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Polyherbal formulation as an anti-atherosclerosis agent by inhibiting foam cell formation and TNF-α expression in a hypercholesterolemic mice model

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Abstract

Atherosclerosis is a progressive inflammatory disease characterized by lipid accumulation in arterial walls. This study investigates the potential of a polyherbal formulation as an anti-atherosclerosis and anti-inflammatory agent in a hyperlipidemic mice model. An *in vitro* and *in vivo* observational experimental design was employed, using a randomized post-test control group design with twenty-five hyperlipidemic mice. The hyperlipidemia model was induced by a high-fat diet of 10% beef fat and 20% duck egg yolk for thirty days. Results showed that increased cholesterol levels, TNF-α levels, and foam cells contribute to atherosclerotic lesions formation via lipid metabolism modulation, inflammation, and foam cell regulation. The polyherbal formula significantly inhibited plaque development by reducing foam cell formation($p<0.05$) with foam cell counts in P1: 8±1.0; P2: 52±6.5; P3: 30±14.5; P4: 13±7.1; and P5: 34±10.2. Additionally, it lowered TNF-α levels (P1: 72.218±8.770; P2: 141.362±63.645; P3: 56.314±10.010; P4: 57.164±24.328; P5: 89.646±7.189). The polyherbal formulation containing *Brassica rapa* L. (quercetin, kaempferol, isorhamnetin), *Apium graveolens* L. (phytosterol, 3-n butylphthalide (3nB), apigenin, apiin, tannin, saponin), and *Ananas comosus* L. Merr (bromelain) effectively reduces foam cell number and TNF-α levels, demonstrating potential as an anti-atherosclerosis agent.

1. Introduction

According to the World Health Organization (WHO), more than 17 million people worldwide die from heart and vascular diseases. Cardiovascular diseases (CVD) are the leading cause of death globally, claiming approximately 17.9 million lives each year. CVD is a group of heart and vascular disorders that include coronary heart disease, cerebrovascular disease, rheumatic heart disease, and other conditions (World Health Organization, 2021). CVD, a clinical manifestation of atherosclerosis (Tjong *et al*., 2021), remains a global health issue and is the leading cause of death to this day (Lasanuddin *et al*., 2022). Inflammation, oxidative stress, and dyslipidemia are major factors contributing to the development of atherosclerotic cardiovascular disease (Tietge, 2014). A recent study provides significant contributions in linking inflammation and dyslipidemia. The research, involving 60,608 individuals, demonstrated that increased cholesterol levels lead to ischemic heart disease and inflammation, while elevated LDL cholesterol levels result in ischemic heart disease without an inflammatory component (Varbo *et al*., 2013). Hyperlipidemia leads to the accumulation of cholesterol and triglycerides in the walls of blood vessels, often causing atherogenesis and atherosclerosis.

Hyperlipidemia and vascular inflammation are not only independently associated with atherosclerosis but also represent interconnected processes. Among these, lipoproteins act as damage-associated molecular patterns, triggering early innate immune responses. If unresolved, this leads to chronic inflammation, which ultimately causes arterial damage and organ infarction due to thrombosis. To date, the innate immune response in atherosclerosis is believed to be largely mediated by monocytes and macrophages. This involves increased hematopoiesis, enhanced recruitment of immune cells into the vascular wall, and activation that is partly mediated by the interaction between macrophages and oxidized LDL (Rodriguez-Garcia and Alcaide, 2021).

