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**Cardamonin and Alpinetin Mitigate Fructose-Induced Endothelial Damage: An *In Vitro*
Evaluation Using HUVEC Model**

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ABSTRACT

Background: Endothelial dysfunction is a pivotal mechanism in hypertension and cardiovascular disease. Excessive dietary fructose accelerates vascular injury by reducing nitric oxide (NO) bioavailability, activating inflammatory cascades, and upregulating adhesion molecules. Natural phytochemicals such as cardamonin and alpinetin possess antioxidant and vasoprotective properties, yet their efficacy against fructose-induced endothelial stress remains insufficiently characterized.

Objective: To evaluate and compare the prophylactic and therapeutic effects of cardamonin and alpinetin against fructose-induced endothelial dysfunction in human umbilical vein endothelial cells (HUVECs), using losartan as a clinical benchmark.

Methods: HUVECs were exposed to fructose-induced metabolic injury and treated under pre-conditioning (prophylactic) and post-conditioning (therapeutic) regimens. Cytotoxicity was assessed by microscopy and MTT assays to define safe concentration ranges. Cytoprotective efficacy was quantified via viability assays, while mechanistic insights were obtained through RT-qPCR and agarose gel electrophoresis of eNOS, RAGE, Endothelin-1, and ICAM-1 transcripts.

Results: Alpinetin and losartan exhibited broad cytocompatibility, whereas cardamonin showed a narrower safety margin. In prophylactic assays, alpinetin and losartan preserved endothelial monolayers, while cardamonin provided limited cluster protection. In therapeutic assays, cardamonin demonstrated potent low-dose rescue, whereas alpinetin and losartan achieved recovery at higher concentrations. Molecular analyses confirmed that post-conditioning more effectively restored eNOS and suppressed RAGE and Endothelin-1, while pre-conditioning strongly reduced ICAM-1.

Conclusion: Cardamonin, alpinetin, and losartan confer distinct cytoprotective benefits against fructose-induced endothelial injury. Cardamonin excels in post-injury rescue, whereas alpinetin and losartan act as robust prophylactic stabilizers. These findings highlight the translational potential of phytochemicals as complementary strategies for mitigating metabolic endothelial dysfunction.

SIGNIFICANCE STATEMENT

This study demonstrates that cardamonin and alpinetin, two natural phytochemicals, confer distinct protective effects against fructose-induced endothelial dysfunction, complementing the

clinical efficacy of losartan. By delineating their prophylactic and therapeutic strengths, the work highlights phytochemicals as promising adjuncts for mitigating metabolic vascular injury. These findings broaden translational strategies for cardiovascular prevention and treatment, underscoring the potential of dietary bioactives to address endothelial stress in the context of modern metabolic disorders.

KEYWORDS: Cardamonin, Alpinetin, Fructose, Endothelial damage, Cardiovascular disorders

INTRODUCTION

Cardiovascular diseases (CVDs) remain the leading global health challenge. Global CVD mortality rose from 13.1 million in 1990 to an estimated 19.2 million deaths in 2023, accompanied by a 1.4-fold increase in disability-adjusted life years (DALYs)[1-3]. Projections for 2050 predict a 90% surge in CVD prevalence and 35.6 million annual deaths[4-6]. While demographic growth and aging drive this absolute escalation, stark regional disparities exist, with low- and middle-income countries bearing a disproportionate burden. Crucially, nearly 80% of cardiovascular DALYs stem from modifiable risk factors like hypertension, obesity, and poor diet, emphasizing the preventable nature of this crisis [7-9].

Endothelial dysfunction is a primary mechanism initiating and driving hypertension. The vascular endothelium regulates vascular tone, leukocyte adhesion, and platelet aggregation by synthesizing nitric oxide (NO) via endothelial nitric oxide synthase (eNOS)[10-13]. Impaired eNOS activity reduces NO bioavailability, triggering vasoconstriction and arterial stiffening. Oxidative stress accelerates this damage; excessive reactive oxygen species (ROS) degrade NO and uncouple eNOS, shifting the vascular environment toward a pro-inflammatory and pro-thrombotic state that exacerbates hypertension[14-19].

