

ACALYPHA INDICA ATTENUATES OBESITY-INDUCED ADIPOSE AND VASCULAR INFLAMMATION VIA MODULATION OF ADIPOKINES AND ENDOTHELIAL ADHESION MOLECULES IN OBESE RATS

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Abstract

Obesity-induced chronic inflammation plays a central role in metabolic syndrome progression through adipokine-mediated systemic inflammation and endothelial dysfunction. *Acalypha indica* (Ai), a polyphenol-rich medicinal plant, has demonstrated anti-inflammatory and metabolic regulatory properties. This study investigated the combined effects of Ai on adipose and vascular inflammation in a high-fructose cholesterol diet (HFCD)-induced rat model. Male Sprague-Dawley rats were fed HFCD for 16 weeks and treated with Ai extract (400 mg/kgBW/day) for 8 weeks. Evaluated parameters included body weight, Lee index, adipokines (TNF- α , IL-6, leptin, adiponectin), adipose histology through crown-like structures (CLS), and vascular inflammation markers (VCAM-1 and ICAM-1). Molecular docking assessed interactions between Ai compounds and the inflammatory targets TNF- α , VCAM-1, and ICAM-1. Ai significantly reduced TNF- α , IL-6, leptin, and CLS formation. Molecular docking identified nicotiflorin and related flavonoids as key compounds with strong binding affinity toward inflammatory and adhesion molecules. These findings suggest Ai as a potential therapeutic agent for obesity-associated vascular and metabolic complications.

Rezumat

Studiul a investigat efectele combinate ale *Acalypha indica* (Ai) asupra inflamației sistemice și disfuncției endoteliale, într-un model experimental indus prin dietă îmbogățită cu fructoză și colesterol (HFCD) la șobolan. Șobolani Sprague-Dawley masculini au primit dietă HFCD timp de 16 săptămâni, fiind tratați ulterior cu extract de Ai (400 mg/kgc/zi) timp de 8 săptămâni. Parametrii urmăriți au inclus greutatea corporală, indicele Lee, adipokinele (TNF- α , IL-6, leptina, adiponectina), aspectul histologic al țesutului adipos, apreciat prin structurile de tip coroană (CLS), precum și markerii inflamației vasculare (VCAM-1 și ICAM-1). Prin docking molecular au fost evaluate interacțiunile dintre compușii din Ai și TNF- α , VCAM-1 și ICAM-1. Administrarea Ai a redus semnificativ nivelurile TNF- α , IL-6 și leptinei, precum și formarea CLS. Prin docking molecular s-au identificat nicotiflorina și flavonoidele înrudite drept compuși importanți, cu afinitate ridicată de legare pentru moleculele inflamatorii și de adeziune. Rezultatele obținute susțin potențialul terapeutic al Ai în complicațiile vasculare și metabolice asociate obezității.

Keywords: *Acalypha indica*, obesity, adipokines, CLS, vascular

Introduction

Obesity, particularly visceral obesity, is a major component of metabolic syndrome, a cluster of interrelated conditions including hyperglycemia, hypertension,

hypertriglyceridemia, and low high-density lipoprotein cholesterol that increase the risk of cardiovascular disease and diabetes (1). According to the World Health Organization (WHO), the global prevalence of overweight and obesity has continued to increase

over time. In 2016, more than 1.9 billion adults were classified as overweight, including over 650 million adults living with obesity. By 2022, the number of overweight adults had risen to 2.5 billion, while the number of adults living with obesity increased to 890 million (2). In Indonesia, the prevalence of adult obesity increased from 14.8% in 2013 to 21.8% in 2018 based on *RISKESDAS* data (3, 4). The latest findings from the 2023 Indonesia Health Survey (*SKI*) indicate that obesity prevalence among adults aged > 18 years has further increased to 23.4%, demonstrating a continuing upward trend in obesity among Indonesian adults (5). The increasing prevalence of obesity is strongly associated with modern dietary patterns, excessive caloric intake, high fructose consumption, and sedentary lifestyles, which contribute to the development of metabolic syndrome and cardiovascular complications (6).

Adipose tissue is no longer viewed merely as an energy storage organ but is now recognized as a complex endocrine organ involved in metabolic and inflammatory regulation. Adipocytes actively secrete adipokines, including tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), leptin, and adiponectin, which regulate appetite, glucose metabolism, lipid metabolism, immune responses, and vascular homeostasis. In obesity, excessive visceral fat accumulation causes adipose tissue dysfunction characterized by adipocyte hypertrophy, macrophage infiltration, extracellular matrix remodeling, and chronic low-grade inflammation. These alterations lead to dysregulated adipokine secretion, increased insulin resistance, and progression of obesity-related complications (7).

One histopathological feature of adipose inflammation in obesity is the formation of crown-like structures (CLS), which consist of macrophages surrounding dead or hypertrophic adipocytes. These macrophages release pro-inflammatory cytokines that perpetuate systemic inflammation and contribute to metabolic and vascular dysfunction. Elevated levels of pro-inflammatory adipokines such as TNF- α , IL-6, and leptin, along with decreased adiponectin levels, are closely associated with endothelial dysfunction and progression of atherosclerosis (1, 7).

Endothelial dysfunction is considered the earliest stage of atherosclerosis and plays a critical role in cardiovascular disease development. It is characterized by increased expression of vascular adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which mediate leukocyte adhesion, migration, and infiltration into the vascular wall (8). The upregulation of VCAM-1 and ICAM-1 in response to inflammatory stimuli and lipid accumulation promotes plaque formation and vascular inflammation (9). Metabolic syndrome-associated conditions such as hyperlipidemia, obesity, and hyperglycemia further exacerbate

endothelial dysfunction and accelerate atherosclerotic progression (10).

Current pharmacological therapies for obesity and metabolic syndrome-related atherosclerosis often involve multiple drugs targeting dyslipidemia, hyperglycemia, hypertension, and obesity (11). Although effective, polypharmacy increases the risk of adverse drug reactions, drug interactions, and poor patient compliance. Therefore, there is growing interest in complementary and alternative therapies, particularly medicinal plants rich in polyphenols and flavonoids, due to their multi-target anti-inflammatory and metabolic regulatory properties (12).

Acalypha indica (Ai), locally known as *akar kucing* or anting-anting in Indonesia, is a medicinal plant from the *Euphorbiaceae* family with promising therapeutic potential against metabolic disorders. Previous studies showed that Ai extract could improve obesity-related parameters, reduce triglyceride levels, improve insulin resistance, and attenuate inflammatory adipokines in rats fed a high-fructose cholesterol diet (HFCD). Phytochemical analysis revealed flavonoids, alkaloids, tannins, and triterpenoids, compounds known for antioxidant and anti-inflammatory activities (13, 14).

Several flavonoid compounds from Ai, particularly nicotiflorin and kaempferol glycosides, have shown strong anti-inflammatory and endothelial protective properties. Molecular docking studies showed that nicotiflorin has favourable binding affinity for inflammatory and endothelial adhesion targets, including TNF- α , IL-6, VCAM-1, and ICAM-1 (15, 16). Nicotiflorin has also been reported to possess antioxidant, anti-inflammatory, hepatoprotective, and endothelial nitric oxide synthase (eNOS)-enhancing activities, suggesting its potential role in suppressing endothelial dysfunction and vascular inflammation (17). Despite these findings, the integrated effects of Ai on both adipose inflammation and vascular endothelial dysfunction in obesity-associated atherosclerosis remain unclear. Therefore, this study aimed to investigate the effects of Ai on adipokine dysregulation, adipose tissue inflammation, and endothelial adhesion molecule expression in HFCD-induced obese Sprague-Dawley rats, and to explore the molecular interactions of its bioactive compounds with inflammatory and vascular targets through molecular docking analysis.