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Several methods to halt atherosclerosis include reducing risk factors, using statins, blockers, angiotensin -converting enzyme (ACE) inhibitors, antithrombotic drugs, and the appropriate use of estrogen and antioxidants. Despite these approaches, there is still no best pharmacological method to treat atherosclerosis (Bays *et al*., 2021). Due to this condition, researchers aim to investigate the most optimal alternative medicines from natural ingredients to prevent and treat the occurrence of atherosclerosis. The current research on natural ingredients as anti-atherosclerosis is mainly focused on single herbs (Kim and Shim, 2019), to improve the benefit a polyherbal formula with benefits for treating inflammation and oxidative stress was formulated. This formula consists of three water-soluble medicinal plants that have undergone phased testing and have been used in several studies, namely bok choy (*Brassica chinensis* L.) which was tested to reduce oxidative stress on rats (Azra Riaz *et al*., 2022), pineapple (*Ananas comosus* L. merr) which have bromelain compound that works as an antioxidant and anti-inflammatory agent (Lee *et al*., 2018), and celery (*Apium graveolens* L.) was already tested in the form of juice on rats is able to increase antioxidant capacity (Kooti and Daraei, 2017). Each of the natural ingredients is expected to have better anti-atherosclerosis and anti-inflammatory benefit in the form of this polyherbal mixture on a hyperlipidemic mice model. The objective of the research was to investigate the potential of a polyherbal formulation (consisting of *Brassica rapa* L., *Apium graveolens* L. and *Ananas comosus* L. Merr.) as an anti-atherosclerotic and anti-inflammatory agent. Specifically, to evaluate the efficacy of this polyherbal formulation in reducing foam cell formation and TNF-α expression in a hypercholesterolemic mice model, thereby assessing its potential therapeutic benefit against atherosclerosis.

2. Materials and methods

2.1 Materials

The materials used in this study include bok choy, pineapple, and celery that was stated in this determination number: 91/In.08/LB.1.1/PP.009/09/2024 honey, yogurt (Biokul®), water, duck egg yolk, beef fat, simvastatin 10 mg (OGB dexa), test animal feed, propylthiouracil (PTU) 100 mg (OGB dexa), Vitamin D3 (Prove D3-1000), chloroform (Bratachem), 0.9% physiological NaCl, 10% Neutral Buffered Formalin (BNF), alcohol 70% (Bratachem), xylene, Hematoxylin Eosin (HE), CHOD-PAP reagent (DiaSys), ELISA reader kit (BT Lab). The equipment used in this study includes a set of glassware (pyrex), surgical instruments, histological reading tools, incubator (Biobase), centrifuge (Ohaus 2456) and spectrophotometer (Shimadzu UV-1800 Spectrophotometer UV-Vis).

2.2 Methods

This study was approved by the Research Ethics Committee with number: DP.04.02/F. XXXII.25/0737/2023. The research method used was an experimental observational *in vitro* and *in vivo* using a post-test randomized control design with CHOD-PAP method, Quantitative sandwich direct Enzyme-linked immunosorbent assay (ELISA), and Hematoxylin eosin (HE) staining. This study used twenty-five hyperlipidemic mice models without other clinical conditions and was divided into five groups. The normal control group (P1) was given only a normal diet. The negative control group (P2) was given an atherogenic diet for 4 weeks + 1% Na-CMC for 2 weeks. Na-CMC was used because it does not affect cholesterol levels in rats. The positive control group (P3) was given an atherogenic diet for 4 weeks + Simvastatin at a dose of 1.3 mg/kg BW for 2 weeks. The treatment group F I (P4) was given an atherogenic diet for 4 weeks + poly herbal F I for 2 weeks. The treatment group F II (P5) was given an atherogenic diet for 4 weeks + poly herbal F II for 2 weeks. Eligible experimental animals included male, white-colored, weighing 20-30 grams with hypercholesterolemia defined as a total blood cholesterol level > 130 mg/dL in mice. The exclusion criteria for the samples were mice that did not have a cholesterol level > 130 mg/dL and the dropout criteria were the mice that died during the study.

2.2.1 Formulation and standardization of polyherbal

The formulation started with the selection of ingredients that met the standard quality requirements for consumption. The bok choy (*Brassica chinensis* L.) used had a taproot system with long, round lateral roots that spread in all directions at a depth of 30-50 cm. Its stem was short and jointed, with broad and sturdy leaf stalks. The pineapples (*Ananas comosus* L. Merr) used were elongated and round in shape with light yellow flesh. The celery (*Apium graveolens* L.) used had compound leaves with long petioles and short stems.

