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Investigating the therapeutic potential of esculetin in mitigating myocardial infarction-associated gut dysbiosis through targeted NLRP3 gene; an experimental animal study

Zahraa Jabbar Mujbel[1*], Gaith Ali Jasim[2], Huda Jaber Waheed[1]

[1]Department of Pharmacology and Toxicology, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq
[2]Department of Pharmacology, Al-Bayan University, Baghdad, Iraq

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ABSTRACT

Introduction: Myocardial infarction (MI) is a major cause of death and illness. Gut dysbiosis and NLR family pyrin domain containing 3 (NLRP3) inflammasome worsen post-MI inflammation and metabolic issues. Esculetin, an anti-inflammatory antioxidant, may help by modulating NLRP3 and restoring metabolic balance.

Objectives: This study aims to investigate the therapeutic effect of esculetin on MI-induced gut dysbiosis via NLRP3 inflammasome and inflammatory/metabolic markers in rats.

Materials and Methods: In this experimental study, thirty male Wistar albino rats were randomized into five groups (n=6) for a study period of 21 days. MI was induced in all but the control group by subcutaneous injection of isoproterenol (100 mg/kg) on two consecutive days. From day three to day 21, three treatment groups received daily oral doses of esculetin at 20, 40, or 60 mg/kg, while control and MI-only groups received standard chow and water or isoproterenol alone, respectively. On day 22, rats were anesthetized, and blood was collected for serum biomarker analysis, including trimethylamine N-oxide (TMAO), short-chain fatty acids (SCFAs), and interleukin-1 beta (IL-1β), while intestinal tissues were processed for NLRP3 gene expression analysis. Biomarker parameters and NLRP3 gene expression were compared across the five groups.

Results: Isoproterenol elevated TMAO and NLRP3 in rats. Esculetin normalized TMAO at all doses, with no dose-related differences. Esculetin 20 mg/kg normalized NLRP3, with higher doses partially effective. Isoproterenol increased IL-1β, dose-dependently reduced by esculetin, without significant dose-group differences. SCFA analysis showed healthy controls differed from all treatments. Isoproterenol animals matched low-dose esculetin only, with esculetin showing dose-dependent SCFA effects between low and higher doses, which were similar.

Conclusion: Esculetin (20 mg/kg) showed anti-inflammatory and metabolic benefits in isoproterenol-induced conditions, normalizing NLRP3 and TMAO similar to controls and significantly reducing IL-1β. Lower esculetin doses appear therapeutically effective for inflammation and metabolic dysregulation, and no need for higher doses.

Original

Implication for health policy/practice/research/medical education:
In an animal experimental study, esculetin demonstrated optimized therapeutic efficacy at 20 mg/kg for managing NLR family pyrin domain containing 3 (NLRP3)-driven inflammatory conditions, effectively normalizing NLRP3 and trimethylamine N-oxide levels while reducing interleukin-1 beta to near-healthy ranges. Higher doses may be reserved for cases requiring incremental interleukin-1 beta (IL-1β) or short-chain fatty acids (SCFAs) modulation, though without significant superiority over intermediate doses, suggesting a plateau effect. These findings advocate prioritizing lower doses to minimize potential side effects while maintaining anti-inflammatory and metabolic benefits, particularly in pathologies like myocardial infarction (MI) or gut dysbiosis linked to NLRP3 activation. Esculetin's dual action on inflammasome suppression and SCFAs restoration positions is as a promising adjunct therapy.

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***Corresponding author**: Zahraa Jabbar Mujbel, Email: zahraa1996j@gmail.com