Materials and Methods

Study design

This study used an experimental *in vivo* design combined with *in silico* molecular docking analysis to evaluate the integrated effects of Ai on adipose and vascular inflammation in a HFCD-induced obesity model. The study investigated obesity-related metabolic parameters, adipose tissue inflammation, endothelial dysfunction markers, and the molecular

interaction of Ai bioactive compounds with inflammatory and adhesion molecules.

Preparation of Ai extract

The roots of *Ai L.* were collected in 2021 from the Rawa Denok area, Depok, West Java, Indonesia. The Herbarium Bandungense, School of Life Sciences and Technology (SITH), Institut Teknologi Bandung (ITB), Bandung, Indonesia, taxonomically identified the plant material. The identification was confirmed in a determination certificate (No. 360/IT1.C11.2/TA.00/2022), issued on 21 January 2022. The roots were washed, dried, and ground into powder before extraction. The powdered root material (2,850 g) was extracted by maceration using 70% ethanol at a plant-to-solvent ratio of 1:5 (w/v) for 24 h at room temperature. The extraction process was repeated for three consecutive cycles. The combined filtrates were concentrated under reduced pressure using a rotary evaporator at 45 - 50°C to obtain a viscous ethanolic extract. We obtained 122.9 g of extract, corresponding to a yield of 4.31%. The extract had a moisture content of 25.5% and a purity of 74.5%. The extract was stored at 4°C until use. Before oral administration, the extract was reconstituted in distilled water.

Animal model

Male Sprague-Dawley rats aged 7 - 8 weeks (weighing 150 - 200 g) were obtained from the National Institute of Health Research and Development (NIHRD),

Ministry of Health, Republic of Indonesia. Standard cages (2 - 3 rats/cage) were used to house the rats, maintained at $25 \pm 2^{\circ}\text{C}$ with a 12:12 h light/dark cycle. Thirty rats were initially enrolled in the study. After 16 weeks of HFCD induction, the surviving animals were randomly allocated to four experimental groups. The minimum sample size *per* group was determined using Federer's formula:

$$(t - 1)(n - 1) \geq 15,$$

where, t represents the number of treatment groups and n represents the number of animals *per* group. Based on four treatment groups, the minimum required sample size was six rats *per* group. To compensate for anticipated attrition due to illness or mortality, 30 rats were initially enrolled. During the HFCD induction and treatment periods, some animals died; therefore, only 20 rats completed the study and were included in the final analyses.

The normal control group (Normal) was fed normal chow. Ai group was administered Ai extract at 400 mg/kgBW/day (HFCD + Ai group). The gemfibrozil group was administered gemfibrozil at a dose of 31 mg/kgBW/day (HFCD + Gemfibrozil group). The HFCD group received HFCD only as a positive control (HFCD group) (Table I).

Table I
Experimental groups

Group	n	Diet	Treatment
Normal	5	Standard chow	None
HFCD	5	HFCD	None
HFCD + Ai	5	HFCD	Ai extract (400 mg/kgBW/day, orally)
HFCD + Gemfibrozil	5	HFCD	Gemfibrozil (31 mg/kgBW/day, orally)

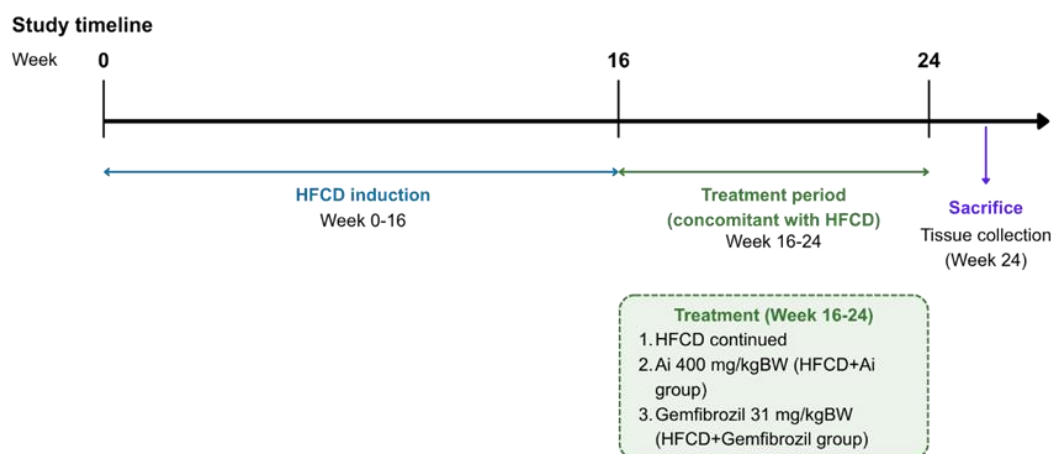


Figure 1.

Study timeline for the total duration of 24 weeks

Ai extract and gemfibrozil were administered orally for 8 weeks. Treatments were given concomitantly with HFCD (Figure 1).

The normal chow consisted of 500 g of Voor 511, 250 g of Spaz fish feed, 150 g of FF 999 fish feed,

100 g of skim milk, one vitamin B-complex tablet, and distilled water added to a final volume of 1 L *per* kilogram of feed. The HFCD was administered by providing 20 - 30 g of standard chow supplemented with 10% cholesterol derived from quail egg yolk *per*

rat, together with 1.5 mL of a 55% fructose solution administered by oral gavage twice daily (equivalent to 1.65 g fructose/day) for 16 weeks. Following the induction period, HFCD administration was continued once daily for an additional 8 weeks concurrently with the treatment regimen. The composition of the HFCD is presented in Table II.

Table II
Results of rat feed analysis (HFCD diet)

Parameter	Unit	Result
Cholesterol	mg/100 g	314.94
Sugar	%	3.41
Glucose	%	1.36
Fructose	%	< 0.17
Vitamin A	mcg/100 g	76.77
Vitamin B3 (Niacinamide)	mg/100 g	13.22
Vitamin B6	mg/100 g	8.42
Vitamin C	mg/100 g	Not detected
Magnesium	mg/100 g	140.62
Iron	mg/100 g	25.34
Calcium	mg/100 g	737.16
Potassium	mg/100 g	691.06
Sodium	mg/100 g	145.94
Moisture Content	%	8.26

Both the normal and HFCD chows were prepared by a research assistant in the Animal Laboratory, Faculty of Medicine, Universitas Indonesia. The nutritional composition of the experimental diets was verified according to the feed composition certificate (Report of Analysis No. SIG.LHP.III.2021.037678) issued by PT Saraswanti Indo Genetech, Bogor, Indonesia.