2.2.2 Preparation of polyherbal formulation

The formulation preparation started with measuring the ingredients based on the reference. The bok choy and celery were weighed, while the ripe pineapple was peeled. All ingredients were then cut into smaller pieces and placed in a blender. Water, yogurt, and honey were added according to the specified formulation to make the juice. The mixture was blended until smooth and then strained to complete the polyherbal formulation, making it ready for consumption.

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2.2.3 Preparation of hyperlipidemic animal models

The hyperlipidemic model was created by giving mice an atherogenic diet of 10% beef fat and 20% duck egg yolk. The beef fat was heated to a liquid state and mixed with animal feed, then combined with weighed egg yolks, Propylthiouracil (PTU), and Vitamin D3 until homogeneous. Mice were fed this high-fat diet daily for 30 days. Cholesterol levels were measured using the CHOD-PAP method. Finally, the blood samples were prepared into a serum by adding it to a microplate with reagent, incubated at 37°C for 10 mins, and the absorbance was measured at 500 nm using a spectrophotometer.

2.2.4 Preparation of TNF-α levels analysis

To determine TNF-α levels, a quantitative sandwich enzyme immunoassay (ELISA) was used. The test was performed at room temperature with prepared reagents, standards, and samples. First, 50 µL of standard or sample was added to the wells, followed by 40 µL of sample, 10 µL of anti-TNF-α, and 50 µL of streptavidin-HRP. The wells were incubated at 37°C for 60 mins, then washed 5 times with wash buffer. After drying, 50 µL of substrate solution A and 50 µL of substrate solution B were added. The plate was incubated for 10 mins at 37°C in the dark. Finally, 50 µL of stop solution was added, turning the blue color yellow. The optical density (OD) at 450 nm was measured within 10 mins using a microplate reader.

2.2.5 Determination of foam cell count

To measure the number of foam cells in mice after treatment, the mice were first anesthetized with chloroform and dissected to expose the heart. The heart and aorta were then isolated and washed with physiological NaCl solution. These organs were fixed in 10% neutral formalin buffer (BNF) and prepared for histology using the paraffin method and Hematoxylin-Eosin (HE) staining. Foam cells were visualized by staining, appearing with a pink nucleus and a clear edge in the tunica intima. The number of foam cells was counted manually under a microscope at 400× magnification using Olyvia software and Screen Calipers.

2.2.6 Data analysis

Statistical analysis was conducted using SPSS version 23. Results were presented as mean ± SD. The Shapiro-Wilk test was used to assess the analysis of constant variance distribution for normality, and the One Way Anova test assesses differences in means between groups. As data was not normally distributed, the non-parametric Kruskal-Wallis test was continued to compare statistics between the two groups. Statistically, A comparison test between groups was also performed using the Mann-Whitney method with a P value <0.05 considered to indicate a significant difference.

3. Results

The data characteristics of total cholesterol levels, TNF-α levels, and foam cell count for each control group are presented in Table 2. It can be observed that the three treatment groups of hypercholesterolemic mice with atherosclerotic lesions, namely the positive group, formula 1, and formula 2, show cholesterol levels, TNF-α levels, and foam cell counts nearly equivalent to the normal group, which consists of healthy mice. As illustrated in Table 2, the TNF-α levels in group P4 (polyherbal formula 1) and group P3 (positive control) show a notable similarity, with group P5 (formula 1) exhibiting a similar trend. There are no significant differences between the normal control group and group P4, indicating that polyherbal formula 1 which is presented in Table 1 can suppress TNF-α levels to those of normal mice. Similarly, the foam cell comparative test shows that group P4 has comparable results to the normal group (P1), effectively reducing foam cell numbers in hyperlipidemic mice with atherosclerotic lesions to normal levels. Table 3 further supports these findings, showing that groups P3, P4, and P5 (positive control, formula 1, and formula 2) exhibit significant

Table 1. Polyherbal formulation.

