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In Vitro Evaluation of Cholesterol-Lowering Activity of Acetyleugenol Synthesized via Esterification of Eugenol

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ABSTRACT: Hypercholesterolaemia is a significant risk factor for cardiovascular disease that necessitates alternative therapies instead of statins due to their adverse effects. Eugenol, a phenolic molecule that has the ability to lower cholesterol levels, is found in clove oil, which is derived from Syzygium aromaticum. The chemical stability and biological activity of its derivative, acetyleugenol, are both significantly higher. The purpose of this research was to determine whether or not acetyleugenol, which was produced by esterifying eugenol with acetic anhydride, have the ability to be effective in decreasing cholesterol levels. In order to determine the anticholesterol activity, UV-Vis spectrophotometry with the Liebermann-Burchard method was utilised at a range of concentrations (50, 75, 100, 125, and 150 ppm). The EC50 value was equal to 94.413 ppm, and the maximum cholesterol decrease was 27.97% when the concentration was 150 ppm. Considering these data, it appears that acetyleugenol possesses a considerable amount of potential as a natural cholesterol-lowering medication.

Keywords: cholesterol ; acetyleugenol; esterification; eugenol; EC_{50}

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INTRODUCTION

Hyperlipidemia, especially hypercholesterolemia, is one of the main risk factors for cardiovascular disease, which is the leading cause of death globally. Death can occur at a young age, which is contrary to the health development target to be achieved in 2025 in the form of increasing life expectancy, which has started from 2005 to 2025 (Pashar et al., 2025). Efforts to treat hypercholesterolemia generally involve the use of statin drugs, but long-term side effects such as myopathy and hepatic disorders encourage the search for safer alternative therapies, especially from natural ingredients (Khatiwada & Hong, 2024). Clove is a fragrant spice made from the dried flowers of the clove tree. Cloves are used in cosmetics, medicine, gastronomy, and agriculture due to the presence of many bioactive components such as gallic acid, flavonoids, eugenol acetate, and eugenol (Hema Krishna, 2024)

Clove oil (*Syzygium aromaticum*) contains eugenol, a phenolic compound with established anticholesterol properties (Djamaluddinet al., 2024). Clove leaf infusion at a dose of 0.18 g/head/day can lower blood cholesterol in mice (Siauta & Pentury, 2021). Early studies have shown that eugenol and its derivatives can interact with key enzymes in lipid metabolism, such as HMG-CoA reductase, which is the main target in hypercholesterolemia therapy (Harb et al., 2019). Eugenol derivative compounds, such as acetyleugenol, show increased chemical stability and higher bioactivity potential than eugenol itself (Ardiansah et al., 2024). Acetyl eugenol shows anti-inflammatory effects in vitro, as evidenced by its ability to inhibit protein denaturation by 32.20% (Dinurrosifa & Indriyanti, 2022). Additionally, the study suggests acetyl eugenol may act as an HIV-1 protease inhibitor. While further research is needed, these findings suggest the potential of acetyl eugenol in a variety of biological applications, including its potential to affect cholesterol levels (Sururi et al., 2023).

Cholesterol levels in pharmaceutical preparations can be tested using the Liebermann-Burchard method using UV-Vis spectrophotometry (Novyanti et al., 2025). This method is specific in measuring cholesterol compounds, which are included in one of the steroid groups (Luhurningtyas et al., 2019). The direct spectrophotometric method is used in the analysis of strongly absorbing analytes, while the indirect approach, involving derivatization, is used for weakly absorbing compounds. Cholesterol (cholesterol-5-en-3β-ol) has a weakly absorbing chromophore, which can be derivatized through the Liebermann-Burchard reaction to obtain a chromophoric group that can absorb strongly at the maximum wavelength (Anggraini & Nabillah, 2018). However, its derivative, acetyleugenol, is reported to have enhanced stability and bioactivity. This study aims to investigate the in vitro cholesterol-lowering potential of acetyleugenol.

