RESEARCH ARTICLE

Quercetin Stabilizes Atherosclerotic Plaques by Reducing Matrix Metalloproteinase-9 Expression and Enhancing M2 Macrophage Activity in Wistar Rats

Romi Ermawan[1,2], Budi Susetyo Pikir[3,*], Mulyanto[2], Budi Utomo[4], Widjiati[5], Yudi Her Oktaviono[3]

[1]Doctoral Program of Medical Science, Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo 47, Surabaya, Indonesia
[2]Faculty of Medicine, Universitas Mataram, Jl. Majapahit 62, Mataram, Indonesia
[3]Department of Cardiology and Vascular Medicine, Soetomo Academic General Hospital/Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo 6-8, Surabaya, Indonesia
[4]Faculty of Medicine, Universitas Airlangga, Jl. Prof. Dr. Moestopo 47, Surabaya, Indonesia
[5]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Mulyorejo, Surabaya, Indonesia

*Corresponding author. Email: bsp49@fk.unair.ac.id

Abstract

BACKGROUND: Quercetin has been shown to alleviate and prevent atherosclerosis. However, its role in stabilizing atherosclerotic plaques to prevent plaque rupture remains unclear. Therefore, this study was conducted to investigate the effects of quercetin on stabilizing atherosclerotic plaques.

METHODS: Thirty-two Wistar rats were objected to a high-fat diet, along with an endothelial injury procedure conducted during the second week to create atherosclerotic plaque models. After six weeks, the subjects were randomly assigned to five groups consist of two control groups and three treatment groups treated with different quercetin dosages. Following the treatment, all subjects were euthanized to collect the left common carotid artery. The stability of the atherosclerotic plaques was evaluated by measuring the expression of matrix metalloproteinase-9 (MMP-9) using real-time polymerase chain reaction, assessing the activity of M1 and M2 macrophages along with the M1/M2 ratio using an enzyme-linked immunosorbent assay, and determining the maximum intima thickness through histopathological examination.

RESULTS: Quercetin significantly reduced the expression of MMP-9, increased the activity of M2 macrophages, and lowered the M1/M2 ratio at doses of 10 and 50 mg/kg. However, there was no effect on M1 macrophage activity or maximum intima thickness. Path analysis indicated that quercetin primarily enhanced atherosclerotic plaque stability by reducing MMP-9 expression ($p<0.001$) and subsequently enhancing M2 macrophage activity ($p=0.002$).

CONCLUSION: Quercetin administration significantly decreased the expression of MMP-9, enhanced the activity of M2 macrophages, and lowered the M1/M2 ratio at specific doses. These findings emphasize the significance of quercetin in stabilizing atherosclerotic plaques.

KEYWORDS: atherosclerotic plaque, quercetin, stability, Wistar rats

Introduction

Atherosclerosis is a complex, long-term inflammatory process that starts with the retention of lipoproteins in the artery walls. This is followed by the recruitment of inflammatory cells, leading to the formation of foam cells. Subsequent processes include apoptosis, necrosis, the proliferation of smooth muscle cells (SMCs), and the synthesis of the extracellular matrix. Collectively, these factors contribute to increased intimal thickness.(1) Additional pathological processes may occur during this

progression, including calcification, angiogenesis, arterial remodeling, rupture of the fibrous cap, and thrombosis. The stability of a plaque is crucial for preventing its rupture. Vulnerable plaques are characterized by a thin fibrous cap, microcalcifications, a large necrotic core, and significant infiltration of inflammatory cells, particularly macrophages, with minimal SMCs.(2)

The fibrous cap thinning occurs because of reduced SMCs and decreased collagen content, which naturally provide structural support. Additionally, macrophage infiltration further degrades the collagen-rich matrix of the fibrous cap. Macrophages express matrix metalloproteinase-9 (MMP-9) which actively degrades various extracellular matrices, including those found in the fibrous cap.(3) Macrophages play a dual role in plaque stability, depending on their subtype. They can be categorized into pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages. The balance between these two subtypes is critical for maintaining the stability of atherosclerotic plaques.(4,5)