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide. CVD refers to a group of conditions that affect the heart and blood vessels, including coronary heart disease (such as heart attacks), stroke, heart failure, and other disorders of the circulatory system (1). When blood flow to the heart muscle is reduced or blocked, it can cause heart attacks, such as myocardial infarction (MI) (2). After a heart attack, the heart undergoes major changes in its muscle tissue, which can weaken its ability to pump blood effectively (3,4). Emerging evidence highlights that renal dysfunction exacerbates post-MI remodeling by amplifying neurohormonal activation, uremic toxin accumulation, and systemic inflammation, ultimately accelerating heart failure progression (5,6). In addition to heart tissue death caused by an imbalance between the heart's oxygen supply and demand, the body's inflammatory response also plays a key role in the development of a MI (7). In rat models, MI was induced using isoproterenol, a beta-adrenergic agonist that triggers severe myocardial stress and leads to infarct-like necrosis of the heart muscle (8). Research demonstrates that this isoproterenol-induced injury closely mimics the pathological features of human MI, including oxidative stress, inflammation, and structural changes in the myocardium (8,9). Gut microbiota influences coronary heart disease pathophysiology, evidenced by a clear correlation between microbiota composition and MI severity in rats. Microbiota likely contribute through maintaining intestinal barrier integrity, modulating immune responses, preventing pathogen invasion, and affecting nutrient absorption and metabolism (10,11). Despite the well-established link between diet and CVD, the role of the gut microbiota has often been overlooked (12,13).

Coumarins are a class of organic compounds that have recently attracted significant attention because of their diverse biological activities (14). Both coumarin itself and its derivatives, such as esculetin (6,7-dihydroxy coumarin), exhibit a wide range of beneficial effects, including antiviral, antifungal, anti-inflammatory, antitumor, antioxidant, and antibacterial properties (15). Coumarins, historically recognized for their broad pharmacological properties, show promise in reducing CVD risk, with studies linking higher intake to improved outcomes (16). Their effects may stem from interactions between gut microbiota and host immunity, which jointly regulate intestinal inflammation and maintain gut tissue homeostasis by balancing immune responses, nutrient metabolism, and pathogen defense (17). Despite coumarins' documented anti-inflammatory and cardioprotective potential, few in vivo studies have explored how their derivatives specifically influence gut inflammation, leaving a critical gap in understanding their full therapeutic scope (18). In this study, we aim to evaluate the effects of coumarin derivatives on intestinal inflammation and to investigate their role in modulating the gut microbiome in a rat model experimental study.

Objectives

The objective of this study is to evaluate the therapeutic potential of esculetin in mitigating gut dysbiosis associated with MI by targeting the NLR family pyrin domain-containing 3 (NLRP3) inflammasome pathway. Specifically, the study aims to determine whether esculetin administration can restore gut microbial balance, reduce pro-inflammatory markers such as trimethylamine N-oxide (TMAO) and interleukin-1 beta (IL-1β), and suppress NLRP3 expression in a rat model of isoproterenol-induced MI. By assessing changes in serum gut metabolites, inflammatory activity, and NLRP3 expression across different treatment groups, the research seeks to elucidate the mechanistic link between esculetin's anti-inflammatory effects and its role in improving both gut and cardiac health following myocardial injury.

Materials and Methods

Study design and samples

This experimental animal study was conducted at the animal house of the college of pharmacy, Mustansiriya University, over a 21-day treatment period, with biochemical, histological, and genetic analysis performed at 3 weeks. Thirty male Wistar albino rats (aged 8 weeks, initial weight 150–180 g) were acclimatized for 10 days under standardized conditions (12-hour light/dark cycle, 22–24 °C, 50%–60% humidity) until reaching a target weight of 200–230 g. Rats were randomized into five experimental groups (n=6/each group) for intervention. Post-acclimatization, groups received daily treatments per protocol, with body weight and health parameters monitored throughout the study.

Animal preparation

Thirty male Wistar albino rats, with no prior treatments, were obtained from the animal house at the college of pharmacy, Mustansiriya University. All procedures were conducted following the ethical guidelines established by the college's animal care committee. The rats were housed in spacious, quiet, temperature-controlled cages with unrestricted access to food and water. To ensure their well-being and acclimatization, the animals were maintained in a healthy state for three weeks before the study under stable environmental conditions, including a 12-hour light/dark cycle and regulated temperature. An air ventilation system was used to maintain optimal air quality, adjustable as needed for environmental changes. Before beginning the experimental procedures, the rats were given a ten-day acclimation period. At the start of the study, their body weights ranged from 200 to 250 g. Before the start of the study, the animals were housed under controlled conditions with a constant temperature of 22 ± 2 °C and a 12-hour light/dark cycle. They had free access

to standard chow and tap water at all times and were kept in well-maintained cages measuring 20×25×35 cm.