METHODS

This research was conducted in the pharmaceutical chemistry laboratory of Sekolah Tinggi Ilmu Farmasi Yayasan Pharmasi Semarang. The equipment and laboratory tools used in this study are as follows: beakers, Erlenmeyer flasks, droppers, measuring pipettes, volume pipettes, analytical balances, watch glasses, ovens, sonicators, water baths, 50 mL measuring flasks, 10 mL measuring flasks, test tubes, filter paper, and Buchner pumps. The materials used in this study were obtained from Nitra Kimia, Yogyakarta, as follows: eugenol (for synthesis sigma aldrich), NaOH (analytical grade), Acetic Acid Anhydride (analytical grade), ethanol (analytical grade Smartlab), chloroform (analytical grade

Smartlab), H_2SO_4 (analytical grade Merck), cholesterol standard (analytical grade Sigma Aldrich), and acetic acid (analytical grade Merck).

Synthesis of acetyl eugenol

Eugenol 5 mL mixed with 13 mL of 10% NaOH. Sonicate for 15 minutes at 70-80°C. Add 9.2 mL of Acetic Anhydride. Sonicate for 150 minutes at 70-80°C. Extraction with 20 mL of chloroform twice. Two phases are formed, chloroform and water phases. The chloroform phase is stored overnight in the refrigerator until the temperature is below .

Anticholesterol Test

Preparation of standard solution

Weigh the cholesterol standard as much as 100 mg, dissolve it in ethanol p.a. up to 100 mL, and heat it in a water bath at 45°C.

Determination of maximum wavelength

An aliquot of 0.5 mL from the standard solution was pipetted into a test tube and diluted with ethanol to a final volume of 5.0 mL. Subsequently, 2.0 mL of acetic anhydride and 0.1 mL of concentrated sulfuric acid (H_2SO_4) were added. The reaction mixture was allowed to stand for 15 minutes at room temperature. After the incubation period, the absorbance was measured within the wavelength range of 400–700 nm using a UV-Vis spectrophotometer.

Determination of Operating Time

To evaluate the optimal reaction time, 0.5 mL of the standard solution was diluted with ethanol (p.a.) to a final volume of 5.0 mL. After the addition of 2.0 mL acetic anhydride and 0.1 mL of concentrated H_2SO_4, absorbance measurements were taken at 2-minute intervals from 10 to 30 minutes. The reaction was maintained at ambient temperature during this period.

Preparation of cholesterol standard curve

To construct the calibration curve, varying volumes of the standard solution (0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 mL) were each diluted with ethanol to a total volume of 5.0 mL, resulting in final concentrations of 40, 50, 60, 70, 80, 90, and 100 ppm, respectively. Each solution was then treated with 2.0 mL of acetic anhydride and 0.1 mL of concentrated H_2SO_4. After incubation for the optimized reaction time, the absorbance was measured at the predetermined wavelength.

Preparation of the sample standard curve

The synthesized acetyl eugenol standard solution was prepared at a concentration of 1000 ppm each 0.5, 0.75, 1, 1.25, and 1.5 mL, then ethanol up to 10 mL. taken 5 mL each and added 5 mL of 200 ppm cholesterol standard and mixed. Each of the solutions was taken 5 mL, then added with 2 mL of acetic anhydride and 1 mL of H_2SO_4. Measure the absorbance according to the wavelength obtained.

Preparation of negative control

Measured 5 mL of 100 ppm cholesterol standard, added 2 mL of acetic acid, and 0.1 mL of H_2SO_4.

Data Analysis

The results obtained from the sample measurement are absorbance, which is then compared with a standard cholesterol solution to determine the level of cholesterol reduction in percentage form. The formula used is as follows:

$$\% \text{ reduction} = \frac{abs\ standard - abs\ sample}{abs\ standard}\ x\ 100\%$$

Information:
% reduction = reduction in cholesterol levels (%)
Standard abs = initial cholesterol standard absorbance
Sample abs = cholesterol absorbance after treatment

RESULT AND DISCUSSION

The method used in this study is the esterification method between alcohol and carboxylic acid derivatives. The structure of eugenol contains an -OH group bound to the benzene ring (Maurya et al., 2020). Acetyl eugenol synthesis is carried out by reacting eugenol and acetic anhydride. The esterification process is very slow without a catalyst (Dinurrosifa & Indriyanti, 2022), so the addition of a catalyst aims to accelerate the acetylation reaction. The catalyst used is NaOH because its basic properties can increase the reactivity of nucleophiles through nucleophilic acyl substitution reactions, so that the reaction takes place faster and the yield obtained is higher (Tursiloadi et al., 2015).