Ingredient	Composition (%)	
	F1	F2
Bok choy (*Brassica rapa* L.)	6.2	8.3
Pineapple (*Ananas comosus* L.)	24.9	33.2
Celery (*Apium graveolens* L.)	0.6	0.8
Honey	5.2	6.9
Yoghurt	13.1	17.4
Water	49.9	33.2

Table 2. Total cholesterol levels, TNF-α count, and foam cell count per group (mean±SD)

Group	Cholesterol count (mg/dL)	TNF-α count	Foam cell count (Cells)
Normal Control Group (P1)	97.294±13.610	72.218±8.770	8±1.0
Negative Control Group (P2)	144.906±14.665	141.36±63.645	52±6.5
Positive Control Group (P3)	91.397±4.118	56.314±10.010	30±14.5
Treatment Group F I (P4)	103.526±0.905	57.164±24.328	13±7.1
Treatment Group F II (P5)	115.294±12.336	89.646±7.189	34±1.2

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differences compared to the negative control group (P2) with p-values less than 0.05. These results suggest that both polyherbal formulas may possess anti-atherosclerosis properties, as evidenced by the reduction in TNF-α levels and foam cell numbers.

Table 3. Comparative test results between groups.

TNF- α			Foam Cell		
Group		p-value	Group		p-value
P1	P2	0.009*	P1	P2	0.009*
	P3	0.076		P3	0.015*
	P4	0.175		P4	0.238
	P5	0.016*		P5	0.009*
P2	P3	0.009*	P2	P3	0.021*
	P4	0.016*		P4	0.009*
	P5	0.028*		P5	0.012*
P3	P4	0.602	P3	P4	0.036*
	P5	0.009*		P5	1.000
P4	P5	0.076	P4	P5	0.016*

* Statistically significant difference ($p < 0.05$).

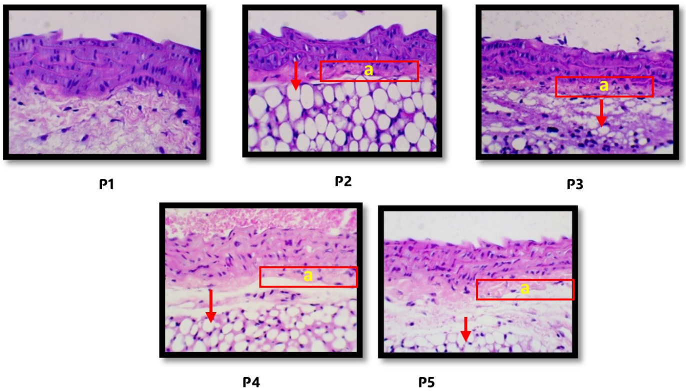

Figure 1. Aortic sub-endothelium in each group was stained with Hematoxylin-Eosin (HE) at 400× magnification.

Histological examination was conducted to observe the progressive reduction in the number of aortic foam cells which can be seen in Figure 1. Additionally, the image reveals the presence of fat streaks, indicating the occurrence of the atherosclerosis stage in the hyperlipidemic mice model given a high-fat diet. The (a) code indicates yellowish fat streaks, while the arrow (→) indicates foam cells with white round shapes among the empty spaces in the *tunica intima* layer of the aorta.