Treatment group classification

The animals were divided into five groups (n=6 per group) for the study. Group 1 served as the healthy control and received only standard chow and water. Group 2 was the isoproterenol-induced group, in which rats were administered isoproterenol at a dose of 100 mg/kg dissolved in normal saline, given subcutaneously once every 24 hours for the first two consecutive days. Groups 3, 4, and 5 followed the same isoproterenol induction protocol as group 2, but starting on day 3 to 21, they received esculetin orally once daily at doses of 20 mg/kg, 40 mg/kg, and 60 mg/kg, respectively, each dissolved in 1 mL of normal saline.

Experimental design

Myocardial infarction was induced in the treatment groups by subcutaneous injection of 100 mg/kg isoproterenol dissolved in normal saline, administered once daily for the first two consecutive days (19). In the esculetin treatment groups (20, 40, and 60 mg/kg), rats received daily oral doses of esculetin from day 3 to day 21. On day 22, after a fasting period, rats were anesthetized via intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) until loss of corneal and toe-pinch reflexes (20). Blood was collected via cardiac puncture using a 5 mL syringe, and serum was separated by allowing samples to clot at room temperature for 15 minutes, followed by centrifugation (3000 rpm for 15 minutes) (21). The resulting serum was aliquoted into microcentrifuge tubes and stored at -20 °C until biomarker analysis, adhering to standard laboratory protocols.

Data collection (Biomarker measurements and NLRP3 expression)

Serum biomarkers, including the metabolic parameters TMAO and short-chain fatty acids (SCFAs), along with the inflammatory cytokine IL-1β, were quantified using enzyme-linked immunosorbent assay (ELISA) techniques. Concurrently, NLRP3 gene expression levels in intestinal tissues were assessed via quantitative reverse transcription polymerase chain reaction (RT-qPCR). This process involved homogenizing intestinal samples, extracting total RNA using TRIzol reagent, and synthesizing complementary DNA (cDNA) via reverse transcription. RT-qPCR amplification was performed using SYBR Green master mix, with GAPDH serving as the endogenous control for normalization. Cycle threshold (Ct) values were analyzed using the ΔΔCt method to determine relative NLRP3 expression.

Outcomes

The outcomes of this study include assessing normalization of serum TMAO levels, IL-1β, restoration of SCFA profiles, and suppression of intestinal NLRP3 gene expression in esculetin-treated rats compared to isoproterenol-induced controls and the evaluation of dose-response relationships of esculetin on these biomarkers. These outcomes support esculetin's therapeutic potential in mitigating MI-associated gut dysbiosis through targeted modulation of the NLRP3 inflammasome pathway.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics v27 (IBM Corp., USA). Data normality was confirmed via the Shapiro-Wilk test. Both parametric and non-parametric approaches were initially applied, but identical *P* values across methods led to the selection of parametric tests for their greater accuracy in hypothesis validation. Intergroup differences were evaluated using one-way analysis of variance (ANOVA), with post hoc Scheffé test to pinpoint specific variations of the levels of TMAO, SCFA, IL-1β, and NLRP3 expression between the five study groups, including healthy rats, the isoproterenol-induced group, and three esculetin dosage groups of 20, 40, and 60 mg/kg. A significance threshold of $P<0.05$ was applied uniformly, ensuring robust inference of treatment effects.

Results

This study included 30 male Wistar albino rats, about 8 weeks old and weighing between 200 and 230 g, that had not received any previous treatments. The rats were randomly assigned to five equal groups (6 rats per group): a healthy control group, an isoproterenol-induced group, and three groups treated with esculetin at doses of 20, 40, and 60 mg/kg. The analysis of serum biomarkers across treatment groups revealed significant variation in gut metabolites, inflammatory activity, and NLRP3 expression, with all comparisons demonstrating statistically significant differences. The TMAO levels were markedly elevated in isoproterenol-induced rats compared to healthy controls, while esculetin treatment at increasing doses progressively reduced TMAO concentrations, approaching baseline values at the highest dose. Conversely, SCFAs were severely depleted in the induced group but showed dose-dependent restoration with esculetin administration. NLRP3 expression and IL-1β levels, both indicators of inflammasome activation, were substantially elevated post-induction. Esculetin treatment suppressed NLRP3 expression and IL-1β secretion in a dose-responsive manner, with near-complete normalization observed at higher doses (Table 1 and Figure 1).

