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Multilevel evaluation of the anticoagulant and antiplatelet effects of clopidogrel, *Melilotus officinalis*, and *Cinnamomum verum* in an experimentally induced rabbit thrombosis model: integration of histopathology, immunohistochemistry, and in silico molecular docking: a randomized controlled animal study

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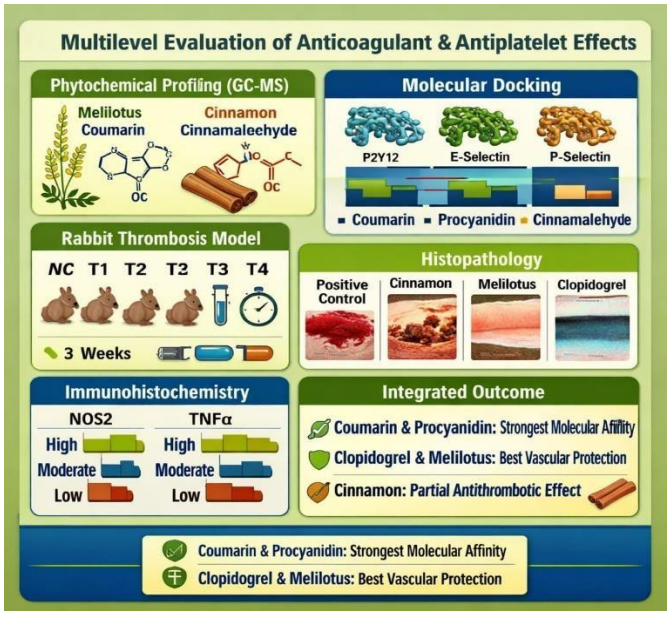
Abstract

The molecular docking analysis demonstrated that coumarin consistently exhibited the strongest binding affinities across all thrombo-inflammatory targets (P2Y12 receptor, E-selectin, and P-selectin), with binding energies ranging from –8.1 to –8.7 kcal/mol and multiple hydrogen bonds and hydrophobic contacts. Procyanidin showed intermediate efficacy, forming extensive hydrogen-bond networks and favorable binding energies (–7.2 to –7.9 kcal/mol), while cinnamaldehyde displayed the weakest interactions (–5.7 to –6.3 kcal/mol), limited by its smaller molecular size. Histological studies confirmed that clopidogrel and *Melilotus officinalis* reduced thrombus formation and collagen deposition, while cinnamon exhibited partial protective effects but still showed significant thrombus presence. Immunohistochemistry revealed that clopidogrel suppressed NOS2 and TNF-α expression, Melilotus reduced oxidative stress and inflammation, and cinnamon moderately decreased TNF-α but maintained high NOS2 activity. Overall, coumarin and procyanidin demonstrated superior predicted binding affinity, while clopidogrel and Melilotus provided the strongest histological and molecular protection against thrombosis and vascular inflammation.

Keywords: Clopidogrel. *Melilotus officinalis*. *Cinnamomum verum*. Rabbit thrombosis model. Molecular docking.

Graphical Abstract



Source: Own authorship.

Introduction

Thrombosis represents a complex pathological process in which disturbances in vascular integrity, blood flow, and coagulation interact to promote clot formation. Although venous and arterial thrombosis are traditionally classified as distinct clinical entities, accumulating evidence indicates that both share common biological pathways driven largely by endothelial dysfunction **[1].** When the vascular endothelium loses its regulatory capacity, oxidative stress increases and nitric oxide availability declines, creating a pro-inflammatory microenvironment that favors platelet activation and leukocyte adhesion. These events accelerate thrombin generation and ultimately lead to fibrin deposition, linking endothelial injury directly to clot development **[2].**

Under physiological conditions, the coagulation system maintains a delicate equilibrium between procoagulant factors and natural anticoagulants to ensure effective hemostasis. Coagulation proteins act sequentially through a cascade that culminates in thrombin formation, which converts fibrinogen into fibrin and stabilizes the developing clot **[3].** This balance is safeguarded by endogenous inhibitors such as protein C, protein S, and antithrombin. Disruption of these mechanisms—whether genetic or acquired—predisposes individuals to bleeding tendencies or thrombotic complications **[4].**

Pharmacologic modulation of platelet activity remains a cornerstone in preventing thrombotic events. Clopidogrel is widely used for this purpose; it requires hepatic biotransformation to its active metabolite, which irreversibly blocks the platelet P2Y12 receptor. By preventing ADP-mediated activation of the IIb/IIIa glycoprotein complex, clopidogrel effectively reduces platelet aggregation and thrombus formation **[5].**

In parallel with synthetic antiplatelet agents, natural products with antioxidant and anti-inflammatory properties have gained increasing scientific interest. Sweet clover (Melilotus officinalis) contains coumarins and related phytochemicals known for their anticoagulant potential and long-standing use in circulatory disorders **[6].** Cinnamon (Cinnamomum verum), rich in compounds such as coumarins and cinnamaldehyde, exhibits anticoagulant, antioxidant, and anti-inflammatory activities and has been traditionally used to manage bleeding and vascular complaints **[7-9].**

Materials and Methods

Methods

GC-MS analysis for Melilotus officinalis and Cinnamomum verum.

Fresh leaves of *Melilotus officinalis* and dried bark of *Cinnamomum verum* were ground into fine powder and extracted using 70% aqueous ethanol at a 1:10 (w/v) ratio. The mixture was macerated at room temperature for 48–72 hours with intermittent stirring and repeated three times to ensure complete extraction. Filtrates were combined, filtered, and concentrated using a rotary evaporator at 40–50°C, then adjusted to a final volume of 100 mL. The extraction procedure followed the maceration methods described by **[10,11]**.

Preparation of Protein Targets and Ligand Structures for Molecular Docking

The three-dimensional crystal structures of the target proteins, specifically the platelet P2Y12 receptor (PDB: 4NTJ), E-selectin (PDB: 1ESL), and P-selectin (PDB: 4DRT), were obtained from the RCSB Protein Data Bank (www.rcsb.org) in PDB format. To prepare each receptor structure for docking, co-crystallized water molecules, native ligands, and non-essential heteroatoms were removed to define the binding site. Subsequently, polar hydrogen atoms were added, and Kollman united-atom partial charges were assigned. The receptor files, once prepared, were then converted into the PDBQT format necessary for docking calculations using Auto Dock. The three-dimensional configurations of the ligands—coumarin, cinnamaldehyde, and procyanidin— were sourced from the PubChem database in SDF format. These structures underwent energy minimization using the MMFF94 force field to reach their lowest-energy states. Gasteiger partial charges were applied to each ligand, non-polar hydrogens were combined, and rotatable bonds were specified to permit torsional flexibility during docking simulations. The ligand files were then saved in PDBQT format.