Quercetin is a potent antioxidant recognized for its numerous therapeutic benefits. Various studies have confirmed its bioactive properties, showing effects such as lowering lipid levels, reducing blood pressure, and managing diabetes.(6-9) Research indicates that quercetin can alleviate atherosclerosis by suppressing oxidized-low-density lipoproteins-induced senescence in plaque macrophages and attenuating atherosclerosis by inhibiting the p38 mitogen-activated protein kinase/p16 pathway. (10) Additionally, another study suggests that regular consumption of dietary quercetin plays a crucial role in preventing atherosclerosis due to its regulatory effects on subunits of nicotinamide adenine dinucleotide phosphate oxidase. Quercetin metabolites likely exert an anti-atherosclerotic effect by reducing caveolin-1 expression in endothelial cells.(11)

However, up to date the role of quercetin in stabilizing atherosclerotic plaques to prevent plaque rupture remains unclear. Despite this, there is significant potential for quercetin to be developed as a stabilization agent for atherosclerotic plaques. Therefore, this study was conducted to investigate the effects of quercetin on the stabilization of atherosclerotic plaques by modulating MMP-9 expression, the activity of M1 and M2 macrophages, the M1/M2 ratio, and maximum intima thickness. While plaque formation occurs more readily in the aorta and proximal large vessels of experimental rats (12), the atherosclerotic plaque models in this study were developed in the carotid artery.

Methods

Study Design

This experimental study employed a post-test-only control group design to investigate the effects of quercetin on stabilizing atherosclerotic plaques. The parameters used to assess plaque stability included the expression of MMP-9, the activity of M1 and M2 macrophages, the M1/M2 ratio, and the maximum intima thickness. To achieve an alpha error of 0.05 and an estimated effect size of 0.03, a combined standard deviation of MMP-9 levels of 0.02, a desired statistical power of 90%, and anticipating a potential dropout rate of 20% based on preliminary research, the required sample size was determined to be 30 subjects. The study was conducted at the Stem Cell and Cancer Research (SCCR) animal laboratory in Semarang, Indonesia, from August 19 to October 30, 2024.

Animal Model and Ethical Considerations

The subjects were male white Wistar rats (*Rattus norvegicus*), aged 3 to 4 months and weighing at least 200 grams. The male sex was selected to minimize bias from hormonal influences such as estrogen, which can have a protective effect against atherosclerosis. These rats were obtained from the Animal Model Research Center, SCCR, in Semarang, Indonesia. They were housed in groups of 3 to 4 individuals per cage and had unlimited access to food and drinking water. The cages were maintained at a room temperature of 25 to 26°C, with a 12-hour light and dark cycle. Prior to the research treatments, all subjects underwent a 2-week acclimatization period on a standard diet. Any rats that lost more than 10% of their body weight were excluded from the study. The rats' body weight and behavioral changes were monitored and recorded weekly. If any of the rats became ill during the study, they received appropriate treatment from a veterinarian overseeing the research. All subjects were terminated at week 8, except for Group C1 which was euthanized early at week 6, using an intraperitoneal injection of a lethal dose of >200 mg/kg ketamine and >30 mg/kg xylazine. The protocol of this study was approved by The Animal Ethics Commission of the Faculty of Veterinary Medicine, Universitas Airlangga University (Approval No. 2.KEH.117.08.2024).

Induction of Atherosclerotic Plaque Model Using a High-Fat Diet and the Endothelial Injury Procedure

All subjects underwent treatment for atherosclerotic plaque model formation, which included a high-fat diet for the

duration of the study, along with an endothelial injury procedure conducted during the second week. The standard diet consisted of pellet-shaped animal feed known as BR A594K (PT. Charoen Pokphan Indonesia, Tbk, Demak Regency, Central Java, Indonesia). This feed has a calorific value of 320 kcal per 100 grams. In contrast, the high-fat diet was based on the composition used as described in previous study.(13) It included 68% of the standard diet, 20% Dutch Lady instant milk powder, 6% Krystal corn oil, and 6% Crispo ghee, resulting in a calorific value of 414 kcal per 100 grams. These ingredients were mixed and formed into livestock pellets.

This study adopted and modified a technique used in previous research that involved an injury balloon catheter and a wire.(14) The procedure was conducted in the second week using a 26-G intravenous catheter from GEA (Jiangsu Webest Medical Product, Xuyi, China). After a two-week high-fat diet, the catheter was inserted through a puncture in the proximal section of the left common carotid artery (LCCA) and advanced to the distal section. A pulling and pushing motion were performed three times to rub the entire LCCA (Figure 1). Throughout, all subjects received intraperitoneal anesthesia with ketamine and xylazine, which allowed for spontaneous breathing. After the procedure, 3 mL of 0.9% sodium chloride was injected subcutaneously to replace the volume lost due to bleeding.