The results indicated that for TMAO, a statistically significant difference was observed between healthy control rats and the isoproterenol-induced group, while no significant differences were detected between healthy controls and any of the esculetin treatment groups, regardless of dosage. Notably, all three esculetin treatment concentrations (20 mg/kg, 40 mg/kg, and 60

Table 1. The distribution of serum gut metabolites, inflammatory activity, and NLRP3 expression among rats' treatment groups

Biomarker	Treatment Group					*P* value
	Healthy rats Mean ± SD	Isoproterenol-induced Mean ± SD	Esculetin dosage (20 mg/kg) Mean ± SD	Esculetin dosage (40 mg/kg) Mean ± SD	Esculetin dosage (60 mg/kg) Mean ± SD	
TMAO (IU/L)	131.28 ± 16.94	573.03 ± 208.98	223.09 ± 51.78	207.69 ± 29.77	148.45 ± 30.00	<0.001*
SCFA (IU/L)	11.78 ± 1.93	0.03 ± 0.01	1.91 ± 1.21	5.91 ± 1.70	8.39 ± 1.33	<0.001*
NLRP3 (fold)	1.00 ± 0.14	2.19 ± 0.40	0.71 ± 0.65	0.09 ± 0.06	0.06 ± 0.05	<0.001*
IL-1β (IU/L)	103.23 ± 20.20	728.73 ± 134.75	256.41 ± 85.65	190.25 ± 36.77	128.34 ± 33.51	<0.001*

SD, Standard deviation; TMAO, Trimethylamine N-oxide; SCFA, Short-chain fatty acids; IL-1β, Interleukin-1 beta; NLRP3, NLR family pyrin domain containing 3. *One-way ANOVA.

mg/kg) demonstrated significant ameliorative effects when compared to the isoproterenol-induced group, suggesting potential therapeutic benefits. No significant differences in TMAO levels were observed between the various esculetin dosage groups, indicating similar efficacy across the tested concentrations. Regarding SCFA levels, healthy control rats exhibited significantly different values compared to all other experimental groups. The