The experimental animals

The experiment was employed on Thirty Healthy adult rabbits (weight 2–2.5 kg), randomized into 5 groups and 5 to 6 months of life were used in the experiment. The rabbits were housed in the College of Veterinary Medicine/Al-Qassim Green's University animal house, which has a 12-hour light and 12-hour dark cycle and a moderate temperature. With the department's ethics committee's agreement, the animals were housed in sawdust-filled, mesh plastic

cages, fed fresh vegetables (carrot, lettuce, and cucumber), with free access to tap water throughout the experimental period. The animals were left to adapt for 14 days before starting the experiment following the acclimatization procedures described by **[12]** for the period from November 2025 to December 2025. It was followed ARRIVE guidelines.

Induction of thrombosis.

Thrombosis was induced according to the collagen–epinephrine protocol described by **[13].** Baseline bleeding time (BT) and clotting time (CT) were measured before induction and reassessed afterward to confirm hemostatic alteration. BT was evaluated using the filter-paper ear-incision method **[14],** while CT was determined by the slide method **[15].** Following treatment with clopidogrel, *Melilotus officinalis*, and *Cinnamomum verum*, BT and CT were re-measured at the end of weeks 1, 2, and 3 to assess therapeutic response. Following treatment with Clopidogrel, *Melilotus officinalis*, and Cinnamon, bleeding time and clotting time were measured again at the end of the first, second, and third weeks to evaluate therapeutic response.

Determination of the doses

The effective dose for clopidogrel was determined as 25 mg/kg per day orally for three weeks according to **[16].** The effective dose for (*Melilotus officinalis*) extract was determined as 200 mg/kg per day orally for three weeks according to **[17].** The effective dose for cinnamon (*Cinnamomum zeylanicum*) extract was determined as 200 mg/kg per day orally for three weeks according to **[18].**

Dividing of experimental animals

Thirty Healthy adult rabbits (weight 2–2.5 kg), randomized into 5 groups (6 rabbits for each group). 6 animals represented the control group, while 25 animals divided in to 4 groups. The animals were dosed orally from treatments by gavage to the stomach directly for a period of three weeks, according to the following groups.

Group NC (negative control): animals in this group received physiological saline solution only **Group T1 (positive control):** animals in this group were injected intraperitoneally with collagen (400 µg/kg) and epinephrine (60 µg/kg) to induce thrombosis

Group T2 (clopidogrel): animals in this group received an oral dose of clopidogrel 25 mg/kg daily for three weeks

Group T3 (*Melilotus officinalis*): animals in this group received an oral dose of *Melilotus officinalis* extract 200 mg/kg daily for three weeks

Group T4 (cinnamon): animals in this group received an oral dose of cinnamon (Cinnamomum zeylanicum) 200 mg/kg daily for three weeks.

Animal sacrifice

Following blood collection, cardiac puncture and tissue handling were performed according to previously published procedures in laboratory animals **[19].** the animal was laid on its back and the abdomen were incised longitudinally, then the skin was removed, the thoracic cavity was opened after cutting the sternum caudally to the articulated area and the diaphragm and last three pairs of ribs were removed, then the thoracic cavity was opened to obtain the heart. Finally, heart samples were taken and placed in plastic containers containing 10% neutral buffered formalin for histopathological and immunohistochemical examination.

Histological Study

Preparation of Aortic Tissue Samples

Preparation of aortic sample as according of **[20].**

Immunohistochemistry Protocol for Aortic Tissue Samples

Immunohistochemistry study of aortic tissue sample Preparation according to **[21].**

Results and Discussion

GC-MS Analysis studies

GC-MS analysis for Melilotus extract

Table 1. GC-MS analysis of *Melilotus officinalis* extract.

NO	Compounds	RT (min)	Chemical formula	MW	Area%	BA
1	1,4-Dioxane-2,6-dione	2.041	C4H4O4	116	3.18	**Antibacterial**
2	Cyanamide	2.149	CH2N2	42	4.86	**Antifungal**
3	Methanesulfonylacetonitrile	3.376	C3H5NO2S	119	3.97	**Antimicrobial**
4	2H-Pyran-2-one, tetrahydro-6-methyl-	8.955	C6H10O2	114	42.87	**Antioxidant, Anti-inflammatory**
5	Coumarin	15.24	C9H6O2	146.15	8.40	**Anticoagulant, Antioxidant, anti-inflammatory**
6	Hexanoic acid, 2-ethyl-	17.766	C8H16O2	144	1.02	**Antifungal**
7	1,6,11-Trioxacyclopentadecane	19.041	C12H24O3	216	4.39	**Bioactive agent**
8	1,6,11-Trioxacyclopentadecane	19.483	C12H24O3	216	3.36	**Bioactive agent**
9	N, N-Dimethyl-1,3-dioxan-2-amine	19.559	C6H13N02	131	11.21	**Chemical intermediate**
10	D-Fructose, 3-O-methyl-	19.788	C7H14O6	194	12.63	**Nutritive agent**
11	α-D-Methylglucofuranoside derivative	19.893	C7H12O6	192	12.43	**Nutritive agent**

MW, molecular weight; BA, biological activity; RT, retention. Source: Own authorship.

GC-MS analysis for Cinnamon extract (Cinnamomum)

Table 2. GC-MS analysis of Cinnamomum verum extract.

NO	Compounds	RT (min)	Chemical formula	MW	Area%	BA
1	(E)-Cinnamaldehyde	16.48	C9H8O	132	78.12	Antioxidant, Anti-inflammatory Anticoagulant
2	Eugenol	18.25	C10H12O2	164	6.35	Antioxidant, Anti-inflammatory Anticoagulant
3	Cinnamyl acetate	20.12	C11H12O2	164	6.35	Antifungal, Flavoring
4	Linalool	12.35	C10H18O	154	2.80	Sedative, Antibacterial
5	Beta-Caryophyllene	22.05	C15H24	204	2.15	Anti-inflammatory, Anticancer
6	Benzyl benzoate	25.40	C14H12O2	212	1.45	Antimicrobial
7	Coumarin	24.50	C9H6O2	146	0.45	Antioxidant, anti-inflammatory, anticoagulant - related compound

MW, molecular weight; BA, biological activity; RT, retention. Source: Own authorship.