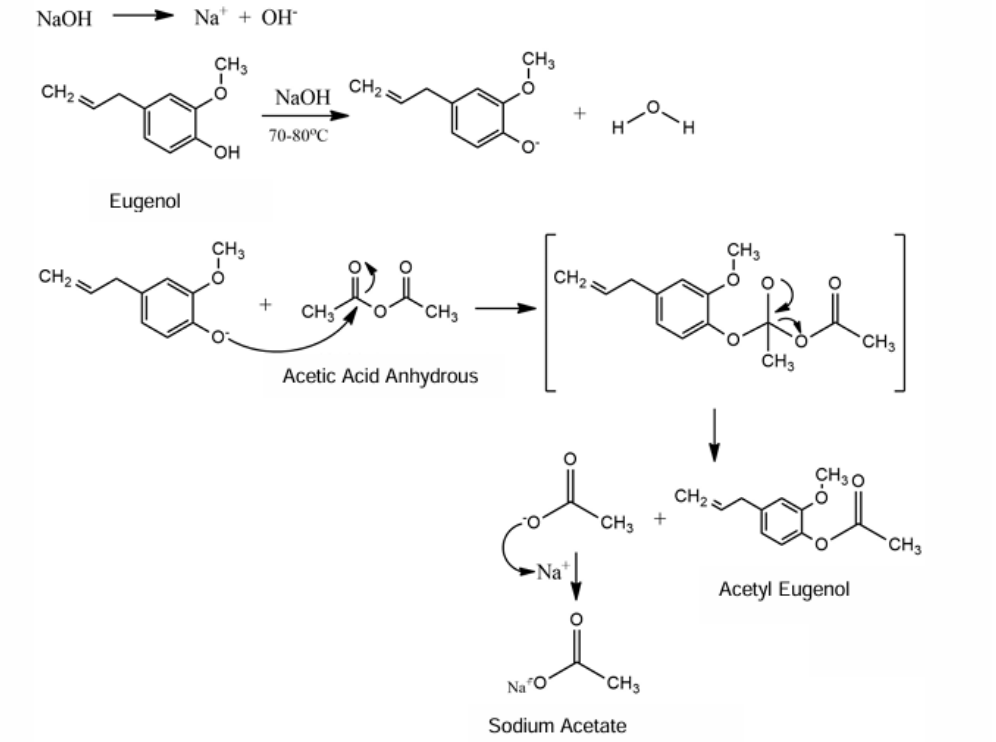

Figure 1. Reaction Mechanism of Acetyleugenol ((Dinurrosifa & Indriyanti, 2022)

This process is also assisted by ultrasonic waves. The sonochemical method is now one of the approaches in green chemistry because it is easy to do, efficient, produces high quantities of products, takes a short time, and does not damage the environment (Patel et al., 2014) and can prevent the use of volatile and toxic solvents by using harmless chemicals (M. Draye, 2020). This method is very important in the synthesis of organic compounds and in the pharmaceutical industry. The use of alkali catalyst sodium hydroxide will cause the

formation of nucleophiles, namely eugenolate ions, so that the reaction can run faster and more effectively. This results in an increase in nucleophilicity, which causes the attack of the carbonyl atom C on acetic acid anhydride to take place more easily. Thus, it is expected that the resulting yield will be greater.