The 400 mg/kgBW/day dose of Ai extract was selected based on a previous study demonstrating its antihyperlipidemic activity. In Wistar rats fed an atherogenic diet for 10 days, treatment with ethanolic Ai leaf extract at 400 mg/kgBW/day for the subsequent 10 days significantly improved dyslipidemia by reducing total cholesterol and triglyceride levels while increasing HDL cholesterol. The antihyperlipidemic effect was comparable to that of simvastatin, supporting the use of this dose in the present study (18). Meanwhile, the dose of 31 mg/kgBW/day for gemfibrozil was selected based on a previous study using an HFCD-induced hypercholesterolemia model in Sprague-Dawley rats. In that study, treatment with 250 mg/kgBW/day of Ai root extract significantly reduced triglyceride levels and improved liver histopathology, with effects comparable to gemfibrozil as the positive control (19). Accordingly, the same gemfibrozil dose (31 mg/kgBW/day) was adopted in the present study to ensure consistency with the established experimental model.

Distilled water was used as the vehicle for the Ai extract, whereas 1% sodium carboxymethyl cellulose (CMC-Na) was used as the vehicle for gemfibrozil. Both the Ai extract and gemfibrozil were administered

by oral gavage once daily for 8 weeks at a dosing volume of 1 mL *per* rat. We recalculated the dose weekly based on each rat's body weight to maintain the target dose, and adjusted the Ai extract concentration accordingly.

Measurement of obesity parameters

Body weight was measured periodically throughout the study. During the 16-week HFCD induction period, body weight was recorded once monthly after a 12-hour overnight fast in the morning. During the subsequent 8-week treatment period (concomitant with HFCD), body weight was measured once weekly, also following a 12-hour overnight fast in the morning. Naso-anal length was measured from the tip of the nose to the anus (cm), and body mass index (BMI) was calculated as body weight (g) divided by the square of the naso-anal length (cm²). Obesity status was evaluated using the Lee index, calculated as:

$$\sqrt[3]{\text{body weight (g)/naso-anal length (cm)} \times 1000}.$$

Lee Index measurements were taken 2 times: before treatment and before termination. Lee Index measurement at termination (before opening the chest) was done under anesthesia.

For lipid profile analysis, rats were fasted for 12 hours before blood collection. At the end of the experimental period, the rats were anesthetized with a ketamine-xylazine combination and euthanized, after which blood samples were collected by cardiac puncture. Total cholesterol and triglyceride concentrations were measured using the Elabsience Rat Total Cholesterol Colorimetric Assay Kit (Cat. No. E-BC-K109-M) and the Elabsience Rat Total Triglyceride Colorimetric Assay Kit (Cat. No. E-BC-K1238), respectively. HDL and LDL cholesterol levels were determined using LipidPro™ Lipid Profile Test Strips.

Histological analysis of adipose tissue

Mesenteric visceral adipose tissue (50 mg *per* animal) was collected immediately after euthanasia and fixed in 10% neutral buffered formalin. For histopathological evaluation, the tissue was processed using the paraffin-embedding method, which included dehydration in graded ethanol (70%, 95%, and absolute ethanol), clearing in xylene, and paraffin impregnation at 56 - 60°C. The processed tissue was embedded in paraffin blocks, sectioned at 4 µm using a rotary microtome, mounted on glass slides, dried on a hot plate, and stained with hematoxylin and eosin (H&E).

We examined the stained sections under a light microscope at 40× magnification and analyzed digital images using ImageJ software (National Institutes of Health, Bethesda, MD, USA). We quantified adipocyte number and cross-sectional area using the AdipoSum3 automated ImageJ plugin after calibrating it with a stage micrometer. We performed measurements in five randomly selected microscopic fields *per* histological section. We analyzed three tissue sections *per*

treatment group. We calculated the mean adipocyte number and area for each experimental group from all analyzed fields.

We evaluated adipogenesis by calculating the percentage of adipocytes with a cross-sectional area of 1,000 - 4,000 μm^2 , defined as the number of adipocytes within this size range divided by the total number of adipocytes in each field and multiplied by 100. We adapted this analytical approach from Lailan *et al.* (2019). We assessed adipose tissue inflammation by quantifying CLS, defined as macrophage aggregates surrounding dead or hypertrophic adipocytes. We identified CLS manually using the multipoint tool in ImageJ and calculated the mean CLS count for each experimental group. For immunohistochemical analysis, we identified CLS as F4/80-positive macrophages surrounding necrotic adipocytes, consistent with methods described by Altinas *et al.* (2011) and Maliniak *et al.* (2020). All histological scoring and image analyses were performed in a blinded manner. We exported the measurement data to Microsoft Excel and analyzed them using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA).

Adipokine measurement

Levels of adipokines and inflammatory cytokines, including TNF- α , IL-6, leptin, and adiponectin, were measured from mesenteric visceral adipose tissue using enzyme-linked immunosorbent assay (ELISA). Following euthanasia, mesenteric visceral adipose tissue was harvested, washed with 0.9% NaCl, weighed, and stored in sealed containers at - 80°C until analysis. For tissue homogenization, 100 mg of adipose tissue was mixed with 500 μL of cold phosphate-buffered saline (PBS; 0.01 M, pH 7.4) and homogenized using a Handy Sonic homogenizer (Model UR216) under chilled conditions. The homogenate was centrifuged at $3,000 \times g$ for 5 min, and the supernatant was collected. We repeated this centrifugation step once to obtain a clarified supernatant, which we aliquoted and stored at - 20°C until analysis.

The total protein concentration of each adipose tissue homogenate was determined using the Bradford protein assay, and adipokine concentrations were normalized to the total protein content. Leptin concentrations were measured using the Rat Leptin (Lep)

ELISA Kit (Elabscience, Cat. No. E-EL-R0582), adiponectin concentrations using the Rat Adiponectin (ADP/Acrp30) ELISA Kit (Elabscience, Cat. No. E-EL-R0329), TNF- α concentrations using the Rat TNF- α ELISA Kit (MyBioSource, Cat. No. MBS2507393), and IL-6 concentrations using the Rat IL-6 ELISA Kit (MyBioSource, Cat. No. MBS175908). All ELISA kits were rat-specific, and the assays were performed according to the manufacturers' instructions.

RNA extraction and quantitative real-time PCR (qPCR)

The abdominal aortic segment was collected immediately after euthanasia, carefully dissected free of surrounding perivascular adipose tissue, immersed in RNAlater[®] stabilization solution, and stored at - 80°C until RNA extraction. Total RNA was extracted using the Promega SV Total RNA Isolation System (Cat. No. Z5200) according to the manufacturer's instructions. RNA purity was assessed using a NanoDrop spectrophotometer by measuring the A260/A280 ratio, with samples exhibiting a ratio > 2.0 considered acceptable for downstream analysis. RNA concentrations ranged from 51 - 97 ng/ μL in the normal group, 46 - 63 ng/ μL in the HFCD group, 50 - 70 ng/ μL in the HFCD + Ai group, and 41 - 73 ng/ μL in the HFCD + Gemfibrozil group.

For reverse transcription, 0.5 μL of total RNA was converted into complementary DNA (cDNA) using the Toyobo ReverTra Ace[®] qPCR RT Master Mix (Cat. No. FSQ-301) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using SensiFAST[™] SYBR[®] Lo-ROX Mix (Meridian Bioscience/Bioline, Cat. No. BIO-94005) on a LongGene Real-Time PCR System (Model 2000C) to determine the mRNA expression levels of VCAM-1 and ICAM-1. GAPDH was used as the housekeeping gene.