Molecular Docking Analysis of Coumarin, Cinnamaldehyde, and Procyanidin against Thrombo-Inflammatory Targets

To assess the antithrombotic and anti-inflammatory efficacy of coumarin, cinnamaldehyde, and procyanidin, a molecular docking study was conducted. These three active compounds were docked against three structurally and functionally diverse target proteins that play a central role in platelet activation and thrombo-inflammatory adhesion. The platelet P2Y12 receptor (PDB: 4NTJ), which mediates ADP-dependent platelet aggregation and is the pharmacological target of clopidogrel-type antiplatelet agents; E-selectin (PDB: 1ESL), a Ca2+-dependent lectin expressed on activated endothelium that initiates leukocyte and platelet rolling; and P-selectin (PDB: 4DRT), which is rapidly translocated from platelet alpha-granules and Weibel–Palade bodies to mediate platelet– leukocyte–endothelial adhesion during thrombus formation and vascular inflammation. The binding free energies, key interacting residues, and total conventional hydrogen bonds and hydrophobic contacts determined for each ligand–receptor complex is compiled in Table 3.

Table 3. Binding energies and interaction profiles of coumarin, cinnamaldehyde, and procyanidin with E-selectin (1ESL), the P2Y12 receptor (4NTJ), and P-selectin (4DRT).

Target (PDB ID)	Ligand	Binding Energy (kcal/mol)	H-Bonds(n)	Hydrophobic Contacts (n)	Key Interacting Residues
E-selectin (1ESL)	Coumarin	-8.7	3	6	Ser33, Asn82, Glu70, Tyr94, Trp97, His105
	Cinnamaldehyde	-5.9	1	5	Ser33, Asn82, Tyr94, Glu70
	Procyanidin	-7.2	2	6	Asn82, Glu70, Tyr94, Trp97
P2Y12 Receptor (4NTJ)	Coumarin	-8.1	2	6	Lys174, Arg256, Tyr105, Phe259, Leu276
	Cinnamaldehyde	-6.3	1	5	Lys174, Tyr105, Phe259, Leu276
	Procyanidin	-7.6	3	7	Lys174, Arg256, Asp107, Ser242, Phe259, Leu276
P-selectin (4DRT)	Coumarin	-8.3	2	6	Asp107, Arg73, Tyr48, Asn74, Glu70, Trp82
	Cinnamaldehyde	-5.7	1	5	Tyr48, Arg73, Asn74, Glu70, Trp82
	Procyanidin	-7.9	2	6	Asp107, Tyr48, Arg73, Glu70, Trp82

These values were extracted from the molecular docking outputs and their corresponding 2D and 3D interaction diagrams. Thermodynamically, more negative binding energy values represent stronger and more favorable predicted binding affinities between the ligands and receptors. Source: Own authorship.

Interaction with the P2Y12 Receptor (4NTJ)

Coumarin produced the most favorable binding energy against the P2Y12 receptor (-8.1 kcal/mol), forming two conventional hydrogen bonds and six hydrophobic contacts within the nucleotide-binding pocket. Procyanidin, although its larger and more flexible polyphenolic scaffold, achieved a comparable binding energy (-7.6 kcal/mol) and the highest number of hydrogen bonds (three) and hydrophobic contacts (seven) while Cinnamaldehyde showed the weakest interaction (- 6.3 kcal/mol, forming only a single hydrogen bond and five hydrophobic contacts. This weaker interaction is primarily due to its smaller molecular size, which limits its ability to simultaneously span and bind to the multiple protein sub-sites that the larger ligands successfully engaged (Figure 1).

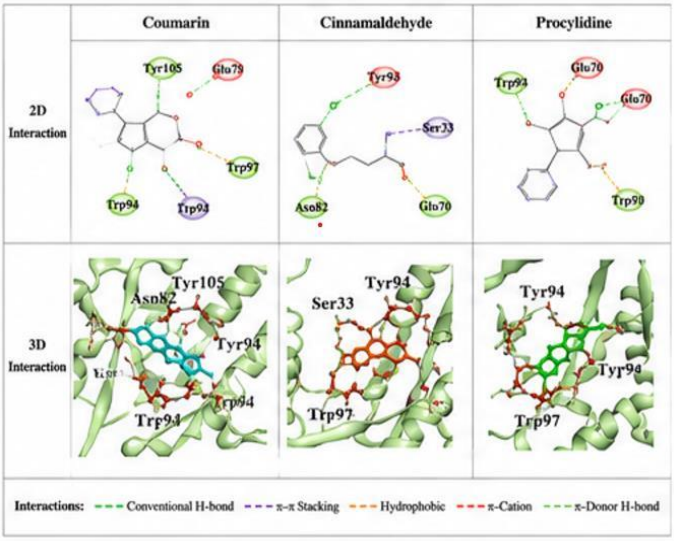


Figure 1. 2D and 3D interaction diagrams of the docked ligands within the binding pocket of the P2Y12 receptor (4NTJ). Source: Own authorship.

Interaction with E-selectin (1ESL)

At the E-selectin lectin domain, coumarin again appeared the strongest predicted affinity (- 8.7

kcal/mol), engaging Ser33, Asn82, Glu70, Tyr94, and Trp97 by three hydrogen bonds and six hydrophobic contacts (Figure 2). Procyanidin formed double hydrogen bonds with Asn82, Glu70, Tyr94, and Trp97 and generated a moderate binding energy (-7.2 kcal/mol), while cinnamaldehyde interacted only weakly (-5.9 kcal/mol) through a single hydrogen bond with Ser33.

