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Identification of Bioactive Compounds in Garlic (*Allium sativum* L.) with Hypocholesterolemic Potential

Aprilya Miftachul Qoyimmah, Nuniek Herdyastuti*

Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Surabaya, Indonesia
*e-mail: nuniekherdyastuti@unesa.ac.id

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Abstract: Hypercholesterolemia is a major risk factor for cardiovascular disease, the leading cause of death worldwide. Although statins remain the standard treatment, their long-term use is associated with side effects, sparking interest in safer natural alternatives. Garlic (*Allium sativum* L.) contains bioactive compounds such as organosulfur compounds (e.g., allicin) and flavonoids (e.g., quercetin and kaempferol), which have been shown to have cholesterol-lowering potential. This study aims to identify bioactive compounds in fresh garlic ethanol extract and powder using liquid chromatography-mass spectrometry (LC-MS), and to evaluate their in vitro anticholesterol activity using the Liebermann-Burchard method. The extracts were prepared using 70% ethanol and tested for their ability to lower cholesterol levels. LC-MS analysis revealed 96 compounds in the fresh garlic extract and 110 compounds in the garlic powder extract. Bioactive compounds such as organosulfur compounds and flavonoids were identified among these compounds. Based on their composition percentages, allicin was more abundant in the garlic powder extract, as were flavonoids such as quercetin and kaempferol, particularly in glycosides. In vitro tests showed cholesterol-lowering effects, with EC_{50} values of 41,349.08 ppm for the fresh extract and 35,462.29 ppm for the powder extract. Garlic powder extract exhibits higher activity. These findings suggest that garlic, particularly powder form, has potential as a natural cholesterol-lowering agent. Further in vivo studies are needed to confirm its efficacy, mechanism of action, and long-term safety.

Keywords: *Allium sativum*; Allicin; Flavonoids; Cholesterol; LC-MS; Liebermann-Burchard.

Introduction

Hypercholesterolemia is a condition characterized by elevated total cholesterol levels in the blood above the normal range (>200 mg/dL), which can increase the risk of cardiovascular disease by up to three times compared to healthy individuals [1][2]. The causes include excessive consumption of saturated fats, lack of physical activity, smoking, alcohol consumption, obesity, certain diseases, and ageing [3]. According to the WHO, cardiovascular disease is the leading cause of death globally, accounting for 32% of deaths in 2019. Hypercholesterolemia can be managed by adopting a healthy lifestyle, including reducing calorie intake from saturated fats, exercising, avoiding or quitting smoking, and taking [5].

The treatment approach for hypercholesterolemia typically involves statin therapy combined with healthy lifestyle changes, such as dietary adjustments and exercise. Statins have been used for over three decades and have proven effective in lowering LDL levels by 20–50% [6], with simvastatin being one of the most commonly used types [7]. However, statin use can also cause side effects such as muscle pain, liver dysfunction, and an increased risk of type 2 diabetes [8]. Therefore, a safer yet effective natural alternative is needed. The use of herbal ingredients is increasingly popular among the Indonesian population, as data from Riskesdas shows that over 59% of adults use traditional medicine as part of their healthcare regimen [9].

One potential natural ingredient is garlic (*Allium sativum* L.), which is known for its ability to lower cholesterol levels from moderate to normal [10]. Its main component, allicin, is an organosulfur compound that plays a role in lowering cholesterol through the inhibition of the HMG-CoA reductase enzyme [11]. In addition to allicin, garlic contains flavonoids supporting its hypolipidemic effects [12].

The bioactive compounds in garlic can be obtained through extraction using solvents such as ethanol, which is safe, affordable, and volatile. A study by Bajac et al showed that the ethanol extract of garlic contains allicin at 4.39–4.56 µg/mL, along with other sulfur compounds in smaller amounts[13]. This extract also contains phenols, flavonoids, flavonols, and proanthocyanidins in significant concentrations [14]. Several in vivo studies have also demonstrated the effectiveness of in significantly lowering blood cholesterol levels, even comparable to simvastatin [15][16][17]. These findings indicate that garlic extract has potential as a natural therapeutic alternative in the management of hypercholesterolemia.