In the synthesis process of acetyleugenol, 5.5 grams were produced using the ultrasonic wave method, where the use of ultrasonic radiation produces a faster reaction rate, energy conversion, and minimizes waste compared to conventional methods. Ultrasonic waves occur at frequencies of 20 kHz to 100 MHz. Ultrasonic waves are known to accelerate various types of organic reactions and are believed to be an important technique in organic synthesis. Next, the sample measurement to see the decrease in cholesterol levels uses the Lieberman-Burchard method. The Lieberman-Burchard method is a very specific method for measuring steroid compounds, one of which is cholesterol. Standard cholesterol is dissolved in chloroform because 1 part of non-polar cholesterol dissolves in a non-polar solvent, namely 4.5 parts of chloroform. The reaction carried out in this method must be free of water because the reaction will be very sensitive and unstable to water. In this method, it is necessary to add anhydrous acetic acid and concentrated sulfuric acid. The addition of anhydrous acetic acid aims to extract cholesterol, ensure that the media is free of water, and form steroid acetyl derivatives which are then dripped with concentrated sulfuric acid through the wall to produce a green color for steroid compounds including cholesterol. In this study, the standard cholesterol concentration used was 200 ppm. Before getting the concentration results used for the study, the results obtained were the actual concentration of 1007 ppm. Then the concentration was diluted to a concentration of 40,50,60,70,80,90, and 100 ppm. Each concentration is put into a test tube and added with a standard cholesterol concentration of 200 ppm. The resulting mixture is added with anhydrous acetic acid solution and also from H_2SO_4. The resulting solution is then left for 15 minutes in a dark place. The goal is for the solution to form a green complex and then read the absorbance value at a maximum wavelength of 668 nm at 15 minutes, in addition the cholesterol solution is photodegradable, unstable to light and will turn into cholestenone (Figure 2).

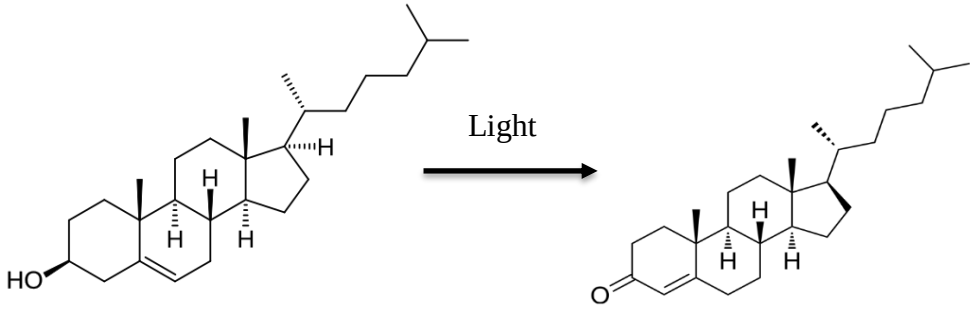

Figure 2. Photodegradation Reaction of Cholesterol to Cholestenone (Dinurrosifa et al., 2022)

The use of anhydrous acetic acid aims to ensure that the media is free of water because in the Lieberman-Burchad method the reaction is very sensitive and unstable to water. If there is water content, then the anhydrous acetic acid in the system can change into hydrated acetic acid and the reaction cannot occur with cholesterol or with concentrated sulfuric acid. In addition, anhydrous acetic acid also plays a role in the formation of acetyl derivative compounds from steroids which when added with concentrated sulfuric acid can produce a green complex, as shown in Figure 3.

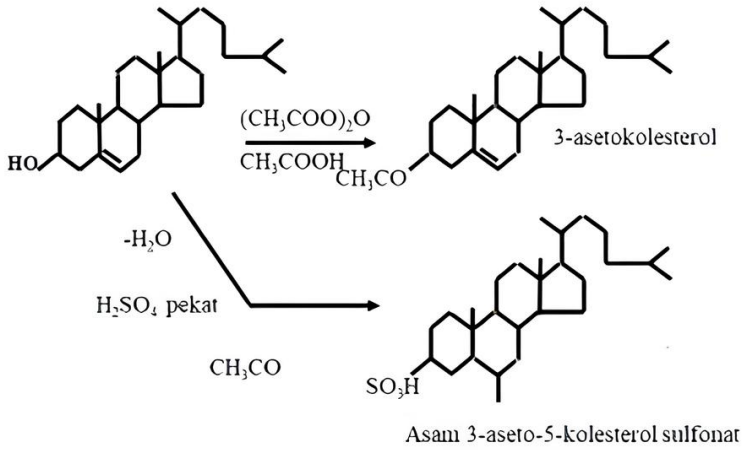

Figure 3. Green Complex Reaction between Cholesterol and Lieberman-Burchard reagent (Anggraini & Nabillah, 2018)