Excessive dietary fructose intake significantly accelerates this endothelial injury. Unlike glucose, fructose metabolism bypasses key glycolytic regulatory steps, triggering rapid ATP depletion, hepatic lipogenesis, and an intracellular surge of uric acid[20-21]. This accumulation of uric acid acts as a primary catalyst for oxidative stress, fueling the generation of advanced glycation end-products (AGEs) and driving the activation of the receptor for advanced glycation end products (RAGE). This uric acid–RAGE cross-talk triggers pro-inflammatory cascades like NF- κ B, which elevates vasoconstrictors like endothelin-1 (ET-1), suppresses eNOS activity, and depleting NO[22-24]. This metabolic breakdown induces insulin resistance, dyslipidemia, mitochondrial dysfunction, and endothelial apoptosis, making fructose-induced injury an ideal translational model for metabolic syndrome and early vascular disease[25-29].

Human umbilical vein endothelial cells (HUVECs) offer a robust, widely accepted *in vitro* model to study these pathways. HUVECs retain key physiological traits of the macrovascular endothelium, including NO synthesis, shear stress responsiveness, and inflammatory signaling regulation. Their reproducibility makes them an excellent platform for

evaluating interventions to restore endothelial homeostasis[30-34]. In experimental designs modelling endothelial injury, the angiotensin II receptor blocker losartan is widely utilized as a positive control; it restores endothelial function by blocking AT1 receptor-mediated oxidative stress, downregulating adhesion molecules, and rescuing NO signaling[35-38]. However, there is growing interest in benchmarking natural phytochemicals against such standard therapies due to their multi-target antioxidant, anti-inflammatory, and vasoprotective properties[39-44].

Among these, the chalcone cardamonin (found in cardamom) and the Alpinia-derived flavonoid alpinetin stand out. Both exhibit potent free radical scavenging, anti-inflammatory, and direct vasodilatory effects[45-50]. However, their specific efficacy in mitigating fructose-induced endothelial dysfunction remains largely unexplored.

Therefore, this study evaluates and compares the protective activities of cardamonin and alpinetin against fructose-induced injury in HUVECs to elucidate their underlying molecular mechanisms and evaluate their potential as therapeutic or prophylactic agents. Utilizing cytotoxicity and cytoprotective assays alongside molecular analyses of eNOS, ET-1, ICAM-1, and RAGE expression, we benchmark these phytochemicals against losartan as a standard reference drug. These findings aim to differentiate between the preventive and restorative capabilities of these compounds, providing novel mechanistic insights to support their future evaluation in *in vivo* models of metabolic hypertension.

2. METHODOLOGY

2.1 MATERIALS AND REAGENTS

The Human Umbilical Vein Endothelial Cell (HUVEC) line along with the corresponding culture media was procured from HiMedia Laboratories. The chemical compounds utilized in this study—cardamonin (CAS No. 19309-14-9), alpinetin (CAS No. 36052-37-6), losartan potassium (CAS No. 124750-99-8), and fructose (CAS No. 57-48-7)—were obtained from Sigma-Aldrich. Unless otherwise specified, all reagents and chemicals employed for cell culture and experimental procedures were of analytical grade.

2.2 CELL CULTURE TECHNIQUE

Human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under standard culture conditions at 37°C in a humidified atmosphere containing 5% CO₂, as previously described [51].

2.3 CYTOTOXICITY EVALUATION BY MICROSCOPIC ASSAY

After 24 h of initial seeding, the growth medium in the 96-well plates was aspirated and replaced with freshly prepared test compounds at serial concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL. Each concentration was tested in triplicate, with untreated cells maintained in parallel as negative controls. Plates were incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO₂. Morphological changes across the wells were examined using an inverted phase-contrast microscope (Olympus CKX41) equipped with a digital imaging system (Optika Pro5 CCD) to capture representative images. Cytotoxicity was qualitatively assessed based on deviations from control morphology, including cell rounding, shrinkage, cytoplasmic granulation, and vacuolization.

2.4 CYTOTOXICITY ASSAY BY MTT METHOD:

Cell viability was quantitatively assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. A stock solution was prepared by dissolving 15 mg of MTT powder (Sigma-Aldrich, Cat. No. M-5655) in 3 mL phosphate-buffered saline (PBS) to yield a concentration of 5 mg/mL, followed by filter sterilization. After 24 h of compound exposure, the treatment medium was aspirated and 30 µL of the MTT solution was added to each experimental and control well. Plates were gently agitated and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 4 h to allow formazan crystal formation. The supernatant was then removed, and 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) was added to solubilize the crystals. Optical density (OD) was measured at 540 nm using a microplate reader [52].