Each PCR reaction contained 10 μL SensiFAST SYBR Lo-ROX Mix, 1.2 μL forward primer (10 μM), 1.2 μL reverse primer (10 μM), 6.1 μL nuclease-free water, and 1.5 μL cDNA template. The amplification protocol consisted of an initial denaturation at 95°C for 2 min (1 cycle), followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 10 s, and extension at 72°C for 10 s.

Table III
Primer sequences

Gene	Primer	Sequence (5'-3')
VCAM-1	Forward	GTG TTT GGC TCC TGA TGT TTA C
	Reverse	GAC TTC TTT GCC ATC TCG TCT A
ICAM-1	Forward	TGT TGG TGT GTT GGT ACT GAT
	Reverse	CTC CTG AGC CTT CTG TAA CTT G
GAPDH	Forward	GAA TGG GAA GCT GGT CAT CAA
	Reverse	CCA GTA GAC TCC ACG ACA TAC T

The primer sequences used were as seen in Table III. Relative gene expression was calculated using the Livak-Schmittgen ($2^{-\Delta\Delta\text{Ct}}$) method. A total of 30 qPCR

reactions were performed, comprising 5 biological samples from each experimental group (Normal, HFCD, HFCD + Ai, and HFCD + Gemfibrozil), 5 no-template

controls (NTCs), and 5 *GAPDH* reactions as the house-keeping gene control.

Molecular docking analysis

Molecular docking analysis was performed to evaluate the interactions between the bioactive compounds of Ai and inflammatory or endothelial adhesion targets associated with obesity-induced vascular inflammation.

A total of 30 bioactive compounds identified from Ai were selected as ligands based on published phytochemical data. Target proteins included TNF- α , VCAM-1, and ICAM-1. Protein structures were obtained from the Protein Data Bank (PDB), including TNF- α (PDB ID: 2AZ5), VCAM-1 (PDB ID: 1VSC) and ICAM-1 (PDB ID: 1IAM).

For protein preparation, we downloaded the receptor structures from the RSCB PDB in .pdb format (20). The receptor chains and native ligands were separated, and all water molecules were removed using AutoDock4. Polar hydrogen atoms were added, non-polar hydrogen atoms were merged, and Gasteiger charges were assigned to the receptors. The prepared receptor structures were subsequently saved in .pdbqt format for docking analysis.

For ligand preparation, the native ligands were isolated from the corresponding receptor-ligand complexes obtained from the PDB. We downloaded three-dimensional structures of the 30 Ai bioactive compounds from ChemSpider and prepared them in .pdb

format using MarvinSketch before docking simulations.

Docking simulations were performed using AutoDock4 on a computer running macOS Mojave version 10.14.6 equipped with an Intel® Core™ i9 processor and 16 GB RAM. Before docking, we redocked the native ligands to validate the docking protocol and parameters. Docking results were evaluated based on binding free energy (ΔG), binding conformation, hydrogen bond interactions, and hydrophobic interactions.

We performed targeted docking for the TNF- α protein using grid boxes centered on known ligand-binding pockets identified from corresponding receptor-ligand crystal structures. We performed blind docking for VCAM-1 and ICAM-1. Because neither VCAM-1 nor ICAM-1 has a co-crystallized native ligand available for validation, we defined the docking grid based on their known protein-protein interaction interfaces. For VCAM-1, we performed molecular docking at the VLA-4 binding site (domain 1), which contains the key amino acid residues involved in VLA-4 recognition. The selected binding site comprised the residues Ile39, Asp40, Lys46, Ser54, and Thr72. For ICAM-1, molecular docking was performed at the LFA-1 binding site (domain 1), which includes the critical amino acid residues responsible for LFA-1 binding. The docking grid was centered on the residues Glu34, Lys39, Met64, Tyr66, Asn68, and Gln73. The grid box parameters were as shown in Table IV.

Table IV

Protein structures and docking parameters

Protein	PDB ID	Resolution (Å)	Organism	Dimension (X × Y × Z)	Center coordinates (x, y, z)	Docking type	Presence of druggable pocket
TNF- α	2AZ5	2.1	<i>Homo sapiens</i>	40 × 40 × 40	(-19.163, 74.417, 33.837)	Targeted	Chain A
VCAM-1	1VSC	1.9	<i>Homo sapiens</i>	50 × 50 × 50	(44.097, 14.639, 14.964)	Blind docking	Domain 1, interface VLA-4
ICAM-1	1IAM	2.1	<i>Homo sapiens</i>	50 × 50 × 50	(16.719, 67.203, 17.063)	Blind docking	Domain 1, interface LFA-1

To validate the docking protocol, we extracted the co-crystallized ligand from TNF- α and redocked it into its original binding site. The docking protocol was considered valid when the root mean square deviation

(RMSD) between the crystallographic and predicted ligand poses was less than 2.0 Å. The RMSD values for TNF- α were below this threshold (Table V).

Table V

Redocking validation

Protein Target	PDB ID	RMSD (Å)	Estimated free energy of binding (kcal/mol)	Estimated inhibition constant/Ki (μM)
TNF- α	2AZ5	1.89	-6.04	37.67
VCAM-1	1VSC	-	-	-
ICAM-1	1IAM	-	-	-

Molecular docking was performed using AutoDock4 employing the Lamarckian Genetic Algorithm (LGA). The docking parameters were set to 100 genetic algorithm (GA) runs, a population size of 150 individuals, and 2,500,000 energy evaluations. All other parameters

were maintained at the AutoDock4 default settings. Compounds with the strongest binding affinities included nicotiflorin, kaempferol glycosides, biorobin, gallic acid, hesperetin, mauritanin, stigmasterol, chebulagic acid, and repandusinic acid. Among these, nicotiflorin

demonstrated the most favorable interactions with VCAM-1 and ICAM-1 and was considered the representative bioactive compound contributing to the anti-inflammatory and anti-atherosclerotic effects of Ai. Redocking of the native ligands was performed to validate the docking protocol before the docking simulations, especially for the protein target that has a co-crystallized ligand; in this study, it was carried out on TNF- α .

Statistical analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). Before statistical analysis, we assessed data normality using the Shapiro-Wilk test and homogeneity of variance using Levene's test. Data that satisfied the assumptions of normality and homogeneity were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Data that did not meet the normality assumption were analyzed using the Kruskal-Wallis

test followed by Mann-Whitney U tests for pairwise comparisons. A p-value < 0.05 was considered statistically significant. Unless otherwise indicated, n = 5 represents the number of biological replicates (animals) *per* experimental group, and error bars in all figures represent the SEM.

Results and Discussion

Effects of Ai on obesity parameters

After 16 weeks of HFCD induction, the mean body weight of HFCD rats was 347.18 ± 13.16 g, compared to the mean body weight of normal diet rats, which was 224.8 ± 8.59 g ($p < 0.0001$). The body weight of HFCD rats increased significantly by 1.5x compared to normal rats. The mean Index Lee of HFCD rats was 281.02 ± 1.97 , compared to the mean Index Lee of rats with a normal diet, which was 252.64 ± 4.07 ($p = 0.008$), as described in Figure 2. We randomized the HFCD rats and divided them into HFCD, HFCD + Gemfibrozil, and HFCD + Ai groups.