Treatment Groups and Administration of Quercetin
After 6 weeks, subjects were randomly allocated into five groups (n=6 per group): Group C1, which underwent early termination and served as a reference model for atherosclerotic plaque formation; Group C2, which continued the high-fat diet for an additional 2 weeks; Group T1, which continued the high-fat diet and received quercetin at 10 mg/kg for 2 weeks; Group T2, which continued the high-fat diet and received quercetin at 50 mg/kg for 2 weeks; and Group T3, which continued the high-fat diet and received quercetin at 100 mg/kg for 2 weeks.

Quercetin (Sigma-Aldrich, St. Louis, MO, USA) was used in powder form under the product name Q4951. It has a molecular weight of 302.24 g/mol and a purity of 96%, as confirmed by high-performance liquid chromatography. The substance was administered once daily using a gavage probe with CMC-Na solvent as the carrier. Subjects in groups T1, T2, and T3 received quercetin for two weeks, starting in the sixth week of the study.
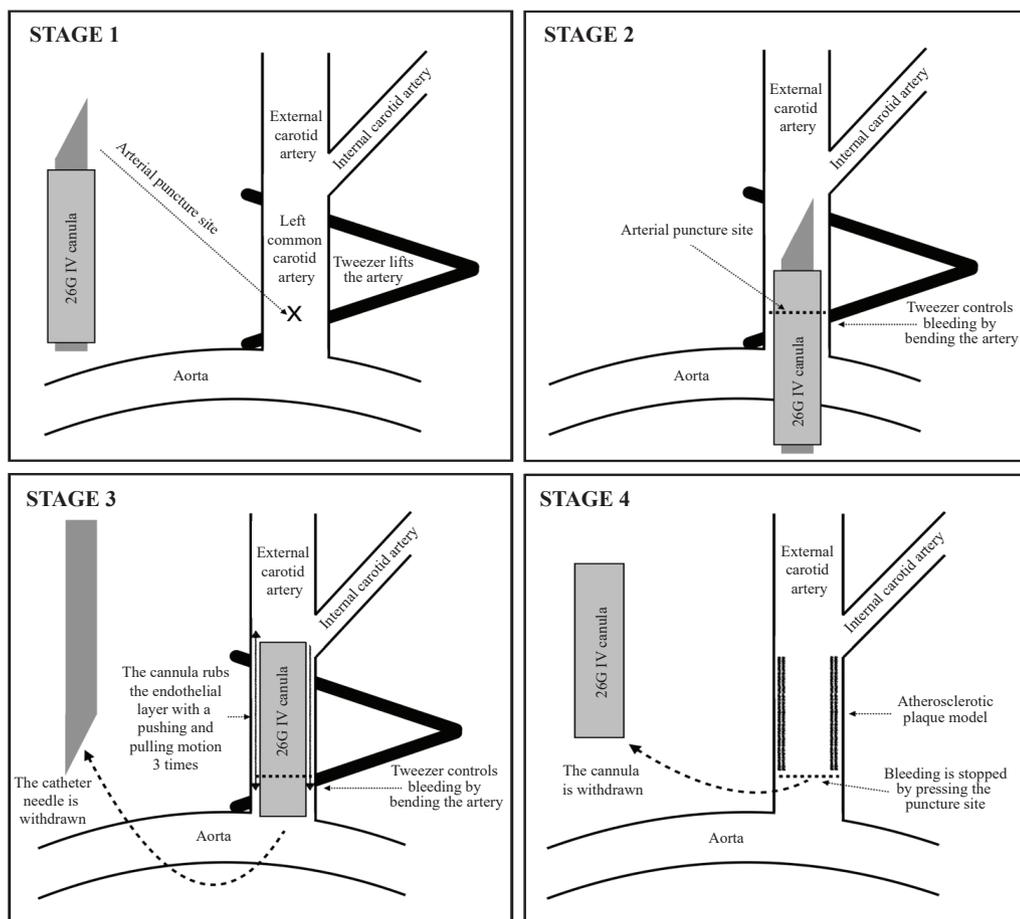

Figure 1. The endothelial injury techniques used in forming atherosclerotic plaque models.