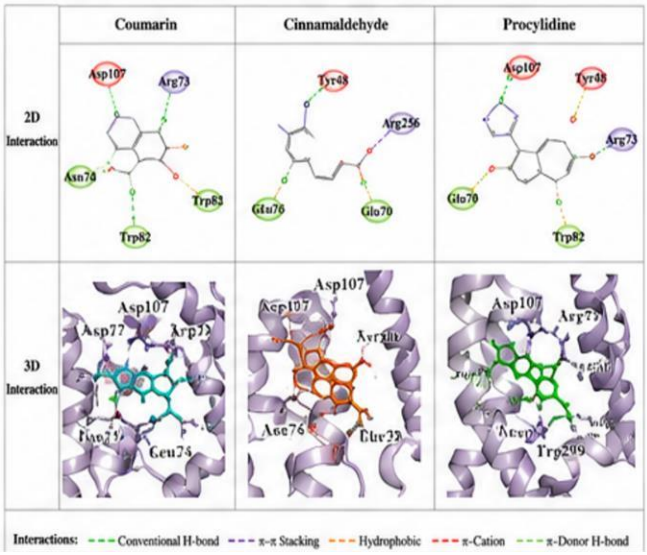


Figure 2. 2D and 3D interaction diagrams of the docked ligands within the lectin domain of E-selectin (1ESL). Source: Own authorship.

Interaction with P-selectin (4DRT)

Regarding the P-selectin interaction, coumarin (-8.3 kcal/mol, two hydrogen bonds), procyanidin (-7.9 kcal/mol, two hydrogen bonds), and cinnamaldehyde (-5.7 kcal/mol, one hydrogen bond) all targeted a shared set of residues. These amino acids—namely Asp107, Arg73, Tyr48, Asn74, Glu70, and Trp82—are situated within the calcium-dependent carbohydrate-recognition domain, which is crucial for mediating the binding of P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1). In the current study (PD B: 4NTJ) was used P2Y12 receptor structure that has acted as the standard docking template in several previous research of antiplatelet compounds **[22].** A recent structure-activity relationship (SAR) study evaluated newly synthesized nitrogen-substituted coumarins against the P2Y12, GPVI, and alpha2-adrenergic receptors. In-silico results predicted that these coumarins act as antagonists at the P2Y12 site, reinforcing the idea that the coumarin-type scaffold is structurally compatible with this specific binding pocket **[23].** Likewise, coumarin-hydras/one derivative tested versus P2Y12 and COX-1 were confirmed to record binding affinities (-7.9 to -8.6) kcal/mol together with interactions at key active-site residues, a range essentially identical to the -8.1 kcal/mol obtained for unsubstituted coumarin in the present docking run **[24].** This divergence suggests that the antiplatelet activity observed for those specific coumarin derivatives may instead stem from the inhibition of the epinephrine pathway. Furthermore, it underscores that the docking outcomes for this core scaffold are highly sensitive to both the specific substitution patterns and the exact docking protocol employed **[25].**

This is consistent with the relatively high hydrogen-bond count (three) and favorable binding energy (-7.6 kcal/mol) obtained for procyanidin against P2Y12 in the present analysis, and supports the broader observation that polyphenolic, multi-hydroxylated natural products tend to form more extensive polar contact networks than smaller monocarbonyl compounds such as cinnamaldehyde. The calcium-dependent carbohydrate-recognition domain is engaged by the natural ligand sialyl-Lewis X and its analogues. Previous research has used the E-selectin lectin/EGF crystal structure (1ESL) as the docking template to model species-specific variations in selectin–ligand binding using a combination of docking and molecular dynamics techniques **[26].** For P-selectin (4DRT), previous docking-based work has established this target as pharmacologically tractable for small-molecule and peptide-based inhibitors: a designed dipeptide-derived inhibitor (THPDTPI) was shown by docking to occupy the P-selectin active site and subsequently demonstrated antithrombotic, anti-inflammatory, and antitumor activity *in vivo*. A designed dipeptide-derived inhibitor (THPDTPI) was appeared by docking to occupy the P-selectin active site and then confirmed antitumor, antithrombotic, and anti-inflammatory activity *in vivo*. Previous docking-based work has established P-selectin (4DRT) as pharmacologically tractable for small-molecule and peptide-based inhibitors **[27].**

The energies of P-selectin binding between -5.9 to -6.1 kcal/mol range were mentioned in more recent articles on sulfated oligosaccharide derivatives derived from sea cucumber **[28],** which is significantly weaker than the -7.9 to -8.3 kcal/mol range found for coumarin and procyanidin in the present study. Moreover, *in vitro* and *in vivo* validation would be necessary before making pharmacological conclusions, this comparison mentioned that the small polyphenolic and lactone ligands studied here may engage the P-selectin recognition site with greater predicted affinity than some previously reported carbohydrate-based inhibitors, at least at the level of predicted binding free energy. From above data study confirmed that docking results indicate that predicted binding affinity of coumarin high than procyanidin and the last was more effective than cinnamaldehyde across all three thrombo-inflammatory targets.

Histological Study

Histopathology of aorta

The photomicrographs of the rabbit's aorta stained with Masson Trichrome demonstrate progressive structural changes across the experimental groups. In the negative control group Figure 3 A and B showed: the tunica intima forms the innermost layer opposing the lumen with a thin lining of endothelial cells, while the tunica media appears as the most substantial layer containing red-stained smooth muscle cells, and the tunica adventitia serves as the outermost coat with densely accumulated blue-stained collagen fibers. In positive group Figures 3 and 4 shows the tunica intima bordering the lumen displays a significant focal plaque (black arrow), where the stain highlights a heavy accumulation of collagen fibers in a distinct blue color. Situated within the lumen is a detached thrombus (yellow arrow) consisting of light-colored, delicate fibrin fibers. Below the lesion, the tunica media is clearly defined, showing a contrast between red-stained smooth muscle cells and the elastic framework of the vessel wall.