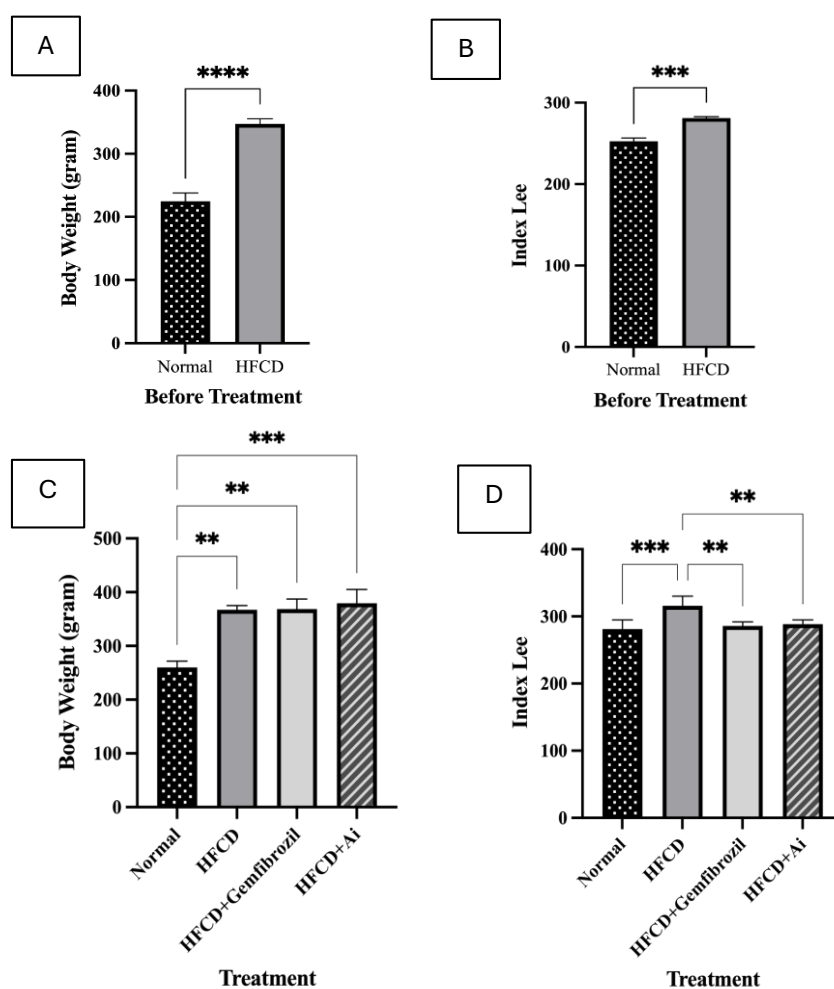


Figure 2.

Body weight and Index Lee of male *Sprague-Dawley* (SD) rats induced by high fructose cholesterol diet (HFCD) before and after administration of Ai roots ethanol extract (400 mg/kgBW) or gemfibrozil (31 mg/kgBW) as a control. A. Body weight (gram) and B. Index Lee of SD rats after induced by HFCD for 16 weeks. C. Body weight (gram) and D. Index Lee of SD rats after treatment by Ai or gemfibrozil along with HFCD

After 8 weeks of treatment, the mean body weight of the Normal, HFCD, HFCD + Gemfibrozil, and HFCD + Ai groups was 271.25 ± 3.11 , 367.4 ± 7.72 , 368.4 ± 18.83 , and 379.00 ± 25.60 grams, respectively. The mean Index Lee values of the Normal, HFCD, HFCD + Gemfibrozil, and HFCD + Ai groups were 281.24 ± 6.14 , 315.92 ± 6.4 , 285.77 ± 2.68 , and 288.36 ± 2.88 , respectively. The index Lee value of the HFCD + Ai group indicated that Ai could prevent the rats from becoming obese, as well as gemfibrozil, as shown in Figure 2. Ai treatment also improved cholesterol, triglyceride, and HDL levels, although some parameters were not statistically significant. The gemfibrozil-treated group showed similar effects.

HFCD-induced obesity is widely used as an experimental model because excessive fructose and cholesterol intake promotes fat accumulation, dyslipidemia, and metabolic disturbances resembling the metabolic syndrome in humans. Increased body weight and Lee index observed in HFCD rats indicated successful induction of obesity (21).

Long-term dyslipidemia contributes to the development of cardiovascular complications associated with obesity (22). The improvement of obesity parameters after Ai administration suggests that Ai possesses metabolic regulatory activity. Previous studies demonstrated that Ai could reduce triglyceride levels and improve insulin resistance, which may be related to its flavonoid and polyphenol contents (23, 24). These compounds regulate lipid metabolism, reduce oxidative stress, and improve metabolic homeostasis. The improvement observed in the Ai-treated group was comparable to gemfibrozil, suggesting that Ai may have beneficial effects against obesity-related metabolic disturbances (25).

Effect of Ai on adipose tissue inflammation

Histological examination of visceral adipose tissue demonstrated increased CLS as an inflammatory-obesity marker in HFCD rats compared with normal rats. CLS were characterized by macrophage accumulation surrounding dead or hypertrophic adipocytes. Ai administration reduced the number of CLS in visceral adipose tissue compared with HFCD rats (Figure 3). Consistent with the histological findings, the lower Lee index in the HFCD + Ai group suggests that Ai may protect against obesity and associated adipose tissue inflammation.

Adipokine analysis showed that HFCD rats had increased TNF- α , IL-6, and leptin levels with decreased adiponectin levels. Ai treatment significantly reduced TNF- α , IL-6, and leptin levels while increasing adiponectin levels compared with untreated HFCD rats (Figure 4).

TNF- α levels in the experimental animals were as follows: Normal group 40.91 ± 16.52 ; HFCD group 58.63 ± 19.53 ; HFCD + Gemfibrozil group 34.60 ± 7.28 ; HFCD + Ai group 25.71 ± 7.25 pg/mg (Figure 4A). IL-6 levels in the experimental animals for the Normal, HFCD, HFCD + Gemfibrozil, and HFCD + Ai groups were as follows: 207.31 ± 83.64 , 239.91 ± 110.70 , 118 ± 20.04 , and 94.07 ± 15.51 pg/mg (Figure 4B). Ai could significantly decrease TNF- α ($p = 0.0081$) and IL-6 levels in experimental animals that received HFCD ($p = 0.0224$).

On the other hand, leptin levels in the experimental animals for the normal, HFCD, HFCD + Gemfibrozil, and HFCD + Ai groups were as follows: 0.314 ± 0.08 , 8.318 ± 0.906 , 0.221 ± 0.06 , and 0.337 ± 0.24 ng/mg (Figure 4D). Ai can improve leptin levels significantly ($p = 0.0008$), the same as gemfibrozil ($p = 0.0001$), in experimental animals that received HFCD. Adiponectin levels in the experimental animal groups that were given a normal diet, HFCD, gemfibrozil, and Ai were as follows: 21.60 ± 4.3 , 16.09 ± 4.14 , 25.48 ± 11.73 , and 30.91 ± 9.72 mg/L (Figure 4C). The data showed that administration of Ai extract could increase adiponectin levels compared to rats that received HFCD only, as shown in Figure 3, although it was not statistically significant.