To determine the wavelength of acetyleugenol, UV-Vis spectrophotometry is used. Ultraviolet and visible spectrophotometry methods are used to determine low levels of a substance, usually in ppm (parts per million) or ppb (parts per billion). The working principle is based on the absorption of light or radiation energy by a solution. The amount of light or radiation energy absorbed allows quantitative measurement of the amount of absorbent in the solution. In this study, the maximum wavelength obtained was 534.60 nm. The Lieberman-Burchard method obtained two maximum absorption wavelengths and stated that the lowest absorption at the maximum wavelength is more stable.

In the process of reducing cholesterol levels, absorbance values and % reduction in cholesterol levels were obtained (Table 1).

Table 1. Effect of Acetyleugenol Concentration on Cholesterol Reduction

Standard absorbance	acetyl eugenol concentration	sample absorbance	% reduction in cholesterol	average (%)	EC_{50}
0,684	50 ppm	0,659	3,65	2,83	94,413 ppm
		0,667	2,49		
		0,668	2,34		
0,684	75 ppm	0,629	8,04	7,50	
		0,632	7,6		
		0,637	6,87		
0,684	100 ppm	0,582	14,91	15,21	
		0,583	14,77		
		0,575	15,94		
0,684	125 ppm	0,533	22,08	22,47	
		0,522	23,68		
		0,536	21,64		
0,684	150 ppm	0,488	28,65	27,97	
		0,499	27,05		
		0,491	28,22		

Based on the table, it can be seen that the higher the concentration used, the higher the % decrease in cholesterol levels and the lower the absorbance value. This means that high concentrations have an effect on reducing cholesterol levels, where the higher concentration provides the highest cholesterol reduction, so that the absorbance value is smaller than the percentage of large anticholesterol activity. Measurements were carried out in triplicate, and the average percentage reduction in cholesterol levels is depicted in the curve in Figure 4.

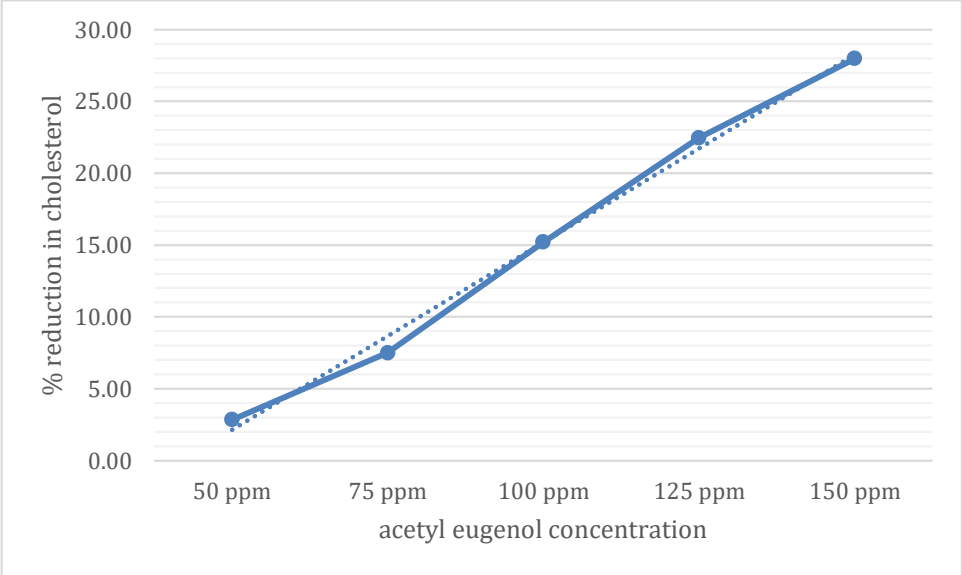

Figure 4. Relationship Curve Between Acetyl Eugenol Concentration and Cholesterol Decrease