The percentage of growth inhibition was calculated using the formula:

$$\text{Percentage of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

2.5 DOSE SELECTION AND EXPERIMENTAL DESIGN FOR CYTOPROTECTIVE ASSAY: PROPHYLACTIC EFFECT AND TREATMENT EFFECT

Based on preliminary cytotoxicity screenings, non-toxic working concentration ranges were selected for each compound by choosing doses below their respective cytotoxic thresholds to assess efficacy against fructose-induced endothelial injury. Experimental groups were designated as follows: a negative control (untreated cells), a positive injury control (cells exposed to fructose), and treatment groups receiving varying concentrations of test compounds in combination with fructose. Once HUVECs reached optimal confluence in multi-well plates,

evaluations were conducted under two distinct regimens: pre-conditioning (prophylactic) and post-conditioning (therapeutic).

- Pre-conditioning model: Cells were pre-exposed for 24 h to optimized concentrations of alpinetin (0.75, 1.5, 3.1, 6.25, 12.5 $\mu\text{g/mL}$), cardamonin (0.18, 0.37, 0.75, 1.5, 3.1 $\mu\text{g/mL}$), or losartan (1.5, 3.1, 6.25, 12.5, 25 $\mu\text{g/mL}$), followed by a 24 h challenge with 2.5 mM fructose.
- Post-conditioning model: Cells were first exposed to 2.5 mM fructose for 24 h to induce endothelial injury, after which the medium was replaced with compound treatments at the same optimized concentrations for an additional 24 h.

To ensure timeline consistency, positive injury control groups were maintained under continuous 2.5 mM fructose exposure for the full 48 h duration[53]. All treatments were performed in triplicate alongside parallel controls. Following completion of the 48 h protocols, monolayer integrity and phenotypic alterations were examined using an inverted phase-contrast microscope (Olympus CKX41) equipped with a high-resolution digital imaging system (Optika Pro5 CCD). Representative photomicrographs were captured to document treatment-induced changes. Cytoprotective and therapeutic effects of alpinetin, cardamonin, and losartan were qualitatively evaluated based on their ability to prevent or mitigate characteristic morphological indicators of fructose-induced endothelial injury, including cell rounding, shrinkage, detachment, and vacuolization[54].

2.6 CYTOPROTECTIVE ASSAY BY MTT

The cytoprotective efficacy of alpinetin, cardamonin, and losartan against fructose-induced endothelial injury was assessed using the MTT cell viability assay. HUVECs were seeded into 96-well plates at a density of 1×10^4 cells per well and allowed to adhere for 24 h. Cells were then subjected to two distinct treatment protocols designed to evaluate both preventive and therapeutic capacities:

- Pre-conditioning (Prophylactic) regimen: Cells were pre-incubated for 2 h with alpinetin (0.75–12.5 $\mu\text{g/mL}$), cardamonin (0.18–3.1 $\mu\text{g/mL}$), or losartan (1.5–25 $\mu\text{g/mL}$), followed by a 24 h challenge with 2.5 mM D-(–)-fructose.
- Post-conditioning (Therapeutic) regimen: Cells were first exposed to 2.5 mM D-(–)-fructose for 24 h to induce endothelial injury, after which the medium was

replaced with the respective compound treatments at the same concentration ranges for an additional 24 h.

Following completion of the exposure periods, cell viability was quantified using the MTT assay as described in Section 2.4, including MTT incubation, formazan solubilization, and spectrophotometric measurement of absorbance at 570 nm[52].

2.7 ISOLATION OF TOTAL RNA TRIzol METHOD

Total RNA was isolated from treated and control HUVECs using TRIzol® reagent (Invitrogen, USA) according to the manufacturer's instructions and the method originally described by Chomczynski and Sacchi [55]. RNA purity and concentration were determined spectrophotometrically before further analysis.

2.8 cDNA SYNTHESIS

First-strand cDNA was synthesized from isolated RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol [56]. The synthesized cDNA was subsequently used as a template for quantitative real-time PCR analysis.

2.9 GENE EXPRESSION ANALYSIS BY qRT-PCR

Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (G BIOSCIENCES, Product code-786-5062) using Lightcycler 96 (Roche). All reactions were performed in triplicates and data were analyzed according to $\Delta\Delta C_t$ method (using Light Cycler 96 SW 1.1 Software)[57]. The primer sequences used were summarized in table 1.