Also, in positive group in Figures 3 and 5 *Large thrombus* (black arrow), stained with light, pink-colored fibrin strands, is firmly attached to the valvular surface. The presence of this mass results in the effacement of the valve leaflet projection, making the normal cusp structure unidentifiable. The underlying valvular stroma (yellow arrow) is highlighted by blue-stained collagen fibers, which transition abruptly into the pale, fibrinous material of the attached thrombus. the cinnamon group in Figures 3 to 6 Thickened aortic wall, particularly in the intimal and medial layers, was observed. The lesion appears as a fibrotic plaque (black arrow), exhibiting intense blue staining consistent with collagen-rich remodeling. A large thrombus (yellow arrow) is observed within the lumen, showing minimal collagen integration at the interface with the vessel wall and partial detachment. the sweet clover group Figures 3 to 7 Mild collagen deposition within the mildly thickened aortic wall (black arrow), indicating limited fibrotic remodeling. The intraluminal thrombus (yellow arrow) exhibits low structural density with scant collagen incorporation, supporting poorly organized/early thrombus (blood aggregation). Attachment to the vessel wall is minimal, suggesting weak adhesion. the clopidogrel group in Figures 3 to 8 Increasing in collagen fiber deposition (black arrow) within the tunica media was evident, contributing to mild wall thickening. The collagen is highlighted by blue staining, confirming early fibrotic remodeling. The vascular lumen is free of thrombus. Thrombus

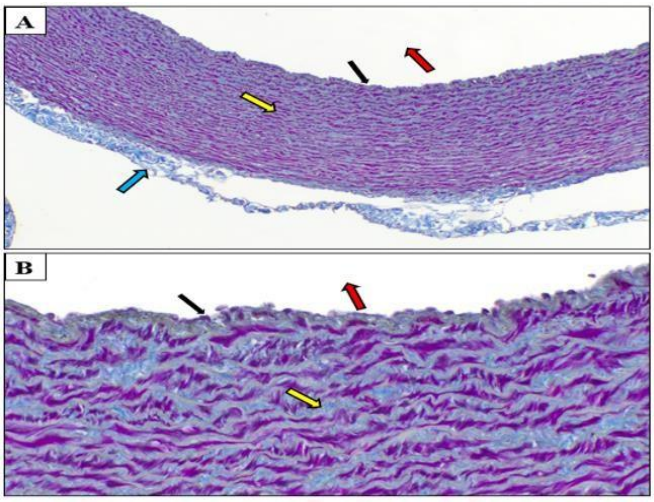


Figure 3. Photomicrograph of aorta of control negative group rabbit Masson Trichrome Stain. Source: Own authorship.

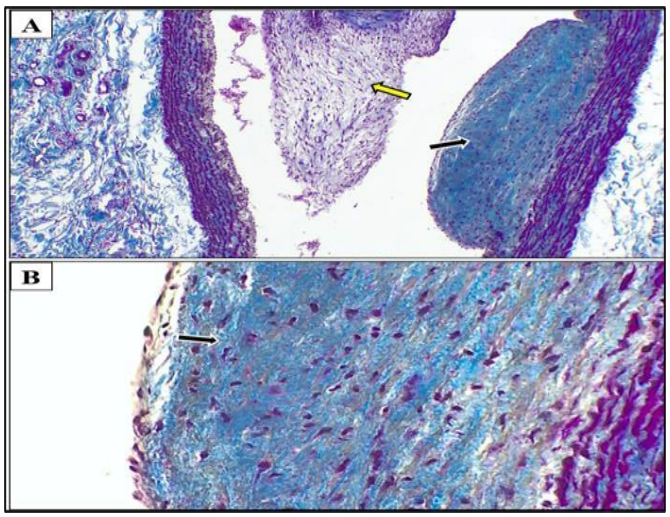


Figure 4. Photomicrograph of aorta of control positive group rabbit. Masson Trichrome Stain. Source: Own authorship.

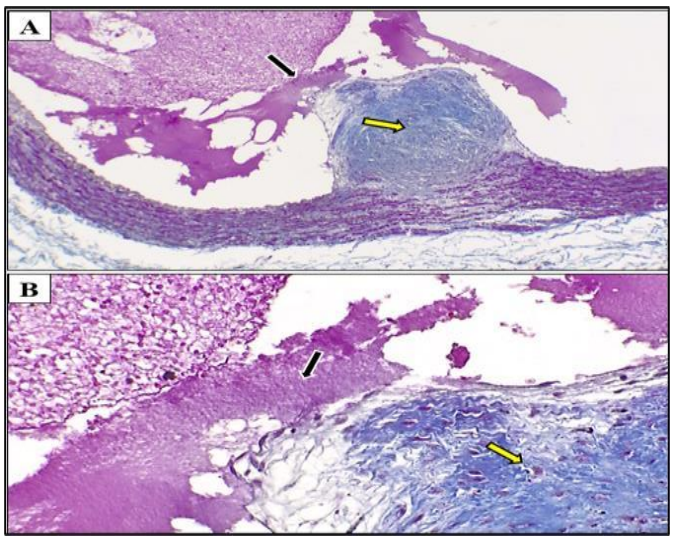


Figure 5. Photomicrograph of aorta of control positive group rabbit. Masson Trichrome Stain. Source: Own authorship.

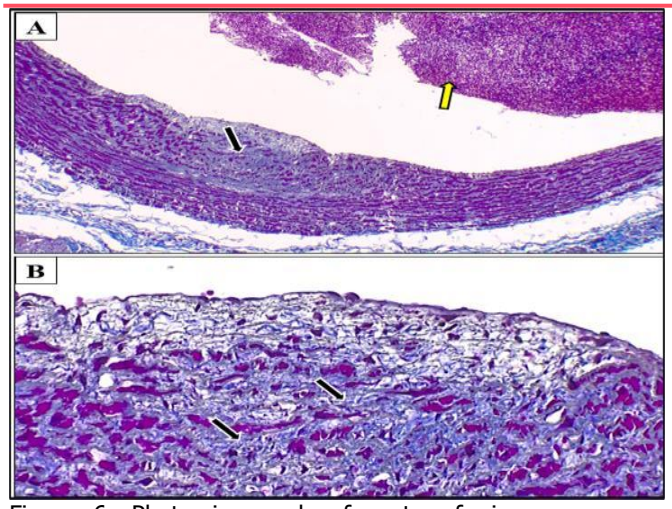


Figure 6. Photomicrograph of aorta of cinnamon group rabbit. Masson Trichrome Stain. Source: Own authorship.

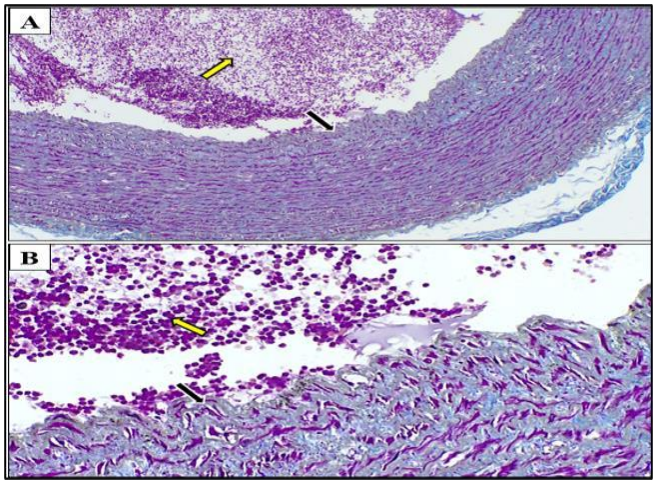


Figure 7. Photomicrograph of aorta of sweet clover group rabbit. Masson Trichrome Stain. Source: Own authorship.