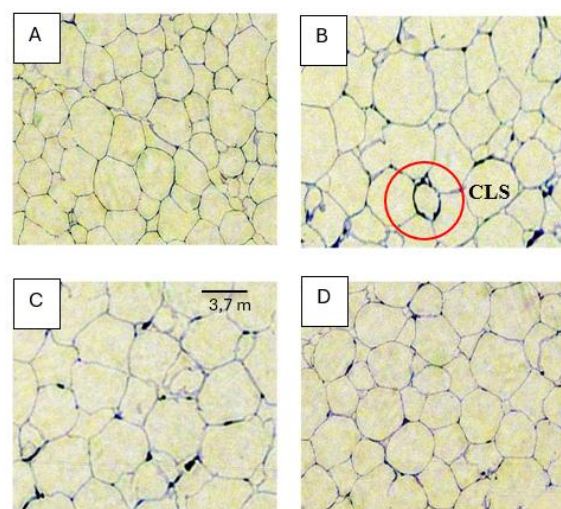


Figure 3.

Histopathologic changes in adipose tissue (HEX40).

A. Control; B. HFCD; C HFCD + Gemfibrozil 31 mg/kgBW; D. HFCD + Ai 400 mg/kgBW. The adipose tissue sections were stained with hematoxylin and eosin (H&E stain). Examined by microscope Olympus CX21, 40x magnification

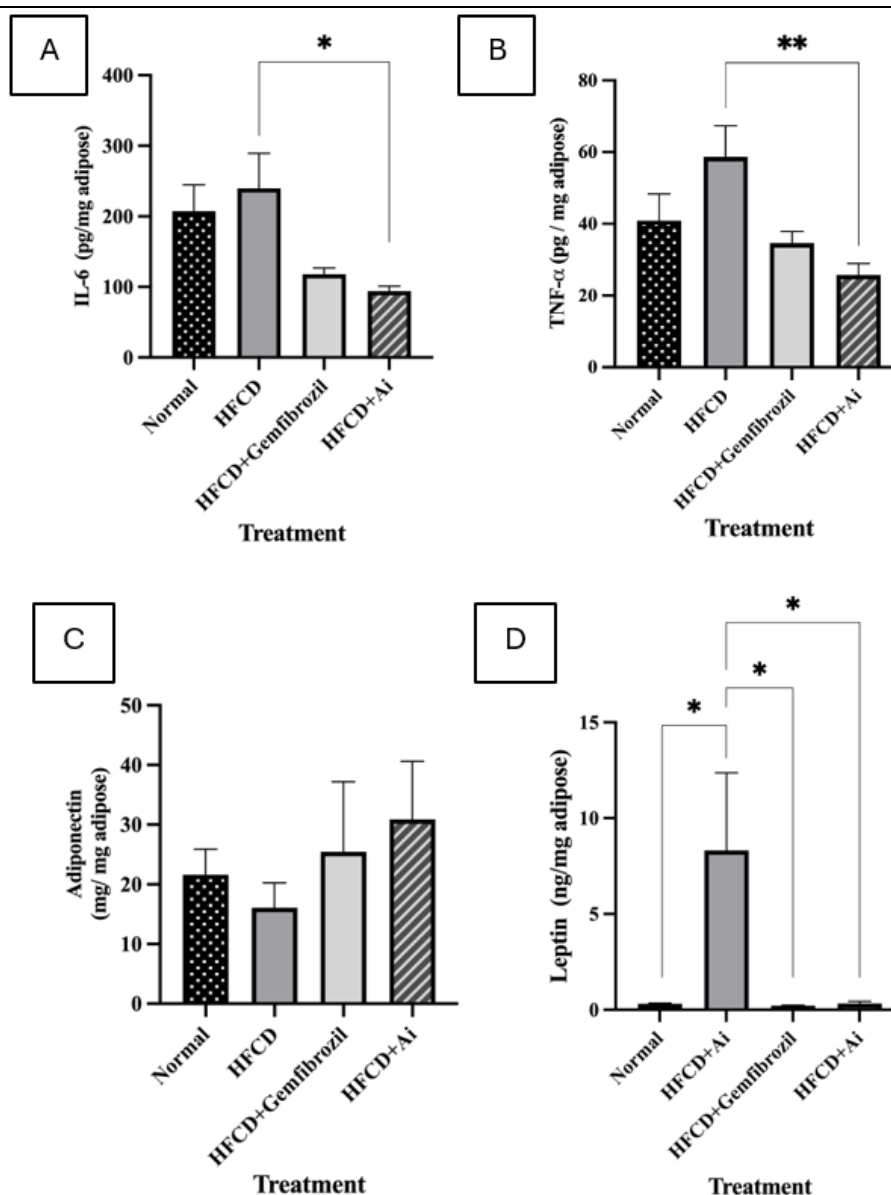


Figure 4.

Adipokines level of visceral adipose in male *Sprague-Dawley* (SD) rats induced by HFCD after administration of Ai roots ethanol extract (400 mg/kgBW) or gemfibrozil (31 mg/kgBW) as a control. A. TNF- level. B. IL-6 level.

C. Adiponectine level. D. Leptin level. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$

Obesity is closely associated with chronic low-grade inflammation. In obese conditions, hypertrophic adipocytes release chemokines that recruit macrophages into adipose tissue, leading to CLS formation. These macrophages produce inflammatory cytokines that perpetuate adipose inflammation and contribute to obesity-related complications (26).

The increased CLS observed in HFCD rats indicated active macrophage infiltration and adipose tissue inflammation. The reduction in CLS following Ai treatment suggests that Ai attenuated inflammatory responses in adipose tissue. Adipokines play an important role in obesity-associated inflammation. TNF- α and IL-6 are pro-inflammatory cytokines involved in insulin resistance and metabolic dysfunction, whereas leptin is associated with adiposity and chronic

inflammation. In contrast, adiponectin exerts anti-inflammatory and insulin-sensitizing effects (27).

Ai's ability to decrease TNF- α , IL-6, and leptin levels while increasing adiponectin suggests it may restore adipokine balance and improve the inflammatory conditions associated with obesity. These effects likely relate to the anti-inflammatory and antioxidant activities of flavonoids and polyphenols in Ai. Higher levels of adiponectin in Ai rats might reflect a protective and compensatory mechanism by certain macrophages in the visceral adipocytes. Additionally, the production and secretion of anti-inflammatory adipokines are more typical of M2 macrophages, while the production and secretion of pro-inflammatory adipokines are more typical of M1 macrophages (25, 27). HFCD intake produced the largest increase in TNF- α , IL-6, and

leptin levels, which have previously been shown to be increased in the adipose tissue of obese animals (28). Therefore, an HFCD-induced increase in pro-inflammatory adipokines in HFCD rats might be the result of the increased prevalence of CLS seen in the visceral fat of those rats. In Ai rats, HFCD exposure was associated with a reduction in the number of CLS. This aligns with the finding that adipokine levels in the visceral fat depots of Ai rats did not deteriorate after HFCD administration; immunohistology results further support this, showing no exacerbation of CLS formation in this tissue. Overall, these results support the notion that Ai rats possess resistance to visceral adipose tissue inflammation when exposed to an HFCD.

Natural products, especially those rich in polyphenols such as flavonoids, have shown great therapeutic value for the prevention and treatment of obesity and its relation to metabolic disorders (17, 29). Anhe *et al.* found that a polyphenol-rich cranberry extract could prevent diet-induced obesity and insulin resistance and alleviate intestinal inflammation (30).

These previous studies are consistent with the fact that Ai is an herb rich in polyphenol and flavonoid compounds, based on the results of its phytochemical tests and HPLC assay.