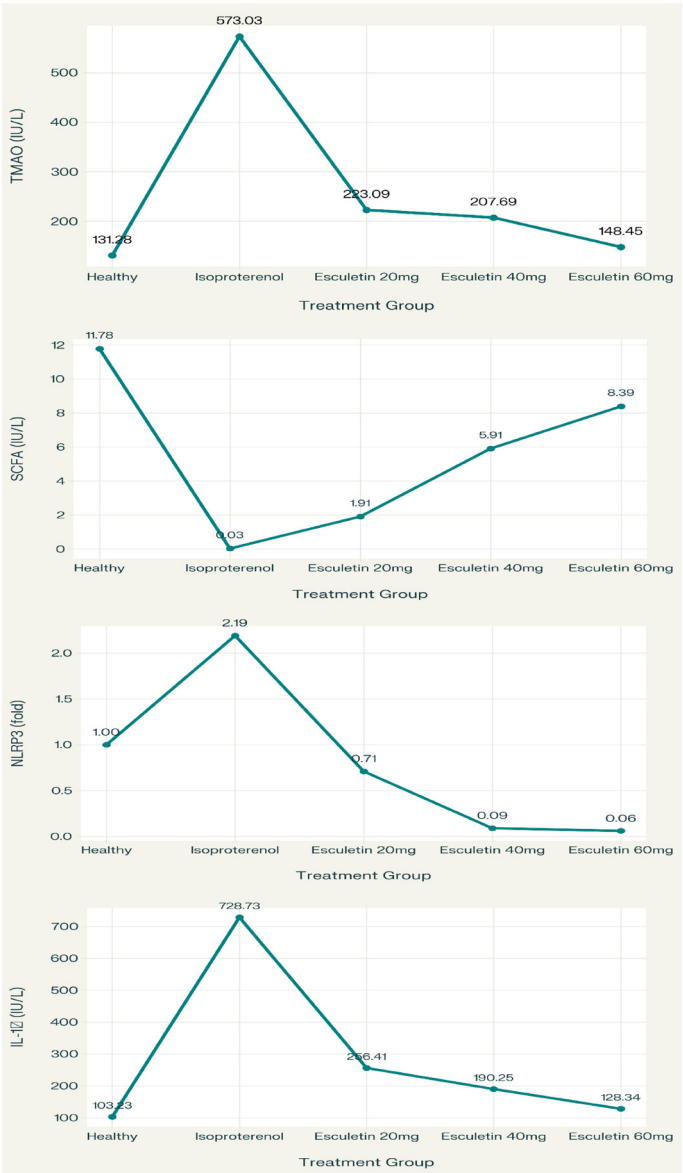

Figure 1. Serum TMAO, SCFA, IL-1β, and NLRP3 levels across healthy, isoproterenol-induced, and esculetin-treated rat groups. TMAO, Trimethylamine N-oxide; SCFA, Short-chain fatty acids; IL-1β, Interleukin-1 beta; NLRP3, NLR family pyrin domain containing 3.

isoproterenol-induced group showed no significant difference compared to the low-dose esculetin group (20 mg/kg), but demonstrated significant differences when compared to higher esculetin doses. Additionally, significant dose-dependent effects were observed among esculetin treatments, with significant differences between the 20 mg/kg group and both higher dosage groups, while the two higher dosage groups (40 mg/kg and 60 mg/kg) did not differ significantly from each other (Table 2).

The comparative analysis revealed significant differences in NLRP3 and IL-1β expression across experimental groups. For NLRP3, isoproterenol-induced rats exhibited a marked increase compared to healthy controls, which was significantly decreased by all esculetin doses. Notably, esculetin at 20 mg/kg showed no significant difference from healthy controls, while higher doses (40 and 60 mg/kg) demonstrated intermediate reductions. Direct comparisons between isoproterenol-induced and esculetin-treated groups confirmed dose-dependent suppression of NLRP3, with all doses achieving statistical significance. However, pairwise comparisons among esculetin doses revealed no significant differences, suggesting no linear dose-response relationship. For IL-1β, isoproterenol induction caused a pronounced elevation relative to healthy controls, with esculetin at 20 mg/kg partially mitigating this effect, while higher doses showed progressively greater reductions. All esculetin doses significantly lowered IL-1β compared to isoproterenol alone, though inter-dose comparisons lacked statistical significance. Both biomarkers displayed parallel trends, with esculetin effectively countering isoproterenol-induced increases, but without significant incremental benefits between intermediate and high doses (Table 3).

Discussion

This study assesses the effect of esculetin on intestinal dysbiosis in isoproterenol-induced MI in male rats. Notably, it addresses dysbiosis resulting from isoproterenol induction, demonstrating that isoproterenol administration exacerbates both cardiac tissue destruction (via increased inflammation) and intestinal dysbiosis. Esculetin treatment improved intestinal health by modulating intestinal biomarkers and reducing inflammation.

The TMAO levels can increase due to diet, altered gut microbiota composition, dysbiosis, and impaired gut barrier function (22). Villa-Rodriguez et al suggest the gastrointestinal tract as a key site for polyphenol-mediated cardioprotection (21). Besides their direct health benefits, dietary polyphenols influence gut microbiota activity and composition (20). In our study, the results demonstrated that isoproterenol administration led to a significant increase in serum TMAO levels in the induction group compared to healthy control rats. However, treatment with esculetin at all three tested doses significantly reduced serum TMAO levels, bringing them closer to normal values. This finding aligns with prior findings demonstrating coumarins' capacity to modulate gut microbiota and mitigate TMAO-driven cardiovascular pathology. For instance, Li et al found that Shenfu injection, a Traditional Chinese medicine containing coumarin-like compounds, similarly reduced serum TMAO in isoproterenol-treated rats by restoring bacteroidota abundance and suppressing