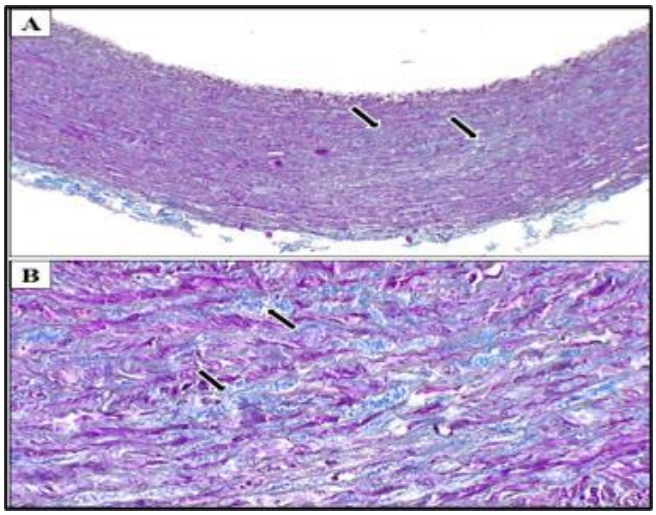


Figure 8. Photomicrograph of aorta of Clopidogrel group rabbit. Masson Trichrome Stain. Source: Own authorship.

The normal aorta has a highly elastic wall composed of a thin intima, thick media rich in elastic lamellae and smooth muscle, and an adventitia containing supportive vessels, giving it strength and flexibility **[29].** In the positive control group, thrombus formation and endothelial injury resulted from intravenous collagen and epinephrine injection, consistent with published findings **[30].** Clopidogrel showed minimal effects on lipid profile but significantly reduced oxidative stress and inflammation. Histological and immunohistochemical analysis confirmed decreased expression of VCAM-1, MCP-1, TNF-α, and IL-17, along with reduced intimal thickness and atherosclerotic lesion development **[31].** In hypertensive rats, clopidogrel improved endothelial function without altering blood pressure, as shown by enhanced aortic relaxation to acetylcholine, supporting its protective vascular role **[32].** Atherosclerosis begins with endothelial dysfunction, and cinnamon extract—through cinnamaldehyde and eugenol—improved endothelial relaxation by increasing nitric oxide, reducing oxidative stress, and limiting damage from AGEs and oxidized LDL **[33].** In hyperlipidemic rats, cinnamon oil reduced aortic wall thickening and improved structural integrity compared to untreated animals **[34].** *Melilotus officinalis* demonstrated strong antioxidant activity by lowering MDA, TOS, and the oxidative stress index while increasing total antioxidant capacity and sulfhydryl groups, helping maintain aortic elasticity and protecting against damage and thrombus formation **[35].** Additional findings showed that *M. officinalis* reduced oxidative stress and inflammation, decreased endothelial adherence, and limited thrombogenesis, thereby enhancing vascular stability **[36].**

Immunohistochemistry study

Immunohistochemistry of aorta

In the negative control group Figure 9 showed: Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for control negative g. Showing low immunoreactivity of NOS polyclonal Ab in all aortic layers. In positive group Figure 10 shows: immunohistochemical image of paraffin embedded section of adult rabbit aorta, for control positive g. Showing high immunoreactivity of NOS polyclonal Ab in all aortic layers. The cinnamon group in Figure 11 shows Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for cinnamon group. Showing high immunoreactivity of NOS polyclonal Ab in all aortic layers. The sweet clover group Figure 12 shows Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for sweet clover group. Showing moderate immunoreactivity of NOS polyclonal Ab in all aortic layers. The clopidogrel group in figure (3-13) show Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for clopidogrel group. Showing low immunoreactivity of NOS polyclonal Ab in all aortic layers.

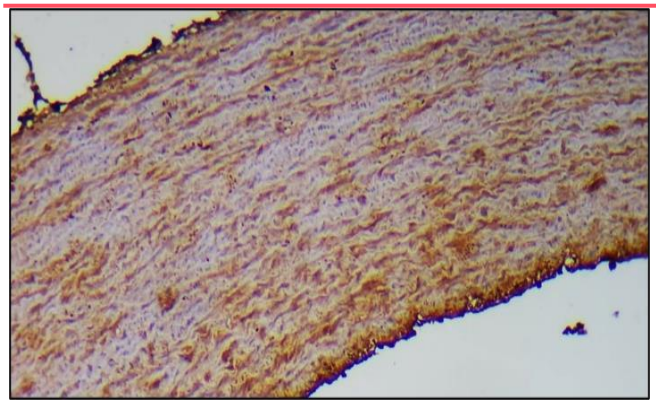


Figure 9. Photomicrograph of aorta of negative group rabbit (Nos2). (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

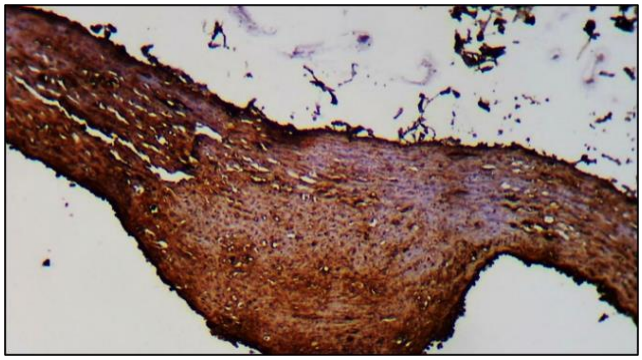


Figure 10. Photomicrograph of aorta of positive group rabbit (Nos2) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

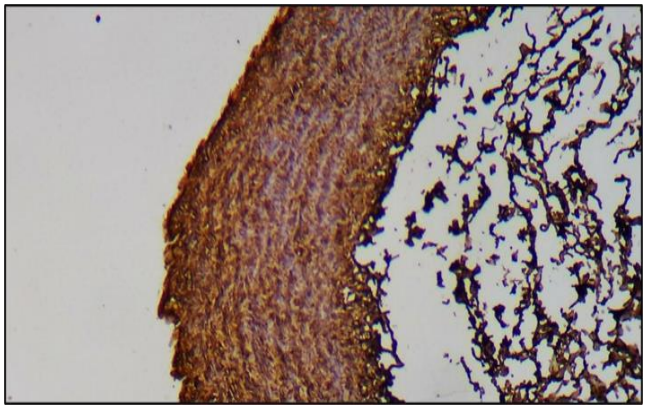


Figure 11. Photomicrograph of aorta of cinnamon group rabbit (Nos2) (DAB chomogen and hematoxylin stains, 10X). Source: Own authorship.