Histopathological Sample Processing and the Measurement of Maximum Intima Thickness

The LCCA was rinsed with 0.9% sodium chloride and placed in a 10% neutral buffered formalin container. Histopathological preparations were then made using hematoxylin and eosin staining. The maximum intima thickness was measured at the sites exhibiting the most severe plaque pathology, with each measurement taken three times to calculate an average value. These measurements were conducted using ImageJ software developed by Wayne Rasband. A single-blinded anatomical pathologist assessed the histopathological tissue to ensure unbiased results.

RT-PCR and ELISA Assays to Measure the Expression of MMP-9, Activity of M1 and M2 Macrophages, and the M1/M2 Ratio

MMP-9 expression was analyzed using real time polymerase chain reaction (RT-PCR) with samples taken from LCCA tissue. RNA was isolated using Genezol (Geneaid Biotech, New Taipei City, Taiwan). cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix, which contains a gDNA remover (Toyobo, Osaka, Japan). For quantitative PCR, SensiFAST SYBR No-ROX Mix (Bioline Meridian Bioscience, Osaka, Japan) was utilized, along with primers sourced from Integrated DNA Technologies (Coralville, IA, USA). The forward primer sequence for MMP-9 is 5'-GAT CCC CAG AGC GTT ACT CG-3', and the reverse primer sequence is 5'-GTT GTG GAA ACT CAC ACG CC-3'. Non-research normal Wistar rats served as a reference group.

Macrophage activity was evaluated through enzyme linked immunosorbent assay (ELISA) using samples from the same tissue, measuring interleukin-1 beta (IL-1β) markers for M1 macrophages and interleukin-10 (IL-10) for M2 macrophages. Intracellular protein extraction was performed using a Radio Immunoprecipitation Assay Lysis Buffer (Servicebio, Wuhan, China). ELISA kits used were Rat IL-1β ELISA Kit (Catalog No: E-EL-R0012; Elabscience, Houston, TX, USA) and Rat IL-10 ELISA Kit (Catalog No: E-EL-R0016; Elabscience). To ensure unbiased results, all samples for analysis were packaged using a blinded method.

Statistical Analysis

All data obtained were statistically analyzed using SPSS Statistics version 29 for Mac (IBM Corporation, Armonk, NY, USA). Normally distributed data are presented as mean±SD, while non-normally distributed data are presented as median (Q1 - Q3). The normality of the data distribution was assessed using the Shapiro-Wilk normality test. The results were considered statistically significant with a *p*-value<0.05. The effect of quercetin administration was analyzed using a One-Way ANOVA test for normally distributed data, followed by either the Bonferroni or Games-Howell post hoc tests. For non-normally distributed data, the Kruskal-Wallis test was applied, followed by the Mann-Whitney post hoc test. Path analysis was performed using Structural Equation Modeling (SEM) with Smart Partial Least Squares (Smart PLS) version 3 for Mac (GmbH, Bönningstedt, Germany).

Results

LCCA Thickening as an Indicator of Atherosclerotic Model

The study included 32 Wistar rats, and none of the subjects dropped out due to weight loss. Based on the histopathological analysis, groups C2, T1, T2, and T3 exhibited LCCA thickening, indicating the formation of an atherosclerotic plaque model (Figure 2).

Quercetin Administration Affected the Expression of MMP-9, M2 Macrophage Activity, and M1/M2 Ratio

This study demonstrated that administering quercetin significantly reduced the expression of MMP-9 across all doses tested. Additionally, it increased M2 macrophage activity for all doses and lowered the M1/M2 ratio at 10 and 50 mg/kg doses. These effects were considered protective for the stability of atherosclerotic plaques. However, the effects of quercetin on M1 macrophage activity and maximum intima thickness were not assessed in this study (Table 1).