4. Discussion

Studies indicate that atherosclerosis results from intimal damage involving various cellular responses from monocytes, smooth muscle cells, and lymphocytes. Early soft lesions consist of foam cells and accumulations of extracellular fat, along with a small number of platelets. As the process progresses, smooth muscle cells proliferate and, in the final stages, intensify hemorrhaging into the plaque (Rafieian-Kopaei *et al.*, 2014). In this study, the process of hyperlipidemia has been documented and the most critical factor, atherosclerotic lesions, characterized by the formation of lipid stripes and foam cells, caused by an atherogenic diet (Cunha *et al.*, 2021). The atherogenic diet consists of providing high-fat feed (Darmi Manggasa, 2017), which induces hyperlipidemia in mice over a period of four weeks. Mice fed a normal diet do not show atherosclerosis or only exhibit mild lesions with significant individual differences. A high-fat and cholesterol diet was used to induce the development of atherosclerotic lesions. In this study, the inducer used was a high-fat diet consisting of 20% duck egg yolk and 10% beef fat, with the addition of PTU and Vitamin D3. This choice of inducer was based on previous research findings, where duck egg yolk contains 35 g of fat, 17 g of protein, and 884 mg of cholesterol per 100 g (Dwi Enggarwati and Qomariyah, 2023). Beef fat contains saturated fatty acids such as laurate, stearate, palmitate, and myristate. Its saturated fatty acid content is 50.3%, and it also contains cholesterol at 9.5 mg/10 g (Gunawan *et al.*, 2018).

Saturated fatty acids can increase LDL levels in the blood. In this study, a high-fat diet was also provided, made by mixing beef fat (saturated fatty acids) and standard food to accelerate the hypercholesterolemia process in mice (Pratiwi *et al.*, 2015). In addition, the high-fat diet contained propylthiouracil (PTU) and vitamin D3. PTU was used to slow down metabolism in the liver, thereby accelerating the rise in blood cholesterol levels in mice; it works by stopping the thyroid peroxidase enzyme. As a result, Diiodotyrosine (DIT) and Monoiodotyrosine (MIT) were not formed (Adhitama *et al.*, 2023). The main function of thyroid hormones in this research was to significantly regulate lipid metabolism. Thyroid hormones stimulate *de novo* hepatic cholesterol synthesis by inducing the coenzyme HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme) reductase and catalyzing HMG-CoA to mevalonate, which is the initial step in cholesterol biosynthesis. In cases of thyroid dysfunction, there is an increase in cholesterol biosynthesis, cholesterol secretion by the liver, the conversion process of HDL (High Density Lipoprotein) to VLDL (Very Low Density Lipoprotein) by the liver, activity of the LPL (Lipoprotein Lipase) enzyme in the cell membrane, and modulation of LDL (Low Density Lipoprotein) receptor activity in body cells (Prumnastianti *et al.*, 2021), whereas vitamin D3 is used to stimulate the proliferation of smooth muscle cells and increase vascular calcification (Krestianto *et al.*, 2020).

The formation of foam cells in high-fat diet administration is due to the oxidation of lipoproteins, particularly LDL. Oxidized LDL (Ox-LDL) is recognized by macrophages as a foreign entity,

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triggering the phagocytosis process and accumulating into foam cells (Singh *et al*., 2014) macrophages to the site of inflammation and adhere to the vascular endothelium (Ganesan *et al*., 2018). Subsequently, macrophages migrate to the subendothelial space, causing thrombosis in the tunica intima of blood vessels, evident by an increased vessel diameter. This mechanism leads to the accumulation of lipids within macrophages, triggering the formation of foam cells. Foam cells then express cytokines and growth factors such as IL-1 and TNF-α, attracting more macrophages to the lesion site and thus enhancing the progression of plaque formation (Prabawa *et al*., 2014). Tumor Necrosis Factor-Alpha (TNF-α) is a cytokine that plays a crucial role in the acute inflammatory response to gram-negative bacteria and other pathogens. TNF-α can be produced in large amounts in response to severe infections, triggering systemic reactions (Supit *et al*., 2015). Additionally, the increase in ROS also plays a role in assisting the modification of LDL into its oxidized form (Ox-LDL), causing vascular inflammation which ultimately leads oxLDL, along with low-level inflammation, to cause chronic inflammation resulting from endothelial injury, triggering the innate immune response and increasing the recruitment of immune cells, particularly monocytes and neutrophils, to the subendothelial space to participate in plaque formation (Javadifar *et al*., 2021).