The effects of acetyl eugenol on cholesterol are influenced by its chemical structure, primarily through the formation of ester bonds and steric effects. Acetyl eugenol, with an acetyl group (-COCH3) in its phenol group, can form ester bonds with cholesterol, modifying the physical and chemical properties of cholesterol. The ester group in the context of cholesterol lowering refers to cholesterol esters. Cholesterol esters are forms of cholesterol bound to fatty acids. Cholesterol esterification, the process of forming cholesterol esters, aids in the storage and transport of cholesterol in the body. Cholesterol esters are more hydrophobic than free cholesterol, so they can be packaged more efficiently in lipoproteins, facilitating the transport of cholesterol through the bloodstream. In addition, stanol esters, which are esterified forms of stanols, may also help lower cholesterol by competing with cholesterol during intestinal absorption. In addition, the structure of acetyl eugenol, specifically the methyl group (-CH3) and aromatic groups, may also cause steric effects, influencing how acetyl eugenol interacts with cholesterol in biological systems.

The effective concentration (EC_{50}) value in this study was 94.413 ppm. This EC_{50} value aims to see the concentration that can reduce total cholesterol levels by 50%. So, from these results, it means that to reduce 50% cholesterol, 94.413 ppm of acetyleugenol is needed.

CONCLUSION

From this study, it can be concluded that acetyleugenol has cholesterol-lowering activity with an EC_{50} value of 94.413 ppm. Additionally, investigations into its mechanism of action at the molecular level, pharmacokinetic properties, and potential for structural optimization are recommended to support its development as a novel hypocholesterolemic agent. Comparative studies with standard lipid-lowering drugs and toxicity assessments will also be essential to validate its therapeutic applicability.

AUTHOR CONTRIBUTION

RSD: design; definition of intellectual content; literature search; experimental studies; data analysis; manuscript preparation.
DFP: Concepts or ideas; design; definition of intellectual content; literature search; experimental studies; data analysis; Manuscript editing; manuscript review.

CONFLICT OF INTEREST

None to declare

REFERENCES

Anggraini, D. I., & Nabillah, L. F. (2018). Activity Test of Suji Leaf Extract (Dracaena angustifolia Roxb.) on in vitro cholesterol lowering. *Jurnal Kimia Sains Dan Aplikasi*, *21*(2), 54–58. https://doi.org/10.14710/jksa.21.2.54-58

Ardiansah, B., Farhan, A., Firdaus, A., Ariyani, T., Nasution, M. A. F., Fadlan, A., Cahyana, A. H., Prabandari, E. E., & Carlos Menéndez, J. (2024). Eugenol derivatives containing 1,2,3-triazole-chalcone hybrids for shikimate kinase inhibition. *Journal of Saudi Chemical Society*, *28*(2). https://doi.org/10.1016/j.jscs.2024.101826

Dinurrosifa, R. S., & Indriyanti, E. (2022). A Green Synthesis of Acetyl Eugenol by Sonochemical Method and Potential as Anti-Inflammatory In-Vitro. *JKPK (Jurnal Kimia Dan Pendidikan Kimia)*, *7*(3), 324. https://doi.org/10.20961/jkpk.v7i3.66630

Harb, A. A., Bustanji, Y. K., Almasri, I. M., & Abdalla, S. S. (2019). Eugenol Reduces LDL Cholesterol and Hepatic Steatosis in Hypercholesterolemic Rats by Modulating TRPV1 Receptor. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-50352-4

Hema Krishna, R. (2024). International Journal of Advanced Chemistry Studies on the phytochemicals of clove and their biological activities. In *International Journal of Advanced Chemistry* (Vol. 12, Issue 1). www.sciencepubco.com/index.php/IJET

Ilham Djamaluddin, M., Dwifauza Suryanto, R., Salsabila, S., Sasikirana Finola Aziz, C., & Alya Setyowati, L. (2024). Studi In Silico Senyawa Bioaktif Daun Sirih (Piper Betle L.) Sebagai Antikolesterol Pada Reseptor HMG Co-A Reduktase. *Jurnal Farmaka*, *22*. https://www.