Reduced Expression of MMP-9 as the Most Significant Factor Contributing to the Stability of Atherosclerotic Plaques

The SEM path analysis conducted in this study included the variable of quercetin administration across groups C2, T1, T2, and T3. All study parameters showed strong convergent validity, discriminant validity, and reliability. The path analysis demonstrated that quercetin administration affected atherosclerotic plaque stability by enhancing M2 macrophages activity (r=0.479, *p*=0.002), decreasing the M1/M2 ratio (r=-0.210, *p*<0.001), increasing M1 macrophages activity (r=0.369, *p*=0.021), and reducing MMP-9 expression (r=-0.810, *p*<0.001) (Figure 3). These effects might be either direct or indirect through other interrelated pathways. Indirect effects were identified through the pathway of quercetin administration, leading
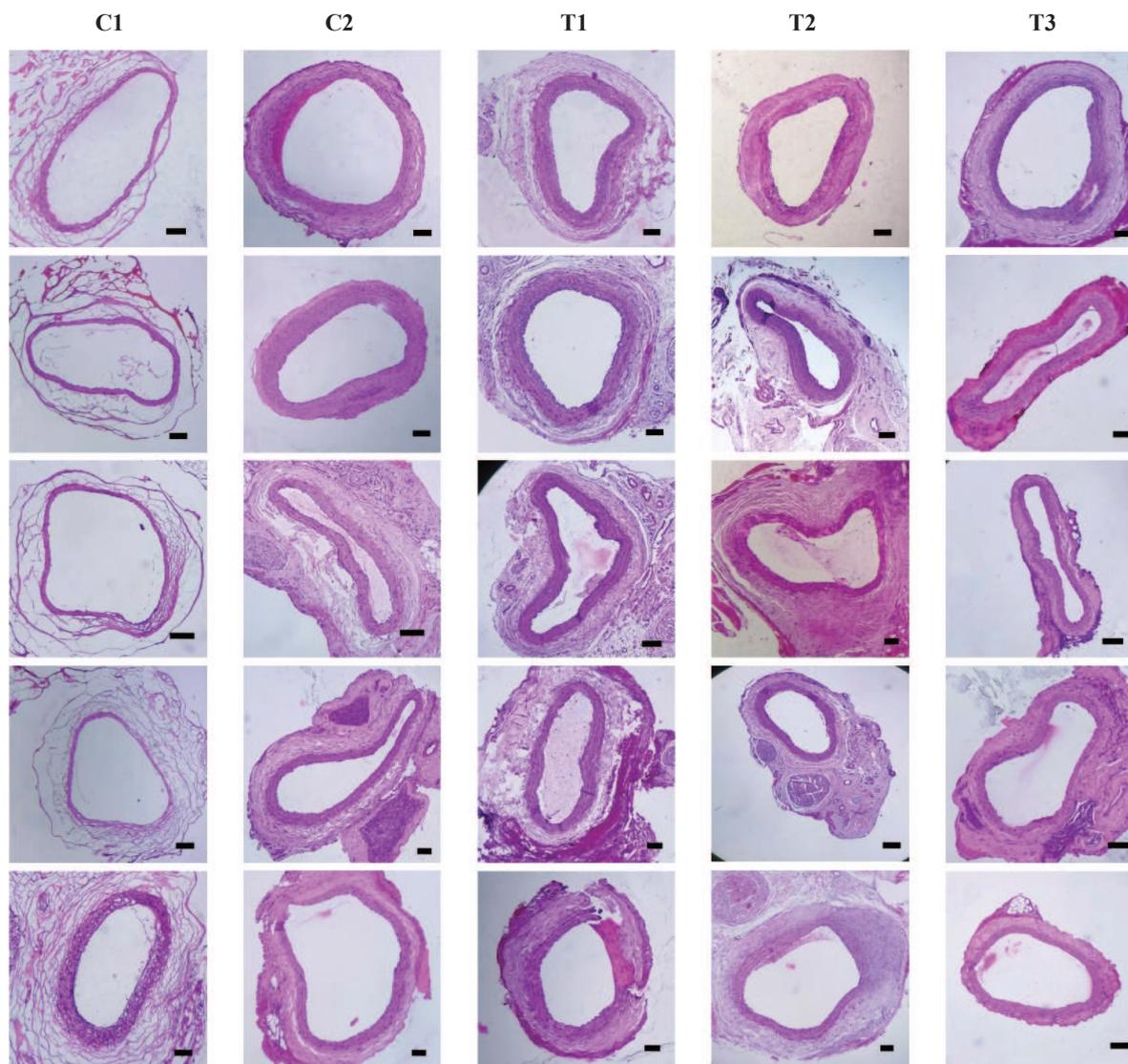
Figure 2. The histopathological findings of the LCCA were presented for each group. After 8 weeks of treatment for endothelial injury, LCCA thickening was observed, indicating the formation of an atherosclerotic plaque model. However, there was no significant difference in maximum intima thickness among groups C2, T1, T2, and T3. The groups are defined as C1: the group that underwent early termination at week 6; C2: the control group that did not receive quercetin; T1: the group that received quercetin at 10 mg/kg; T2: the group that received quercetin at 50 mg/kg; and T3: the group that received quercetin at 100 mg/kg. All histopathological images are shown at 100x magnification. Black bar: 100 μm.