This study combines two complementary approaches, liquid chromatography mass spectrometry (LC-MS) for identifying bioactive compounds and the Liebermann-Burchard in vitro assay for evaluating cholesterol-lowering activity, to provide a more comprehensive understanding of garlic's therapeutic potential. This dual-method approach not only confirms the presence of key compounds like allicin, quercetin, and kaempferol [13][14], but also assesses their functional activity, positioning garlic extract, especially in powder form, as a promising natural alternative for hypercholesterolemia management.

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Research Methods

Extraction of Fresh Garlic and Garlic Powder

Fresh garlic weighing 300 grams was selected, crushed, and soaked in 400 ml of 70% ethanol for 3 × 24 hours at a cold temperature. The filtrate was then evaporated using a vacuum rotary evaporator at a temperature below 50°C [18]. Meanwhile, 800 grams of garlic powder were soaked in 2 liters of 70% ethanol under the same conditions (3 × 24 hours at a cold temperature), then evaporated using the same equipment [17]. The extracts obtained from both materials were then weighed to calculate the yield.

Identification of Chemical Compounds in Garlic

The extraction results from fresh garlic and garlic powder were analyzed using LC-MS. The ethanol extract sample solution was injected into the LC-MS spectrophotometer to determine the bioactive compounds contained in the fresh garlic and garlic powder extracts [19].

Cholesterol Reduction Test

For the anticholesterol test, a 1000 ppm cholesterol stock solution was prepared from 25 mg of cholesterol dissolved in 25 mL of chloroform. The maximum wavelength of cholesterol was determined by reacting a 100 ppm cholesterol solution with Liebermann-Burchard reagent (anhydrous acetic acid and concentrated sulfuric acid, 10:1 then analyzing it using UV-Vis spectrophotometry in the range of 400–700 nm. The operational time was determined by measuring the absorbance every 2 minutes to find the most stable time. A cholesterol standard curve was created from a series of solutions (60, 70, 80, 90, and 100 ppm) that were reacted with the reagent, and then the absorbance was measured to determine the relationship between concentration and absorbance. The anticholesterol activity test was performed by adding garlic extract (30, 60, 90, 120, and 150 ppm) to the reacted cholesterol solution, then measuring the absorbance to calculate the cholesterol reduction using the following calculation formula [20][21].

$$A = \frac{(C - B)}{C} x\ 100\%$$

Explanation:
A = % decrease in cholesterol
B = Absorbance of sample
C = Absorbance of negative control

The controls used included a blank, negative control, and positive control (simvastatin). Absorbance data were used to calculate the percentage decrease in cholesterol and the EC_{50} value based on linear regression between concentration and percentage decrease in cholesterol.

Results and Discussion

Extraction of fresh garlic and garlic powder

Extraction is a method for separating a substance based on the difference in the solubility of the components in a mixture [22]. In this study, the extraction method used was cold extraction, specifically maceration, to avoid damaging compounds that are sensitive to high temperatures [23]. Both fresh garlic and garlic powder were extracted using the maceration method with 70% ethanol as the solvent. The selection of 70% ethanol as the solvent was due to the fact that the use of ethanol combined with water results in differences in the polarity concentration of the extraction solvent. Referring to the theory of similarity and mutual solubility, the more similar the polarity of the solvent to the solute, the faster the solute dissolves from the plant cells [24]. The maceration duration of 72 hours was conducted to optimize the secondary metabolites in garlic dissolved maximally in 70% ethanol solvent [17]. The yield results of the maceration method can be seen in Table 1.

Table 1. Yield of Fresh Garlic and Garlic Powder

Sample	Sample Weight (g)	Extract Weight (g)	Yield (%)
Fresh Garlic	300	67	22.3
Garlic Powder	400	63	15.7

Identification of Chemical Compounds in Fresh Garlic and Garlic Powder

After the extraction process, the extract was identified for chemical compounds using LC-MS instruments. Bioactive compounds are compounds found in animals and plants that can be used as medicinal ingredients with physiological effects on other organisms [25]. In the fresh garlic extract, 96 bioactive compounds were identified, while in the garlic powder extract, 110 bioactive compounds were identified. These compounds were grouped into several categories, including organosulfur compounds, flavonoids, phenolics, amino acids, sterols, saponins, triterpenoids, and others, as presented in Table 2.