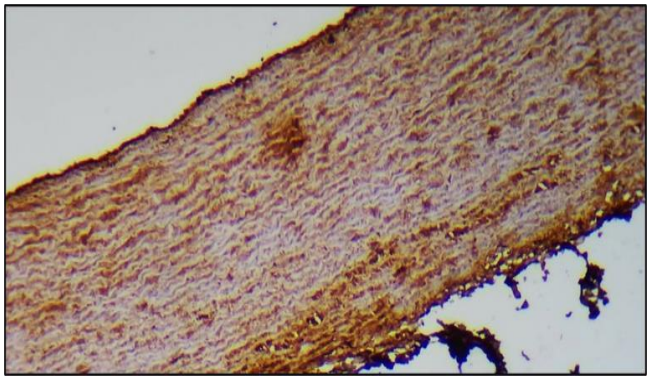


Figure 12. Photomicrograph of aorta of sweet clover group rabbit (Nos2) (DAB chromogen and hematoxylin stains, 10X. Source: Own authorship.

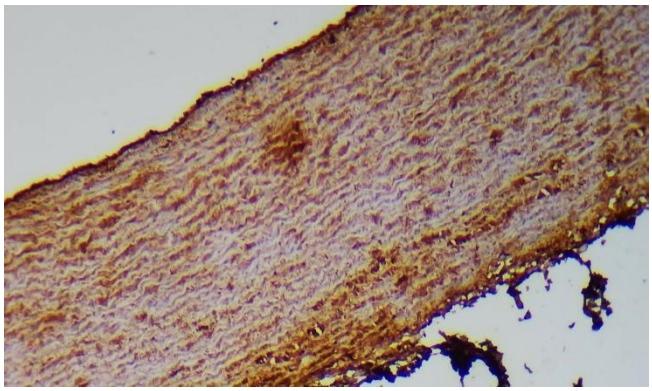


Figure 13. Photomicrograph of aorta of clopidogrel group rabbit (Nos2) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

In the negative control group Figure 14 showed: Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for control negative group. Showing low immunoreactivity of TNF-α Ab in all aortic layers. In positive group Figure 15 shows: Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for control positive group. Showing high immunoreactivity of TNF-α Ab in all aortic layers. The cinnamon group in Figure 16 shows Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for cinnamon group. Showing moderate immunoreactivity of TNF-α Ab in all aortic layers. The sweet clover group Figure 17 shows Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for sweet clover group. Showing moderate immunoreactivity of TNF-α Ab in all aortic layers. The clopidogrel group in Figure 18 show Immunohistochemical image of paraffin embedded section of adult rabbit aorta, for clopidogrel group. Showing low immunoreactivity of TNF-α Ab in all aortic layers.

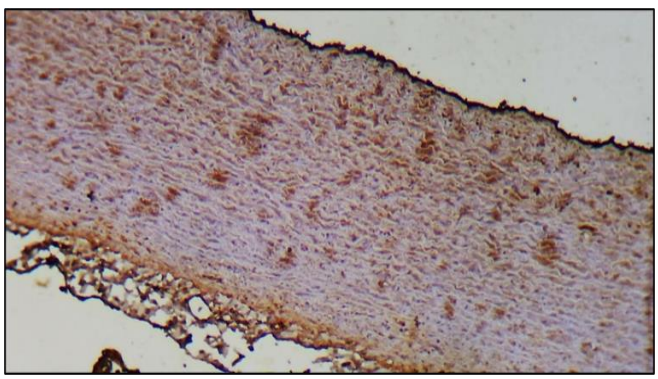


Figure 14. Photomicrograph of aorta of negative group rabbit (TNF) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

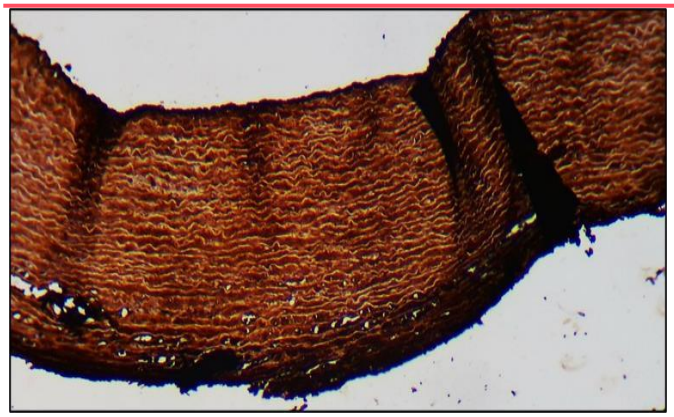


Figure 15. Photomicrograph of aorta of positive group rabbit (TNF) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

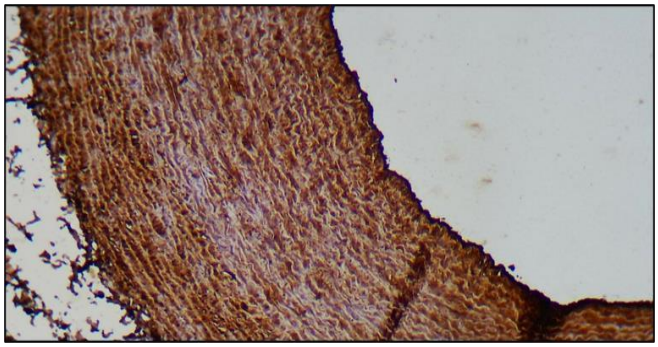


Figure 16. Photomicrograph of aorta of cinnamon group rabbit (TNF) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