to M2 macrophage activity and, subsequently, the M1/M2 ratio (p=0.007) (Table 2). The total effect was primarily driven by the influence of quercetin on MMP-9 expression (p<0.001), followed by its effects on M2 macrophages activity (p=0.002) and M1 macrophages activity (p=0.021) (Table 3).

Discussion

The effect of quercetin on reducing MMP-9 expression in this study is a significant finding in atherosclerosis research,

as it represents the most prominent effect compared to other unstable atherosclerotic plaque parameters. There is growing interest in MMP-9 as a therapeutic target, particularly for patients with coronary heart disease. One previous study found that MMP-9 levels were higher in unstable coronary artery plaques compared to stable ones. (15) Many metabolic and cardiovascular research studies have shown that quercetin enhances anti-atherosclerotic effects and inhibits adipogenesis.(16-19) However, there is still limited research on the effect of quercetin on MMP-9 in the context of atherosclerotic plaque stabilization. To date, the benefits of quercetin in inhibiting MMP-9 activity

Table 1. Effect of quercetin administration on atherosclerotic plaque stability.

Parameters	Mean±SD or Median (Q1-Q3)	*p*-value	Post hoc *p*-value
MMP-9 Expression (Ct value)			
Group C1	1.35±0.19	<0.001[2]	C1-T1 0.003[3]
Group C2	1.64 (1.25-1.75)		C1-T2 0.004[3]
Group T1	0.02±0.01		C1-T3 0.004[3]
Group T2	0.02±0.01		C2-T1 0.002[3]
Group T3	0.01±0.01		C2-T2 0.003[3]
M1 Macrophage Activity (pg/mL)			
Group C1	14.71±0.62	0.592[2]	
Group C2	14.83±0.66		
Group T1	14.73±1.00		
Group T2	15.30±1.03		
Group T3	15.64 (14.33-19.43)		
M2 Macrophage Activity (pg/mL)			
Group C1	12.77±0.45	<0.001[2]	C1-T1 0.003[3]
Group C2	13.02±0.51		C1-T2 0.004[3]
Group T1	22.93±4.54		C1-T3 0.004[3]
Group T2	19.89 (18.46 - 24.42)		C2-T1 0.002[3]
Group T3	20.80±3.53		C2-T2 0.003[3]
M1/M2 Ratio			
Group C1	1.15±0.05	<0.001[1]	C1-T1 <0.001[4]
Group C2	1.14±0.07		C1-T2 <0.001[4]
Group T1	0.66±0.08		C2-T1 <0.001[4]
Group T2	0.72±0.11		C2-T2 <0.001[4]
Group T3	0.85±0.30		
Maximum Intima Thickness (µm)			
Group C1	3.92±1.65	0.003[2]	C1-C2 0.032[3]
Group C2	7.55 (4.57-50.35)		C1-T1 0.003[3]
Group T1	37.47±25.94		C1-T2 0.006[3]
Group T2	13.38 (10.37-98.34)		C1-T3 0.010[3]
Group T3	8.27 (5.42-17.07)		T1-T3 0.032[3]

[1]One-Way Anova, [2]Kruskal-Wallis, [3]Mann-Whitney, [4]Games-Howell, the post hoc results presented are only with *p*<0.05.

have been more clearly demonstrated in non-atherosclerotic studies. For example, quercetin was able to reduces tumor necrosis factor-alpha (TNF-α)-induced MMP-9 expression in the human gastric epithelial cells, further showcasing its anti-inflammatory properties.(20)