Table 2. LC-MS Test Results on Garlic Extract

Compound Type	Number of compounds in garlic powder extract	Number of compounds in fresh garlic extract
Organosulfur	20	20
Flavonoid	19	18
Amino acid	19	19
Phenolic	9	9
Sterol	3	3
Saponin	2	2
Triterpenoid	3	3
Terpenoid	1	1
organoselenium	2	2
carbohydrate	2	-
aldehid	2	-

The compounds obtained include organosulfur and flavonoid groups. Allicin belongs to the organosulfur group, while quercetin and kaempferol are part of the flavonoid group, which are believed to have cholesterol-lowering potential [26][27]. As shown in Table 3, the allicin content in garlic powder extract is higher than that in fresh garlic extract. This is consistent with the statement by Agriawati & Nurmalia, who noted that garlic powder has a more stable

chemical composition than fresh garlic due to its lower water content, which slows down the degradation of bioactive compounds [28]. Storage conditions are one of the factors that can influence allicin levels in garlic. Improper storage can cause both physical and chemical damage [29]. During storage, the activity of allinase enzymes and volatile compounds in garlic can undergo changes that affect allicin levels [30]. In fresh garlic, the levels of flavonoids such as quercetin and kaempferol are higher than in garlic powder. Meanwhile, their glycoside forms, such as quercetin-3-O-malonylglucoside and kaempferol-3-O-glucoside, increase in garlic powder. This is because during processing, the aglycone structure undergoes transformation into glycoside forms [31].

Table 3. Differences in The Levels of Allicin, Quercetin and Kaempferol

Name of compound	Fresh Garlic (%)	Garlic Powder (%)
Allicin	4.23	6.54
Quercetin	2.04	1.48
Quercetin-3-glucoside-7-rhamnoside	2.15	2.37
Quercetin-3-O-D-glucoside	2.57	2.18
Kampferol	2.17	1.94
Kaempferol-3-rhamnoside	1.77	2.11
Kaempferol-3-O-D-glucoside	0.92	1.20

Anticholesterol Activity Test

The anticholesterol activity of fresh garlic extract and powder was tested using the Liebermann-Burchard method. This method was chosen because it is highly sensitive to steroid compounds such as cholesterol and has a relatively simple procedure [32]. The principle of this method is the reaction of cholesterol in chloroform with anhydrous acetic acid and concentrated sulfuric acid in a chloroform solvent. Cholesterol reacts to form a green-colored complex due to the formation of 3-acetyl-5-cholesterol sulfonate [33]. The intensity of the green color is directly proportional to the cholesterol content, allowing it to be quantitatively measured using a UV-Vis spectrophotometer [34]. The Liebermann-Burchard reaction is highly sensitive to water and light. Therefore, anhydrous acetic acid is used to remove moisture, and the test is conducted in a dark place with the tube covered in aluminium foil to prevent degradation caused by light [20][35]. Concentrated sulfuric acid serves as the main reagent in the formation of the green color from cholesterol compounds [36].

In the Liebermann-Burchard method, the initial steps involve determining the maximum wavelength, optimal operating time, and creating a cholesterol standard curve. The maximum wavelength is determined in the range of 400–700 nm, and the results show an absorption peak at 625.4 nm, consistent with previous references [20]. The optimal operating time is set at 8 minutes after reagent addition, when absorbance reaches its highest and stable value, indicating the maximum formation of the 3-acetyl-5-cholesterol sulfonate complex, as shown in Figure 2 [38].

After obtaining the maximum wavelength value and operating time of the cholesterol solution, the research continued with the creation of a standard cholesterol curve. The cholesterol standard curve was prepared using a series of concentrations ranging from 60 to 100 ppm, yielding a linear equation of $y = 0.0046x - 0.1102$ and a correlation coefficient of $R^2 = 0.9934$, indicating good linearity [20].

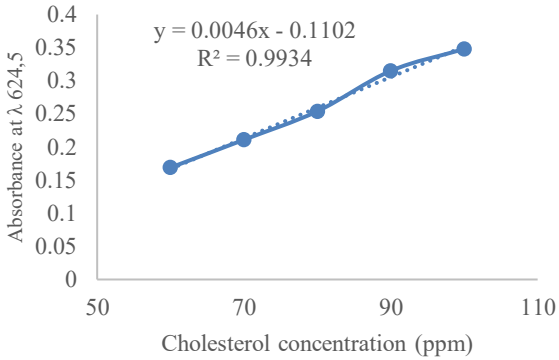

Figure 1. Results of Cholesterol Standard Curve Creation

Anticholesterol activity testing was performed on fresh garlic extract and powder at concentrations of 30, 60, 90, 120, and 150 ppm in chloroform solvent. The reaction was conducted in the dark and covered with aluminium foil to prevent cholesterol degradation due to light [35]. Absorbance measurements were performed at 625.4 nm using a UV-Vis spectrophotometer. The results were calculated as the percentage reduction in cholesterol levels.