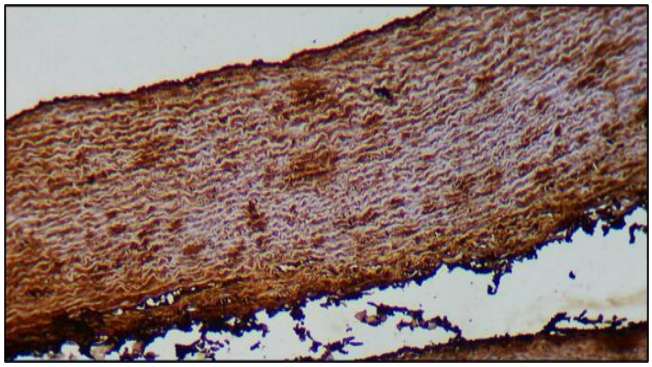


Figure 17. Photomicrograph of aorta of sweet clover group rabbit (TNF) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

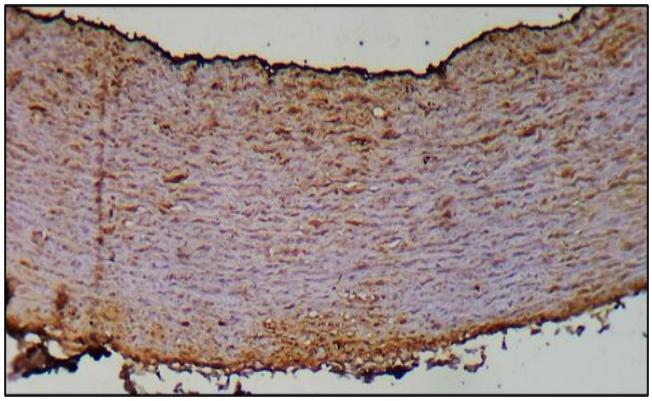


Figure 18. Photomicrograph of aorta of clopidogrel group rabbit (TNF) (DAB chromogen and hematoxylin stains, 10X). Source: Own authorship.

NOS2 is normally found in the aortic adventitia and in neutrophils and monocytes within thrombi, while NOS3 is constitutively expressed in endothelial cells to maintain vascular tone and prevent inflammation. NOS2, however, is induced only by stimuli such as LPS and inflammatory cytokines through NF κB and STAT 1α activation **[37].** Unlike NOS3, TNF α is not constitutively expressed in healthy vessels; it appears during inflammation or injury, increasing endothelial permeability and disrupting junctions, which shifts the endothelium toward a pro inflammatory state

[38]. TNF α also promotes prothrombotic activity by reducing protein C receptor and thrombomodulin, activating complement, and stimulating tissue factor release. These actions sustain inflammation and increase arteriolar thrombosis, while TNF α inhibition reduces venous thrombosis **[39].** Endothelial NO, produced by NOS3 in response to VEGF, estrogen, acetylcholine, bradykinin, and shear stress, increases cGMP, lowers intracellular calcium, stabilizes the endothelial barrier, and prevents smooth muscle proliferation. NO also inhibits platelet activation by blocking GPIIb/IIIa and fibrinogen binding **[40].** Clopidogrel, through P2Y12 inhibition, reduces platelet activation and suppresses NF κB signaling, thereby decreasing pro inflammatory mediators including iNOS **[41].** Cinnamon powder (1 g/day) lowered TNF α levels by blocking NF κB activation and inhibiting JNK, p38, and ERK1/2, with polyphenols contributing to reduced cytokine synthesis **[42].** Cinnamon also suppressed inflammation by decreasing TNF α and iNOS, limiting excessive NO production and oxidative stress **[43].** *Melilotus officinalis* extracts reduced infarct size in cerebral ischemia by lowering neuronal damage, ROS, oxidative stress, and inflammatory cytokines. It also decreased coagulation markers such as TXB2 and 6 keto PGF1α, indicating protection against ischemia related vascular coagulation **[44].**

Limitations

This study faced several limitations. A small number of animals died at the beginning of thrombus induction with epinephrine and collagen, reducing the final sample size. The use of an animal model limits direct extrapolation to human physiology, and the short treatment period (three weeks) may not reflect long-term effects. In addition, variability in the phytochemical composition of natural extracts may affect reproducibility. Finally, the findings are based on in silico docking, histological, and immunohistochemical analyses, which provide valuable mechanistic insights but require further validation in long-term and clinical studies.

Conclusion

GC-MS analysis identified coumarin as a major bioactive compound in *Melilotus officinalis* and cinnamaldehyde as the dominant compound in *Cinnamomum verum*, both associated with anticoagulant and anti-inflammatory properties. In molecular docking, coumarin showed the strongest predicted binding affinity (–8.7 kcal/mol with E-selectin), indicating superior interaction with thrombo-inflammatory targets compared to cinnamaldehyde. Histological evaluation revealed that *Melilotus officinalis* markedly reduced thrombus formation and collagen deposition, while cinnamon extract exhibited partial protection but persistent thrombus presence. Immunohistochemistry confirmed that *Melilotus officinalis* decreased NOS2 and TNFα expression more effectively than cinnamon, which maintained high NOS2 activity despite moderate TNFα reduction. Overall, *Melilotus officinalis* demonstrated stronger protective effects than *Cinnamomum verum*, aligning both experimental and molecular findings, while emphasizing the need for further long-term and clinical validation.

CRediT

Author contributions: All authors contributed to the conception and design of the study. Material preparation, data collection, and laboratory analyses were performed by all authors. The first draft of the manuscript was prepared by the corresponding author, and all authors critically reviewed, edited, and approved the final version of the manuscript for submission.

Acknowledgment

Not applicable.

Ethical Approval

This study was reviewed and approved by the Research Ethics Committee of Al-Qasim Green University, Babylon, Iraq (Approval No. qgec/7/2025, Date: 8 October 2025). All animal experiments were conducted in accordance with institutional and national ethical standards, and followed the ARRIVE guidelines (Animal Research: Reporting of *in vivo* Experiments).

Informed Consent

Not applicable.

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Data Sharing Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request, subject to ethical approval and participant privacy restrictions.

Conflict of Interest

The authors declare no competing interests.

Similarity Check

It was applied by Ithenticate[@].

Application of Artificial Intelligence (AI)

Not applicable.

Peer Review Process

It was performed.

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