Quercetin inhibits MMP-9 activity through at least two pathways. The first is an extracellular pathway, which exerts an inhibitory effect on the TNF receptor (TNFR) located on the cell surface.(21) The second is an intracellular pathway inhibiting nuclear factor-kappa B (NF-κB) activity. (20,21) One study supports the inhibitory effect of quercetin on TNF-α based on an in silico model using Molecular Dynamics simulations. Their findings revealed the lowest binding energy of the protein-ligand complexes at 9.34 kcal/mol.(22) In another approach, a different study examined

how quercetin suppresses lipopolysaccharide-induced TNF-α production. This study also utilized an in-silico model and demonstrated that quercetin binds to Akt within the PI3K/Akt signaling pathway, reporting a binding energy of 8.9 kJ/mol. This indicates a strong affinity between quercetin and Akt.(23)

This study demonstrates that quercetin enhances M2 macrophage activity while decreasing the M1/M2 ratio, although it does not inhibit M1 macrophage activity. Other research indicates that quercetin can significantly reduce the expression of M1 macrophage markers, including IL-6, TNF-α, and IL-1β. Additionally, quercetin has been shown to suppress the production of reactive oxygen species (ROS) in microglia. On the other hand, quercetin also promotes the expression of M2 macrophage markers such as IL-
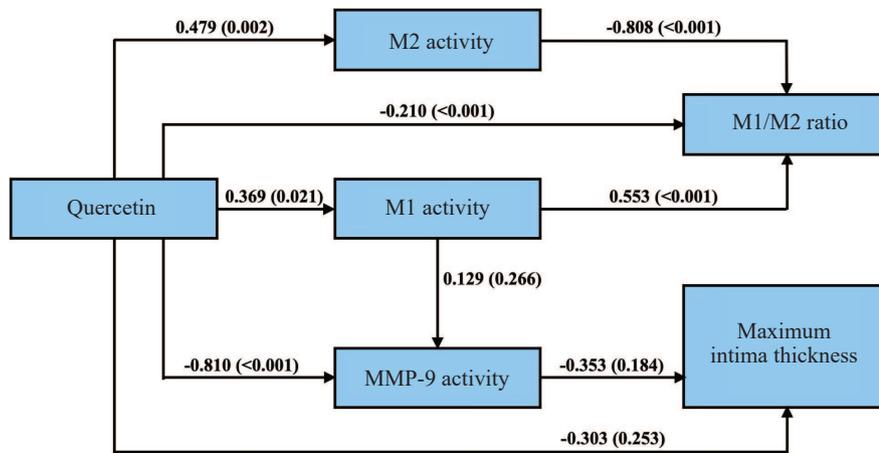
Figure 3. The SEM path analysis using Smart PLS presents both path coefficients and significance values. The value presented as r (*p*-value).

10 and endogenous antioxidants through the adenosine monophosphate-activated protein kinase and Akt signaling pathways.(24)

Various publications indicate that antioxidants, such as quercetin, exhibit dual effects: they can inhibit the activity of M1 macrophages while promoting the activity of M2 macrophages. However, the inhibitory effect of antioxidants on M1 macrophage activity is more pronounced in the context of malignancy. In the contrary, the enhancement of M2 macrophage activity is more commonly observed in cases of chronic inflammation (25), as indicated by the results of this study. One previous study demonstrated that quercetin can reduce oxidative stress and inflammation, promoting a shift in macrophage polarization towards the anti-inflammatory M2 phenotype through the modulation of the sirtuin 1/peroxisome proliferator-activated receptor gamma coactivator 1-alpha (SIRT1/PGC-1α) signaling pathway.(26) Additionally, quercetin has been shown to induce a shift in M1 macrophage polarization to other phenotypes while decreasing the expression of pro-inflammatory cytokines and enhancing the activity of antioxidant enzymes.(27)

This study demonstrated that quercetin reduces MMP-9 expression and enhances M2 macrophage activity, with no significant difference observed based on the dosage. Meanwhile, both low and medium doses of quercetin affected the M1/M2 activity ratio. Antioxidants can have opposing effects, functioning as both antioxidants and pro-oxidants, depending on the dosage and the complex biochemical pathways involved.(28) Excessive amounts of antioxidants may disrupt ROS physiological levels and impair normal cell function.(29) Therefore, understanding and investigating the effects of antioxidants relative to their dosage is essential for studies examining their therapeutic benefits.