OLIGO NAME	FORWARD		REVERSE	
	SEQUENCE (5' ->3')	Tm	SEQUENCE (5' ->3')	Tm
eNOS[58]	GAAGGCGACAATCCTGTATGG C	62.1	TGTTTCGAGGGACACCACGTCAT	62.1
RAGE[59]	CACCTTCTCCTGTAGCTTCAGC	62.1	AGGAGCTACTGCTCCACCTTCT	62.1
Endothelin1[60]	CTACTTCTGCCACCTGGACATC	62.1	TCACGGTCTGTTGCCTTTGTGG	62.1
ICAM-1[61]	TAGAGACCCCGTTGCCTAA	56.7	GTAAGGTTCTTGCCCACTG	56.7
GAPDH[62]	AATGCATCCTGCACCACCAACT GC	64.4	GGAGGCCATGTAGGCCATGAGG TC	67.8

Table 1: Primer sequence of the target genes

2.10 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was performed to separate and visualize DNA fragments. A 1.5% agarose gel was prepared in 1× TE buffer by dissolving agarose in a hot water bath at 90 °C, followed by cooling to 45 °C. Ethidium bromide (6 µL of 10 mg/mL) was added to the molten agarose, which was then poured into a casting tray with a comb. After solidification, the comb was removed and the gel was placed in an electrophoresis tank containing 1× TE buffer. DNA samples were loaded into the wells, and electrophoresis was carried out at 50 V for 30 min[63]. The stained gel was visualized using a gel documentation system (ChemiDoc Imaging System, Bio-Rad).

4. RESULTS

4.1. CYTOTOXICITY EVALUATION BY MICROSCOPIC ASSAY

Phase-contrast microscopy combined with quantitative MTT assays was employed to establish the non-toxic working concentration ranges of alpinetin, cardamonin, and losartan in cultured HUVECs. In all experimental plates, untreated negative control cells exhibited the characteristic cobblestone-like morphology of healthy endothelial monolayers, with intact cell–cell junctions and high confluence (Figures 1A, 2A, 3A). Exposure to alpinetin across concentrations of 6.25–50 µg/mL preserved normal proliferation, spindle-shaped morphology, and stable substratum attachment (Figures 1B–E), indicating negligible cytotoxicity at low-to-moderate doses. However, terminal toxicity was observed at 100 µg/mL (Figure 1F), characterized by pronounced cell rounding and detachment. Cardamonin demonstrated a comparatively narrower safety margin. At 6.25 µg/mL, cells retained normal morphology (Figure 2B), while mild thinning was noted at 12.5 µg/mL (Figure 2C). At concentrations ≥25 µg/mL (Figures 2D–F), distinct cytotoxic features emerged, including cellular shrinkage, loss of intercellular bridges, and widespread detachment. Losartan exhibited intermediate biocompatibility. Cells maintained cytoplasmic integrity and proliferation comparable to controls up to 25 µg/mL (Figures 3B–D). Mild stress responses and thinning appeared at 50 µg/mL (Figure 3E), progressing to advanced cell rounding and partial detachment at 100 µg/mL (Figure 3F). Collectively, microscopic observations corroborated by MTT assay data confirm that all three compounds are biocompatible within their designated downstream experimental ranges. Among them, alpinetin demonstrated the highest threshold of cytocompatibility, followed by losartan and cardamonin.