Table 2. Comparative analysis of TMAO and SCFA across experimental groups

Experimental group			Mean difference	*P* value*
First	Second			
TMAO (IU/L)	Healthy control rats	Isoproterenol-induced	441.75	<0.001
		Esculetin (20 mg/kg)	91.81	0.630
		Esculetin (40 mg/kg)	76.40	0.770
		Esculetin (60 mg/kg)	17.17	0.999
	Isoproterenol-induced	Esculetin (20 mg/kg)	349.94	<0.001
		Esculetin (40 mg/kg)	365.34	<0.001
		Esculetin (60 mg/kg)	424.57	<0.001
	Esculetin (20 mg/kg)	Esculetin (40 mg/kg)	15.40	0.999
		Esculetin (60 mg/kg)	74.63	0.785
	Esculetin (40 mg/kg)	Esculetin (60 mg/kg)	59.23	0.893
SCFA (IU/L)	Healthy control rats	Isoproterenol-induced	11.74	<0.001
		Esculetin (20 mg/kg)	9.86	<0.001
		Esculetin (40 mg/kg)	5.86	<0.001
		Esculetin (60 mg/kg)	3.38	0.008
	Isoproterenol-induced	Esculetin (20 mg/kg)	1.88	0.281
		Esculetin (40 mg/kg)	5.87	<0.001
		Esculetin (60 mg/kg)	8.36	<0.001
	Esculetin (20 mg/kg)	Esculetin (40 mg/kg)	3.99	0.002
		Esculetin (60 mg/kg)	6.47	<0.001
	Esculetin (40 mg/kg)	Esculetin (60 mg/kg)	2.48	0.083

TMAO, Trimethylamine N-oxide; SCFA, Short-chain fatty acids. *One-way ANOVA followed by post hoc Scheffe test.

Table 3. Comparative analysis of NLRP3 and IL-1β across experimental groups

Experimental group			Mean difference	*P* value*
First		Second		
NLRP3 (fold)	Healthy control rats	Isoproterenol-induced	1.19	<0.001
		Esculetin (20 mg/kg)	0.28	0.731
		Esculetin (40 mg/kg)	0.91	0.004
		Esculetin (60 mg/kg)	0.94	0.003
	Isoproterenol-induced	Esculetin (20 mg/kg)	1.47	<0.001
		Esculetin (40 mg/kg)	2.10	<0.001
		Esculetin (60 mg/kg)	2.13	<0.001
	Esculetin (20 mg/kg)	Esculetin (40 mg/kg)	0.62	0.082
		Esculetin (60 mg/kg)	0.65	0.060
	Esculetin (40 mg/kg)	Esculetin (60 mg/kg)	0.03	0.999
IL-1β (IU/L)	Healthy control rats	Isoproterenol-induced	625.50	<0.001
		Esculetin (20 mg/kg)	153.18	0.033
		Esculetin (40 mg/kg)	87.01	0.426
		Esculetin (60 mg/kg)	25.10	0.987
	Isoproterenol-induced	Esculetin (20 mg/kg)	472.32	<0.001
		Esculetin (40 mg/kg)	538.48	<0.001
		Esculetin (60 mg/kg)	600.39	<0.001
	Esculetin (20 mg/kg)	Esculetin (40 mg/kg)	66.16	0.681
		Esculetin (60 mg/kg)	128.07	0.102
	Esculetin (40 mg/kg)	Esculetin (60 mg/kg)	61.91	0.731

IL-1β, Interleukin-1 beta; NLRP3, NLR family pyrin domain containing 3. *One-way ANOVA followed by post hoc Scheffe test.

pro-inflammatory pathways (23), mirroring esculetin's effects on microbial balance and inflammation (24). Esculetin's hydroxyl groups, critical for its free radical-scavenging activity (25), likely disrupt TMAO synthesis by inhibiting microbial choline TMA-lyase (26), a mechanism shared by other coumarin derivatives that attenuate TMAO production through gut microbiota modulation (27). This is consistent with the study by Koay et al, showing that TMAO elevation correlates with plaque instability rather than atherosclerosis extent (28), suggesting esculetin's TMAO-lowering effect may target mechanisms of acute cardiac injury rather than chronic plaque burden. Furthermore, esculetin's dual action, enhancing antioxidant defenses and stabilizing gut barrier integrity (24,27), parallels interventions that decouple the "gut-heart axis" dysregulation seen in isoproterenol models (23). These results collectively underscore coumarins' therapeutic potential in cardio-metabolic disorders mediated by TMAO, though further studies are needed to validate their clinical efficacy in humans (29,30).

In this study, isoproterenol administration led to a significant decrease in SCFA levels in the induction group compared to healthy control rats. However, treatment with esculetin resulted in a dose-dependent increase in serum SCFA levels, significantly improving them compared to the isoproterenol group.