Several factors may explain the lack of significant effects of quercetin on M1 macrophage activity. First, the impact of quercetin is highly dependent on the dosage. Therefore, negative or diminished effects could result from inadequate dosage exposure rather than an absence of pharmacological effect. Some *in vivo* studies have utilized relatively high systemic doses to showcase a polarizing effect.(30,31) To validate this hypothesis, further research using a broader range of dosages is necessary. Second, the formulation and combination of quercetin may play a crucial role in optimizing its anti-inflammatory effects. In certain studies, significant anti-M1 effects were only observed when quercetin was administered through nanoparticles, collagenase-decorated carriers, or sustained-release matrices. This suggests that seemingly negative results may stem from pharmacokinetic or delivery limitations rather than a lack of inherent activity.(32,33)

Table 2. Indirect specific effects of quercetin on atherosclerotic plaque stability.

Indirect Specific Effects	*p*-value
Quercetin → M1 macrophages → MMP-9 expression	0.397
Quercetin → M1 macrophages → MMP-9 expression → maximum intima thickness	0.596
Quercetin → MMP-9 expression → maximum intima thickness	0.217
Quercetin → M1 macrophages → M1/M2 ratio	0.072
Quercetin → M2 macrophages → M1/M2 ratio	0.007*

*Significant, *p*<0.05.

Table 3. Total effects of quercetin on atherosclerotic plaque stability.

Total Effects	*p*-value
Quercetin administration on MMP-9 expression	<0.001*
Quercetin administration on M2 macrophages	0.002*
Quercetin administration on M1 macrophages	0.021*
Quercetin administration on M1/M2 ratio	0.072
Quercetin administration on maximum intima thickness	0.838

*Significant, $p<0.05$.

Currently, there is no evidence demonstrating the histopathological effects of quercetin in inhibiting the progression of atherosclerotic plaque. The closest related research has investigated how quercetin inhibits the proliferation of vascular SMCs through the NF-κB p65/microRNA-17/RB pathway. This mechanism can potentially reduce the risks of restenosis and atherosclerosis.(34) Other studies have primarily shown quercetin's effects at the biomolecular level, such as its ability to inhibit ROS (35,36), and suppress the activity of pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α (37), which may also help slow down the progression of atherosclerotic plaque.

The progression of histopathological changes in atherosclerotic plaques is a long process that begins with an increase in intimal thickness, eventually forming a fibrous cap.(38) However, the development of a fibrous cap is not the sole factor contributing to acute thrombosis. Instead, the composition of the plaque plays a more significant role in the occurrence of thrombosis in the vascular system than just the thickness of the intima or the degree of arterial stenosis. In addition to plaque rupture, the erosion of atherosclerotic plaques is also a major cause of acute arterial thrombosis, particularly in cases of acute coronary syndrome (ACS), even when the arteries show only minor lesions.(39) Pathological processes at the genomic level occur much earlier than the histopathological changes and play a crucial role in plaque stability, which is primarily influenced by inflammatory markers such as the polarization of M1 macrophages and M2 macrophages and the activity of MMP-9.(40)

This study has several limitations that should be addressed in future research. First, the impact of quercetin on histopathological changes cannot be adequately assessed with the experimental treatment duration of 8 weeks, as observing these changes in atherosclerotic plaques typically requires a longer timeframe. Additionally, the age of the experimental animals and the research deadlines further constrain the study's findings. Lastly, this research only examined three different doses of quercetin, which means it did not establish the optimal dose for stabilizing atherosclerotic plaques in Wistar rats.

Conclusion

This study demonstrates that quercetin administration significantly decreased the expression of MMP-9, enhanced the activity of M2 macrophages, and lowered the M1/M2 ratio at specific doses. Pathway analysis revealed that the most prominent effect was attributed to the reduction in MMP-9 expression. These findings emphasize the significance of quercetin in stabilizing atherosclerotic plaques.

Acknowledgments

Authors Contribution

RE was involved in concepting and planning the research, RE, BSP, M, and BU performed the data acquisition/collection, RE, BSP, M, BU, W and YHO calculated the experimental data and performed the analysis, RE, BSP, M, BU, W and YHO drafted the manuscript and designed the figures, RE, BSP, M, BU, W and YHO aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

Conflict of Interest

All authors declare that they have no conflicts of interest.

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