Effect of Ai on VCAM-1 and ICAM-1 expression

Real-time PCR analysis demonstrated increased VCAM-1 and ICAM-1 expression in the aortic tissue of HFCD rats compared with normal rats, indicating endothelial dysfunction and vascular inflammation.

There was an increase in VCAM-1 and ICAM-1 expression in rats fed the HFCD compared with the normal diet. The relative expression of VCAM-1 increased to 3.198 ± 0.84 (approximately 3.0-fold), while ICAM-1 increased to 1.638 ± 0.83 (approximately 1.6-fold) ($p = 0.47$ and $p = 0.8$, respectively). Administration of Ai resulted in changes in VCAM-1 and ICAM-1 expression to 2.390 ± 1.071 and 1.33 ± 0.31 , respectively, although all these results were not statistically significant, as shown in Figure 5. Gemfibrozil treatment also did not appear to significantly reduce VCAM-1 and ICAM-1 expression ($p = 0.99$ and $p = 0.97$, respectively).

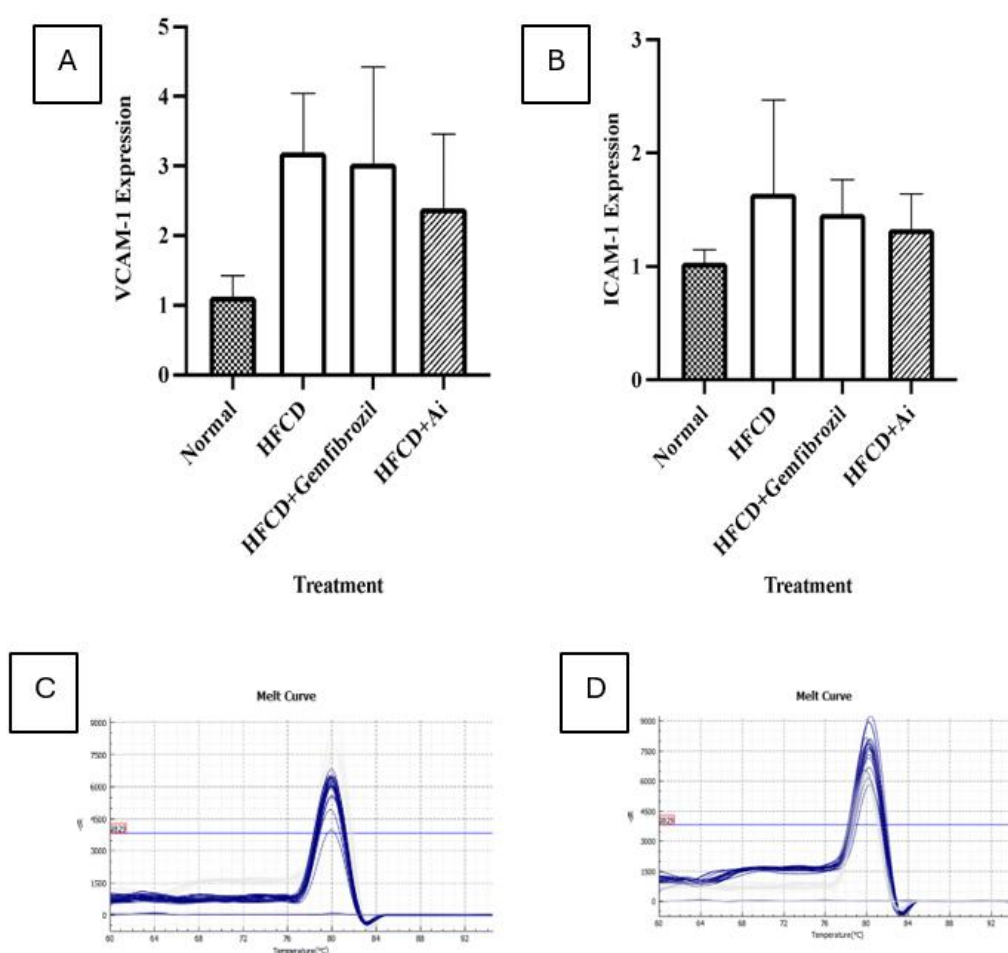


Figure 5.

VCAM-1 and ICAM-1 expression and melting curve from aorta Sprague-Dawley rats which induced by HFCD.

A. VCAM-1 expression; B. ICAM-1 expression; C. Melting curve of VCAM-expression; D. Melting curve of ICAM-1 expression

Endothelial dysfunction is considered the earliest stage of atherosclerosis and is characterized by increased expression of vascular adhesion molecules such as VCAM-1 and ICAM-1. These adhesion molecules mediate leukocyte adhesion and transendothelial migration into the vascular wall during the inflammatory process of atherosclerosis (8).

HFCD administration increased VCAM-1 and ICAM-1 expression, indicating activation of vascular inflammatory pathways. The reduction of VCAM-1 and ICAM-1 expression after Ai treatment suggests that Ai may have endothelial protective activity. From previous studies, we knew that nicotiflorin or kaempferol-3-O-rutinoside, is a flavonoid glycoside that was first extracted from a traditional Chinese medicine, *Flos carthami*, which has anti-glycation and hepatoprotective activity (28). Nicotiflorin is also produced by *Tricyrtis maculata* (D. Don) Machride, mainly distributed in northwest China, and the entire plant has been used as an important folk medicine for the treatment of bruises, cough, tuberculosis, and ischemic cerebrovascular disease. Nicotiflorin also has anti-inflammatory, antioxidant, analgesic, and neuroprotective properties, according to modern pharmacological investigations (17). At the cellular level, nicotiflorin was able to upregulate the expression of mRNA and protein levels by enhancing the catalytic activity of eNOS in endothelial cells (29). Furthermore, nicotiflorin has been demonstrated to diminish the volume of infarction induced by ischemic injury, as well as oxidative stress and inflammatory response in mice (17). These data gave a strong suggestion that Ai may decrease VCAM-1 and ICAM-1 expression, which is mediated by its flavonoid compounds, nicotiflorin. This is presumably because the results of previous studies showed us that nicotiflorin could increase the expression of eNOS, which could improve endothelial dysfunction, leading to VCAM-1 and ICAM-1 suppression (29). The lack of statistical significance

may reflect biological variability and the small number of experimental animals. Nevertheless, the consistent decrease in adhesion molecule expression indicates that Ai has the potential to suppress endothelial dysfunction associated with obesity and metabolic syndrome. The suppression of VCAM-1 and ICAM-1 may be related to the flavonoid compounds contained in Ai, which are known to reduce oxidative stress and inflammatory signaling pathways involved in endothelial activation (30).

Molecular docking analysis of Ai compounds

Molecular docking analysis identified several active compounds from Ai that interacted with inflammatory and endothelial adhesion targets, including nicotiflorin, kaempferol glycosides, biorobin, gallic acid, hesperetin, mauritanin, stigmasterol, chebulagic acid, and repandusinic acid.

Among these compounds, nicotiflorin showed the strongest binding affinity toward VCAM-1 (- 9.22 kcal/mol) and ICAM-1 (- 6.31 kcal/mol). Docking analysis demonstrated that nicotiflorin formed hydrogen bond interactions with Trp35, Asn49, Phe33, Gly45, Leu43, Asn44, Pro42 in VCAM-1, along steric interaction with Ser34, Val47, Leu43, Pro42, Ser41 (Figure 6).