Khatiwada, N., & Hong, Z. (2024). Potential Benefits and Risks Associated with the Use of Statins. In *Pharmaceutics* (Vol. 16, Issue 2). Multidisciplinary Digital Publishing Institute (MDPI). https://doi.org/10.3390/pharmaceutics16020214

Luhurningtyas, F. P., Hasani, N., Aprilliana, M., Saputra, D., & Prayasanti, D. (2019). *Pengaruh Kombinasi Ekstrak Daun Ashitaba (Angelica Keiskei Ito.) Dan Daun Sukun (Artocarpus Communis) Terhadap Kadar Glukosa Dan Kolesterol Secara In Vitro Menggunakan Metode Fotometri*, 9(2).

M. Draye, G. C. and R. D. (2020). Ultrasound for drug synthesis: A green approach. *Pharmaceuticals*, *13*(2).

Maurya, A. K., Agarwal, K., Gupta, A. C., Saxena, A., Nooreen, Z., Tandon, S., Ahmad, A., & Bawankule, D. U. (2020). Synthesis of eugenol derivatives and its anti-inflammatory activity against

skin inflammation. *Natural Product Research*, *34*(2), 251–260. https://doi.org/10.1080/14786419.2018.1528585

Novyanti, E. S., Andina, F. R., Subekti, L. A., & Imtihani, H. N. (2025). In-Vitro Cholesterol Reduction Using Three Dosages of Chitosan from Mangrove Crab Shells (Scylla serrata) in Effervescent Granules Suspension. *Jurnal Ilmiah Medicamento*, *11*(1), 58–66. https://doi.org/10.36733/medicamento.v11i1.9914

Pashar, I., Olsi Rahmi, Y., Islamiyah, T., Studi Keperawatan, P., & Kedokteran dan Ilmu Kesehatan, F. (2025). Faktor-Faktor Risiko Terjadinya Penyakit Jantung Koroner Pada Usia Produktif Risk Factors For Coronary Heart Disease In Productive Age. *Jurnal Ilmiah Manusia Dan Kesehatan*, *8*(1), 174–184. https://jurnal.umpar.ac.id/index.php/makes

Patel, B. R., Desai, D. H., Raval, J. P., & Patel, B. R. (2014). Green Efficient Synthesis of Aryl Thioamides Using Ultrasound: A Comparative Study. *Journal of Pharmacy And Applied Sciences*, *1*(1), 29–33.

Siauta, D., & Pentury, K. (2021). Efek Antihiperkolesterolemia Seduhan Daun Cengkeh (Syzygium aromaticum L.) Terhadap Kolesterol Darah Tikus Model. *Kalwedo Sains (KASA)*, *2*(1), 48–52. https://doi.org/10.30598/kasav2i1p48-52

Sururi, A. M., Kartika Maharani, D., & Wati, A. (2023). Potensi Senyawa Eugenol Dari Cengkeh (Syzygium Aromaticum) Sebagai Inhibitor Protease Hiv-1 (Pr) Potency Of Eugenol Compounds From Clove (Syzygium Aromaticum) As Hiv-1 Protease (Pr) Inhibitors. In *UNESA Journal of Chemistry*, 12(1)

Tursiloadi, S., Artanti, N., & Sulaswatty, A. (2015). *Chemical Catalytic And Biocatalytic Process Of Clove Oil Derivatives Review Turunan Dari Minyak Cengkeh Dengan Proses Katalis Dan Biokatalis, Indonesian Journal of Applied Chemistry*, 17(1). 69-85.