The observed dose-dependent restoration of SCFA levels by esculetin in isoproterenol-induced MI aligns with prior evidence of coumarins' capacity to modulate gut microbiota and mitigate cardio-metabolic dysregulation. For instance, esculetin's structural analog caffeic acid similarly attenuated isoproterenol-induced mitochondrial dysfunction by scavenging reactive oxygen species (ROS) and preserving antioxidant defenses (31), while curcumin, another polyphenol, ameliorated isoproterenol-driven lipid peroxidation and stabilized membrane integrity through analogous anti-inflammatory pathways (32). The SCFA elevation observed here likely stems from esculetin's dual capacity to enhance gut barrier function, as demonstrated in intestinal ischemia-reperfusion models where it activated SIRT3/AMPK/mTOR signaling to reduce permeability and inflammation (33), and to directly scavenge ROS via Nrf2/HO-1 activation (34), thereby creating a microenvironment conducive to SCFA-producing commensals like Lactobacillus. Pharmacokinetic data showing rapid esculetin absorption and hepatic accumulation (35) further suggest that enterohepatic circulation may amplify its prebiotic effects. These findings extend previous reports of esculetin's cardioprotection (24) by implicating SCFA-mediated mechanisms, such as histone deacetylase inhibition and G protein-coupled receptor activation, as novel contributors to its therapeutic efficacy. However, unlike curcumin's primary focus on myocardial salvage (32), esculetin's gut-heart axis modulation represents a distinct multimodal strategy, warranting clinical exploration in cardiorenal syndromes.

This study revealed that esculetin treatment dose-dependently suppressed NLRP3 inflammasome signaling in heart tissue. Compared to rats with isoproterenol-induced MI, esculetin at 20, 40, and 60 mg/kg significantly

reduced mean NLRP3 expression levels. Notably, at the two higher doses (40 and 60 mg/kg), NLRP3 protein concentrations were reduced to levels below those observed in healthy control rats, suggesting that esculetin not only mitigates MI-driven inflammation but may also exert prophylactic NLRP3 inhibition. This finding of the suppression of NLRP3 inflammasome signaling by esculetin in isoproterenol-induced MI aligns with prior studies demonstrating coumarins' anti-inflammatory properties. For instance, Choudhary et al found that 4-methylesculetin, a structural analog, reduced NLRP3 expression in lipopolysaccharide-induced depression models by binding to the NLRP3 PYD domain and inhibiting caspase-1 activation (36), while esculetin itself attenuated pyroptosis in endothelial cells by downregulating NF-κB/NLRP3 signaling (37). The current findings extend these results by showing that higher esculetin doses (40–60 mg/kg) not only normalized but exceeded baseline NLRP3 levels in healthy controls, suggesting a potential prophylactic effect. This contrasts with the study by Toldo et al, which stated OLT1177, a selective NLRP3 inhibitor, reduced infarct size in ischemia-reperfusion models but required administration within 60 minutes of injury (38). The Esculetin's broader mechanism, activating Nrf2/HO-1 to suppress NF-κB (39), may explain its superior NLRP3 inhibition compared to isoproterenol-induced dysregulation. However, the supranormal NLRP3 suppression at higher doses raises questions about off-target effects, as esculetin is known to modulate SIRT3/AMPK/mTOR pathways in intestinal ischemia-reperfusion (33), which could indirectly influence inflammasome activity. These findings underscore the esculetin multimodal cardioprotection but highlight the need for dose-response studies in clinical settings to balance efficacy and safety.

Furthermore, our study found that IL-1β levels increased in rats with isoproterenol-induced MI compared to healthy controls. However, esculetin treatment decreased IL-1β levels, bringing them closer to the baseline of healthy control rats. This finding aligns with prior studies demonstrating coumarins' anti-inflammatory properties. For instance, a study in China by Ju et al found that esculin, a structurally related coumarin, similarly reduced IL-1β in streptozotocin-induced diabetic rats by suppressing NF-κB signaling and NLRP3 inflammasome activation (40). This mechanism mirrors esculetin's ability to inhibit NF-κB-driven cytokine production, as evidenced by its attenuation of TNF-α and IL-6 in isoproterenol-exposed H9C2 cardiomyocytes (24). Furthermore, Alchemilla vulgaris extract, which shares polyphenolic properties with coumarins, mitigated isoproterenol-induced IL-1β elevation in mice by downregulating the NF-κB/p65 pathway, paralleling esculetin's suppression of pro-inflammatory cascades (41). The current findings extend these results by specifically linking esculetin's IL-1β reduction to NLRP3 inflammasome inhibition, a pathway implicated in isoproterenol-mediated cardiac injury. Notably, esculetin's activation of the Nrf2/HO-1 antioxidant axis (40) may synergistically suppress oxidative stress and inflammasome activity, offering a dual mechanism for its cardioprotection. These results corroborate the broader therapeutic potential of coumarins in modulating cytokine networks, though further studies are needed to elucidate dose-dependent effects on inflammasome components.