In ICAM-1, nicotiflorin formed hydrogen bond interactions with Asn68, Gln73, Thr75 and steric interactions with Glu34, Gln73, Tyr66, Thr75, Met64, Lys77 (Figure 7).

Docking analysis also demonstrated favorable interactions between Ai compounds and adipokine-related target including TNF- α (Table VI).

The molecular docking findings support the *in vivo* results showing Ai's anti-inflammatory and endothelial protective effects. Among the tested compounds, nicotiflorin showed the strongest binding affinity for VCAM-1 and ICAM-1, suggesting it may be the major active compound responsible for suppressing endothelial inflammation.

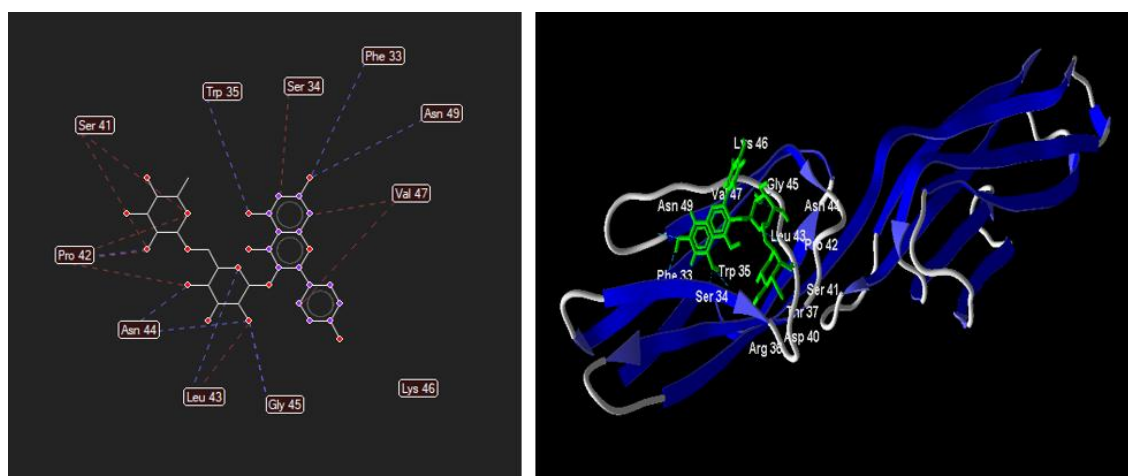
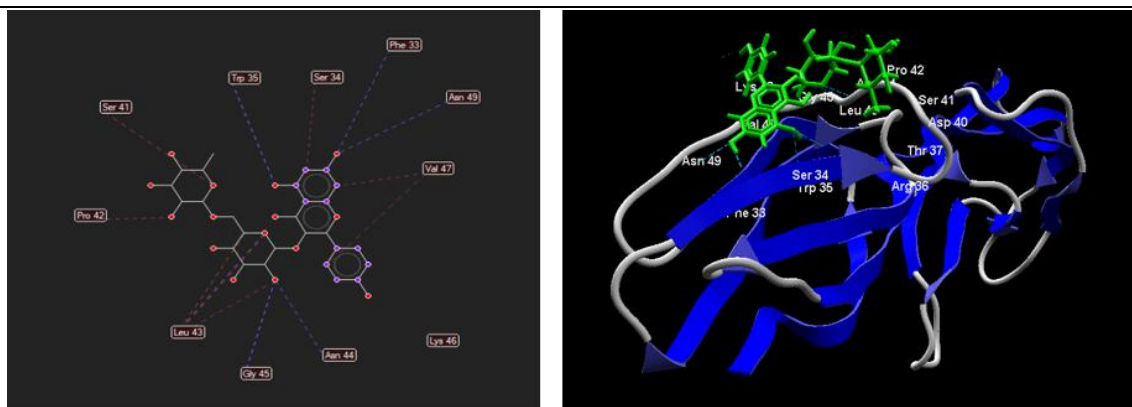


Figure 6.

Visualization interaction 2D and 3D between VCAM-1 and nicotiflorin

**Figure 7.**

Visualization interaction 2D and 3D between ICAM-1 and nicotiflorin

Table VIBonding Energy and Residue Components of Ai compounds that can bind to adiponectin, leptin, TNF- α , IL-6

No.	Molecule	ΔG (kcal/mol)	Hydrogen Bonding with Protein Target
1.	TNF-α		
	Kaempferol	-7.56	Met148, Tyr145
	Mauritanin	-7.64	Tyr145, Thr251
2.	VCAM-1		
	Nicotiflorin	-9.22	Trp35, Asn49, Phe33, Gly45, Leu43, Asn44, Pro42
	Mauritanin	-8.60	Ser34, Leu43, Gly45, Trp35, Phe33, Asn49
3.	ICAM-1		
	Biorobin	-6.91	Val4, Ser3, Gln1, Ser74, Thr75, Gln74
	Nicotiflorin	-6.31	Asn68, Gln73, Thr75

Nicotiflorin, also known as kaempferol-3-O-rutinoside, has previously been reported to possess antioxidant, anti-inflammatory, hepatoprotective, analgesic, and neuroprotective activities. Previous studies also demonstrated that nicotiflorin could increase eNOS activity and reduce oxidative stress and inflammatory responses (17). The interaction between nicotiflorin and I κ B suggests possible modulation of NF- κ B signaling pathways. NF- κ B is a major regulator of inflammatory gene expression, including cytokines and adhesion molecules. Inhibition of NF- κ B activation may therefore contribute to reduced expression of VCAM-1, ICAM-1, TNF- α , and IL-6 observed in this study (31).

Phytotherapy generally modulates multiple biochemical pathways simultaneously. Therefore, the anti-inflammatory effects of Ai may involve combined regulation of adipokines, oxidative stress, inflammatory cytokines, and endothelial adhesion molecules. The integrated modulation of adipose and vascular inflammation observed in this study suggests that Ai has potential therapeutic value against the obesity-atherosclerosis axis (31, 32).

Conclusions

Ai showed beneficial effects against obesity-related inflammation and endothelial dysfunction in HFCD-induced Sprague-Dawley rats. The Ai administration improved the obesity parameters, reduced the crown-

like structure formation in adipose tissue, and improved adipokine balance by decreasing TNF- α , IL-6, and leptin levels while increasing adiponectin levels. Ai treatment also reduced VCAM-1 and ICAM-1 expression in aortic tissue, indicating improvement of vascular inflammation associated with early atherosclerosis. Molecular docking analysis suggested that flavonoid compounds, particularly nicotiflorin, may contribute to these effects through interactions with inflammatory cytokines and adhesion molecules. Overall, the findings indicate that Ai has the potential to modulate both adipose and vascular inflammation through multiple mechanisms. These results support the possible use of Ai as a complementary therapeutic agent for obesity-associated metabolic and vascular complications.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article. The other

data generated in the present study may be requested from the corresponding author.

Ethics approval and consent to participate

All experimental procedures were approved by Ethics Committee of the Faculty of Medicine, Universitas Indonesia (approval No. KET-996/UN2.FI/ETIK/PPM.00.02/2019).

AI use disclosure

During the preparation of this manuscript, the author used ChatGPT for language improvement, specifically to improve spelling, grammar, word choice, readability, and clarity. The authors reviewed and revised all outputs from these tools and takes full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

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