In the anticholesterol activity test, simvastatin was used as a positive control because it is a widely used drug in the treatment of hypercholesterolemia. Simvastatin works by inhibiting the HMG-CoA reductase enzyme, which is a key enzyme in cholesterol biosynthesis in the liver, thereby effectively lowering total cholesterol and LDL levels [39]. Based on Figure 2, garlic powder extract showed a higher percentage of cholesterol reduction compared to fresh garlic extract, although it was still lower than simvastatin. These results were reinforced by the Least Significant Difference (LSD) analysis, which showed that simvastatin provided a significantly higher reduction in cholesterol levels compared to both garlic extract groups. Meanwhile, there was no significant difference between fresh and powdered garlic extracts, indicating that both have relatively equivalent anticholesterol potential.

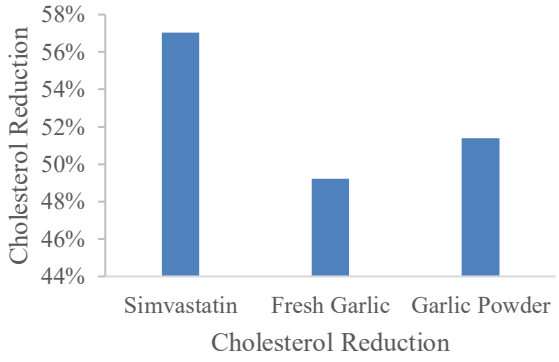

Figure 2. Cholesterol Reduction Test Results

To evaluate the effectiveness of inhibitory compounds in lowering cholesterol levels, the EC_{50} (Effective Concentration 50%) parameter is used. The EC_{50} value indicates the concentration of the compound required to lower cholesterol levels by 50%. The lower the EC_{50} value, the greater the cholesterol-inhibiting potential [40]. Based on the measurement results, garlic powder extract has the lowest EC_{50} value of 35,462.29 ppm compared to fresh garlic at 41,349.08 ppm, indicating higher anti-cholesterol potential. These results are supported by previous studies showing that garlic powder can reduce total cholesterol and LDL levels in patients with hypercholesterolemia [44].

The higher anti-cholesterol potential of garlic powder is likely due to its higher content of organosulfur compounds such as allicin and flavonoids such as quercetin and kaempferol. Allicin is known to inhibit HMG-CoA reductase, the primary enzyme in cholesterol biosynthesis in the liver. The mechanism of action of allicin is thought to involve interaction with the -SH group, which is the functional part of coenzyme A, thereby directly inhibiting the activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase [17]. This type of inhibition is similar to the mechanism of action of simvastatin, which acts as a competitive inhibitor of the enzyme, effectively reducing total cholesterol and LDL levels in the liver [39].

Additionally, flavonoids also play a role in lowering cholesterol levels through various mechanisms, one of which is by inhibiting the digestion and absorption of fats in the digestive tract [41]. Flavonoids can bind to lipid micelles in the small intestine after consuming fatty foods, thereby inhibiting the activity of digestive enzymes such as cholesterol esterase and pancreatin [42][43].

Conclusion

Based on the results of LC-MS analysis, fresh garlic extract contains 96 compounds, while garlic powder extract contains 110 compounds. Among these compounds, bioactive compounds from the organosulfur and flavonoid groups were identified. Based on their composition percentages, allicin is more abundant in powdered garlic extract, as are flavonoids such as quercetin and kaempferol, particularly in the form of glycosides. This difference indicates that the processing method influences the diversity and composition of bioactive compounds in garlic. In vitro, both extracts were able to lower cholesterol levels, with EC_{50} values of 41,349.08 ppm for the fresh extract and 35,462.29 ppm for the powdered extract. The powdered garlic extract showed higher efficacy compared to the fresh extract. These findings support the potential of garlic, particularly in powder form, as a natural cholesterol-lowering agent. However, further in vivo studies are needed to confirm its efficacy, mechanism of action, and long-term safety.

Author's Contribution

Aprilya Miftachul Qoyimmah: Collect data and compile the article; Nuniek Herdyastuti: Responsible person and article compiler.

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