-malonylglucoside, which is a flavonoid compound in black garlic extract with the smallest binding affinity, binds to amino acid residues through hydrogen, hydrophobic, and unfavorable bonding as shown in Figure 2, namely ALA938, ASN988, ARG1279, TYR977, ASP1276, PRO937, ASP985 (hydrogen bond), VAL932, ALA931, ALA 938 (hydrophobic), ARG1279 (unfavorable).

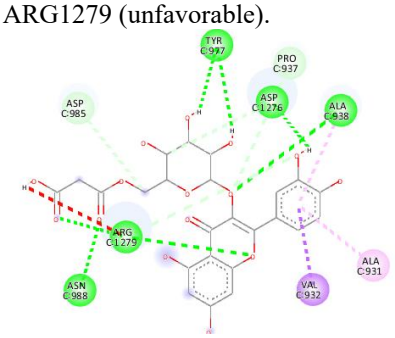

Figure 2. Binding Interactions between Xanthine Oxidase Enzyme and Quercetin-3-O-malonylglucoside Compound

The results of this in silico study align with other research indicating that flavonoid compounds can inhibit the activity of the enzyme xanthine oxidase. Many in silico studies have been conducted, such as studies on apigenin compounds in celery, which showed a binding affinity value of f -10.28 kcal/mol and have the potential to exert an inhibitory effect on xanthine oxidase [14]. Additionally, epicatechin and luteolin in garlic and black garlic may also inhibit xanthine oxidase due to their binding affinity of -9.30 [30].

Xanthine Oxidase Enzyme Inhibition Activity Test by Black Garlic Extract

Xanthine oxidase is an enzyme involved in the catalysis of the oxidation reaction that converts hypoxanthine into xanthine and subsequently into uric acid. In normal amounts, uric acid can act as a natural antioxidant, but elevated uric acid levels can pose various health risks. Gout treatment can be achieved by inhibiting the activity of the xanthine oxidase enzyme using black garlic. Xanthine oxidase enzyme inhibition testing was carried out using the UV-Vis spectrophotometry method at a maximum wavelength of 291.7 nm because uric acid, which is the final product of xanthine oxidation, has a strong chromophore group so that it can absorb ultraviolet light as well as visible light [19]. For the determination of uric acid levels formed in the sample, a standard uric acid curve was used, and a regression equation was obtained, namely $y = 0.0734x+0.0016$ with $R^2 = 0.9998$

The black garlic extract obtained has been tested for its inhibition of the enzyme xanthine oxidase at various concentrations ranging from 2 to 10 ppm. The results indicate that higher concentrations of black garlic lead to increased inhibition percentages, as illustrated in Figure 3. This suggests that the concentration of uric acid formed decreases, with the highest inhibition of 76.352% achieved at a concentration of black garlic extract of 10 ppm. In this study, allopurinol, a commonly used drug for reducing uric acid by inhibiting the xanthine oxidase enzyme, was employed as a positive control at a concentration of 2 ppm, yielding an inhibition of 88.331%. Based on these results, the black garlic methanol extract can inhibit the enzyme xanthine oxidase, but the inhibition value is still lower than that of allopurinol. However, this study has limitations, such as the lack of in vivo tests and bioavailability, because it analyzes the effect of black garlic on inhibiting the xanthine oxidase enzyme through in vitro and in silico approaches.

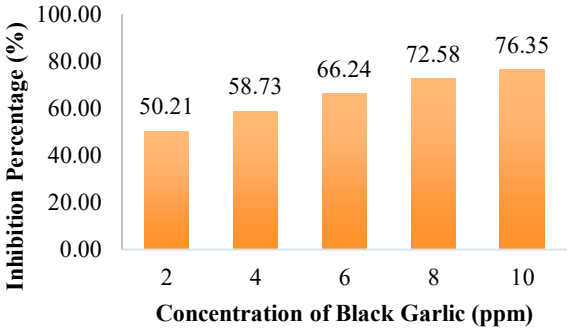

Figure 3. The inhibition percentage of xanthine oxidase enzyme by methanol extract of black garlic

Research on the inhibition of xanthine oxidase enzyme using black garlic is supported by the research [31], which showed the highest inhibition of 68.01% in commercial xanthine oxidase enzymes with black garlic at a concentration of 10 ppm. Other research that supports this research is [32], which showed that onions with a concentration of 100µg/mL provide up to more than 90% inhibition of the enzyme xanthine oxidase [32]. Other research on natural xanthine oxidase inhibitors, such as papaya seed extract and herbal extract. Papaya seed ethanol extract with a concentration of 300 ppm was able to inhibit the enzyme xanthine oxidase up to 86.105% [18], while Peperomia pellucida L. extracts extracted using ethanol or water provided an inhibition percentage of 51.77% at an extract concentration of 50 µg/mL [33].

Determination of Xanthine Oxidase Enzyme Kinetics with the Addition of Black Garlic Methanol Extract

In the kinetics test of the xanthine oxidase enzyme with the addition of black garlic methanol extract, the effect of substrate concentration on the activity of the xanthine oxidase enzyme was analysed. Based on the results of the study, data on the activity of the enzyme xanthine oxidase without inhibitors or inhibitors and data on enzyme activity

with the addition of black garlic extract, as in Table 2, were obtained.