Previous research has shown that bok choy (*Brassica chinensis* L.) can help prevent hypertension and heart disease, and reduce the risk of certain cancers due to its anti-cholesterol properties, which are attributed to compounds such as flavonoids, alkaloids, tannins, and saponins (Andriani and Anggraini, 2023). Bok choy, pineapple, and celery also have anti-inflammatory effects. Bok choy contains flavonoids (Hwang *et al*., 2020) like quercetin, kaempferol, and isorhamnetin derivatives (Paul *et al*., 2019). Celery contains compounds such as 3-n-butylphthalide (3nB), apigenin, apiin, tannins, and saponins, which inhibit inflammatory cytokines and reduce cholesterol levels by preventing the conversion of HMG-CoA to mevalonate (Rusdiana, 2018). Tannins in celery lower cholesterol by excreting it through feces without affecting the enterohepatic cycle, while its vitamin C content helps prevent atherosclerosis by increasing HDL levels (Adrianta, 2020; Sarwindah, 2020).

Pineapple contains bromelain, an enzyme with antioxidant and anti-inflammatory properties that reduces the secretion of pro-inflammatory cytokines and increases anti-inflammatory cytokines like IL-10 through the AMPK-TFEB (Adenosine Monophosphate-activated Protein Kinase-Transcription Factor EB) pathway (Ou *et al*., 2018; Insuan *et al*., 2021). Bromelain also inhibits NF-kB (nuclear factor kappa B) translocation and COX-2 activity, thereby reducing inflammation and slowing atherosclerosis progression (Amalia *et al*., 2017; Chen *et al*., 2023). Additionally, pineapple's flavonoids, including myricetin, kaempferol, luteolin, apigenin, and quercetin, enhance the activity of the paraoxonase enzyme, increasing HDL levels (Sanggih *et al*., 2019).

Each ingredient in the papillary formulation has been tested to be an anti-atherosclerosis agent on its own. Bok choy (*Brassica chinensis L*) was tested to have a cardio-protective role in diabetic subjects by decreasing the risk of dyslipidemia and atherosclerosis (Azra Riaz *et al*., 2022). Pineapple (*Ananas comosus* L. Merr) especially the bromelain compound has significant anti-oxidant activity and anti-inflammatory ability which are crucial to treat and prevent atherosclerosis (Lee *et al*., 2018). Celery (*Apium graveolens* L.) especially the roots was tested in vivo in the form of aqueous extract and was able to increase antioxidant capacity (Kooti and Daraei, 2017). The similar benefit from each ingredient and the available form such as the aqueous extract that has been tested was suitable to finally be formulated as a polyherbal formulation to prevent and treat atherosclerosis by preventing the progression of atherosclerotic plaques in mice model (Dwinanda *et al*., 2019), which can be seen in Figure 1 where the aortic sub-endothelium in each group was stained with Hematoxylin-Eosin (HE) at 400x magnification (Apriani *et al*., 2023). Future research should consider the limitations of this study, including the need for long-term and clinical trials, to validate the efficacy and safety of this formulation in humans.

5. Conclusion

In conclusion, this study demonstrates that hyperlipidemia and inflammation resulting from a high-fat diet can lead to atherosclerosis, characterized by the formation of foam cells and lipid accumulation. The findings highlight the potential of a polyherbal formulation containing polyphenols like quercetin and kaempferol, phytosterols, and bromelain from bok choy, pineapple, and celery to mitigate these effects by reducing cholesterol levels and inflammation. These compounds were shown to inhibit NF-kB activity and lower TNF-α levels, thereby preventing the progression of atherosclerotic plaques. This conclusion was drawn based on *in vitro* and *in vivo* testing results. In this research, it was found that the polyherbal preparation alleviated atherosclerotic lesions, observed through quantitative data such as the low levels of TNF-α and the number of foam cells in the hyperlipidemic mice model compared to the negative control group. These promising results suggest that this polyherbal formula could be further developed for the prevention and treatment of atherosclerosis.

Conflict of interest

The authors declare no conflict of interest.

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