Conclusion

The experimental results collectively underscore esculetin's capacity to modulate inflammatory and metabolic biomarkers in isoproterenol-induced conditions, revealing nuanced dose-dependent relationships. At 20 mg/kg, esculetin demonstrated robust efficacy by normalizing TMAO levels and NLRP3 expression to values indistinguishable from healthy controls, while simultaneously attenuating IL-1β elevations. Higher doses (40–60 mg/kg) exhibited incremental reductions in IL-1β and SCFA perturbations compared to the low-dose group, though the absence of significant differences between intermediate and high doses suggests a therapeutic plateau beyond 40 mg/kg. This non-linear dose-response pattern implies that escalating doses beyond 20 mg/kg yield diminishing returns for NLRP3 and TMAO regulation, while still providing graded benefits for IL-1β and SCFA parameters. The differential effects across biomarkers highlight esculetin's multifaceted mechanism of action, potentially involving distinct pathways for inflammatory versus metabolic modulation. These findings advocate for optimized dosing strategies that balance therapeutic efficacy with potential side-effect profiles, particularly favoring lower doses for NLRP3-related pathologies. The consistent superiority of all esculetin doses over untreated isoproterenol-induced groups reinforces its protective role, positioning it as a viable therapeutic agent for conditions involving inflammatory cascades and metabolic dysregulation. Further investigations into long-term effects and molecular targets are warranted to fully elucidate esculetin's clinical potential.

Limitations of the study

The study has several limitations: First, the exclusive use of a rat animal model may not fully replicate human pathophysiology, as interspecies differences in gut microbiota composition and NLRP3 inflammasome responses could affect translational relevance. Second, the small sample size (n = 6 per group) reduces statistical power and increases vulnerability to type II errors, limiting generalizability. Third, the 21-day intervention period may be insufficient to assess long-term therapeutic effects or sustained microbial changes, particularly given the dynamic nature of gut microbiota. Fourth, while NLRP3 expression and specific biomarkers were measured, the study lacks multi-omics analyses (e.g., metagenomic

sequencing) to comprehensively characterize microbial shifts and their functional metabolic consequences. Fifth, the isoproterenol-induced MI model primarily mimics type 2 MI mechanisms, which differ from atherosclerotic type 1 MI seen in most clinical cases.

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Authors' contribution

Conceptualization: Zahraa Jabbar Mujbel and Huda Jaber Waheed.
Data curation: Zahraa Jabbar Mujbel and Huda Jaber Waheed.
Formal analysis: Gaith Ali Jasim.
Investigation: Zahraa Jabbar Mujbel and Gaith Ali Jasim.
Methodology: Gaith Ali Jasim.
Project management: Zahraa Jabbar Mujbel.
Resources: All authors.
Supervision: Huda Jaber Waheed.
Validation: Gaith Ali Jasim.
Writing—original draft: All authors.
Writing—reviewing and editing: All authors.

Conflicts of interest

The authors declare no conflict of interest.

Ethical issues

This research resulted from a pharmacology student thesis named (Zahraa Jabbar Mujbel) with the thesis number of 49 registered on February 12, 2025, approved by the ethics Committee of the Pharmacy College, Animal House, Mustansiriya University, Iraq. The research and its protocol followed the guidelines of animal studies. We also followed the guidelines related to animal experiments, approved by the United States National Institutes of Health (NIH, 1978). Besides, the authors have ultimately observed ethical issues (including plagiarism, data fabrication, and double publication).

Declaration of generative artificial intelligence (AI) and AI-assisted technologies in the writing process

During the preparation of this work, the authors utilized AI (Perplexity.ai and Grammarly.com) to refine grammar points and language style in writing. Subsequently, the authors thoroughly reviewed and edited the content as necessary, assuming full responsibility for the publication's content.

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