Table 2 Xanthine Oxidase Enzyme Activity Before and After Inhibitor Addition

Substrate Concentration (mM)	Enzyme Activity without Inhibitors (mU/mL)	Enzyme Activity with Black Garlic Extract (mU/mL)
0.05	0.168	0.059
0.10	0.181	0.086
0.15	0.199	0.115
0.20	0.201	0.135
0.25	0.202	0.136

The determination of the type of inhibition of the enzyme xanthine oxidase in this study was analyzed based on enzyme kinetics using the Michaelis-Menten equation, which involves two parameters, namely the V_{max} and K_M values. K_M and V_{max} values can be determined based on variations in substrate concentration to enzyme activity. The concentration of the substrate is proportional to the activity of the enzyme, where the higher the concentration of the substrate, the faster the reaction speed will increase [34]. The determination of the K_M value can be predicted through the Michaelis-Menten equation, but the V_{max} value cannot be known to be the true value. Therefore, to determine the values of K_M and V_{max}, they must be converted into the Lineweaver-Burk equation by using double inverse mapping by channelling between 1/V and 1/[S.

Based on Table 2, the effect of variations in substrate concentration on the activity of the xanthine oxidase enzyme with the addition of black garlic extract as an inhibitor shows that the higher the substrate concentration, the more the activity of the xanthine oxidase enzyme. However, at a substrate concentration of 0.20 to 0.25 mM, the activity of the xanthine oxidase enzyme did not undergo significant changes as the enzyme had reached its maximum speed. The inhibition of black garlic extract can be determined by using the Lineweaver-Burk graph based on K_M and V_{max} values (Figure 4). Based on the results of the Lineweaver-Burk graph, there is a difference in the K_M value, from 0.014 to 0.134, but the V_{max} value does not change significantly, namely, 0.214 to 0.213.

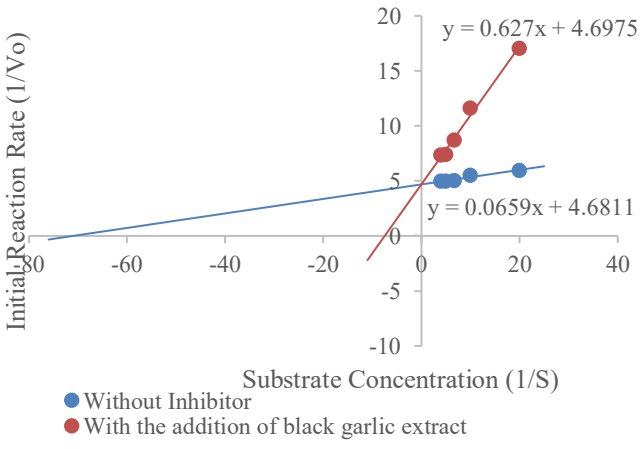

Figure 4. Lineweaver-Burk Graphic of Xanthine Oxidase Enzyme Inhibition by Black Garlic Extract

The analysis results indicate that the inhibition type of black garlic extract on the enzyme xanthine oxidase is competitive. Competitive inhibition occurs when a substrate or inhibitor binds to a specific active site of the enzyme, leading to competition between them for the same active site [35]. In this case, the enzyme's Vmax remains unchanged, while the KM value increases, indicating a decrease in the enzyme's affinity for the substrate due to the presence of competitive inhibitors [10]. The competitive inhibition exhibited by black garlic extract on xanthine oxidase aligns with the findings of [24], which utilized commercially available kaempferol compounds. Additionally, the isolated compound 6,4'-dihydroxy-4-methoxybenzophenone-2-O-β-D glucokopiranoside from the crown of gods, which also demonstrated competitive inhibition with a KM value of 0.033 and Vmax of 0.140 [19].

Conclusion

Black garlic methanol extract contains 133 chemical compounds, including 22 flavonoid compounds that are thought to inhibit xanthine oxidase. According to in silico studies, quercetin-3-O-malonylglucoside exhibits the lowest binding affinity (-9.2 kcal/mol) with the xanthine oxidase enzyme compared to the xanthine substrate (-5.2 kcal/mol) and allopurinol (-5.3 kcal/mol). Inhibition of the xanthine oxidase enzyme by black garlic demonstrated the highest inhibition of 76.352% at a concentration of 10 ppm of black garlic extract. The inhibition type of the xanthine oxidase enzyme by black garlic methanol extract showed a competitive inhibition mechanism, evidenced by an increase in the K_M value from 0.014 to 0.134 without a significant change in the V_{max} value. Therefore, it can be concluded that black garlic extract has the potential to inhibit the xanthine oxidase enzyme, which could be utilized as an herbal supplement or in clinical trials to treat gout or hyperuricemia.

Author's Contribution

Mahrunisa Nur Afifah: Collect data and compile the article; Nuniek Herdyastuti: Responsible person and article compiler.

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