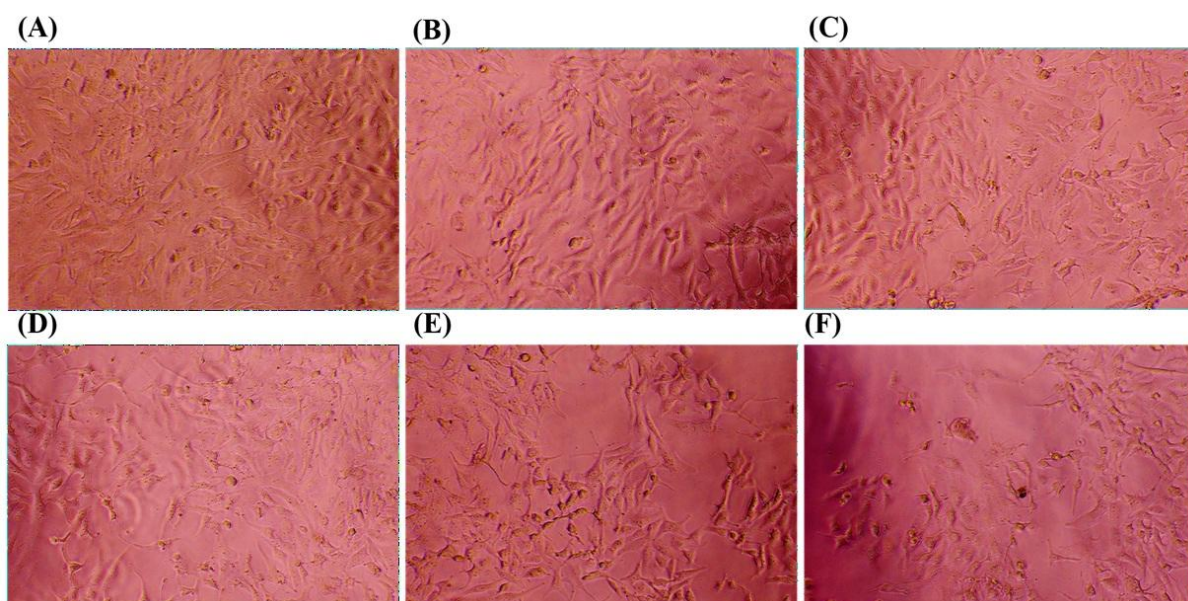


Figure 1: Photomicrographs depicting the cytotoxic effect of alpinetin on cultured HUVEC cells (A) Untreated control cells (B) 6.25 (C) 12.5 (D) 25 (E) 50 and (F) 100 μg .

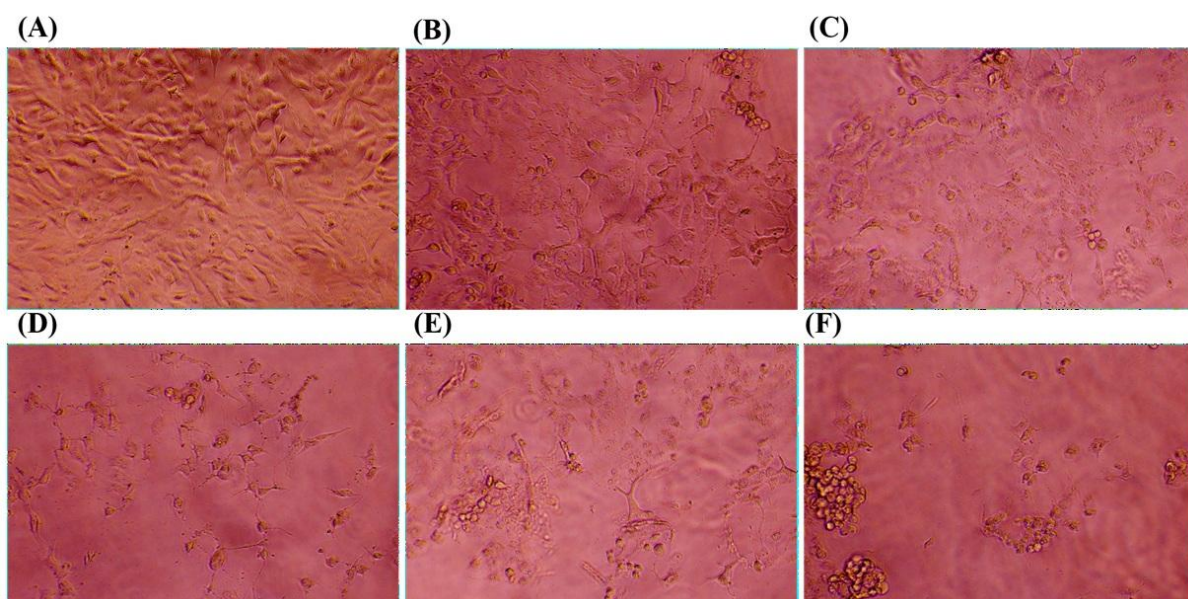


Figure 2: Photomicrographs depicting the cytotoxic effect of Cardamonin on cultured HUVEC cells (A) Untreated control cells (B) 6.25 (C) 12.5 (D) 25 (E) 50 and (F) 100 μg .

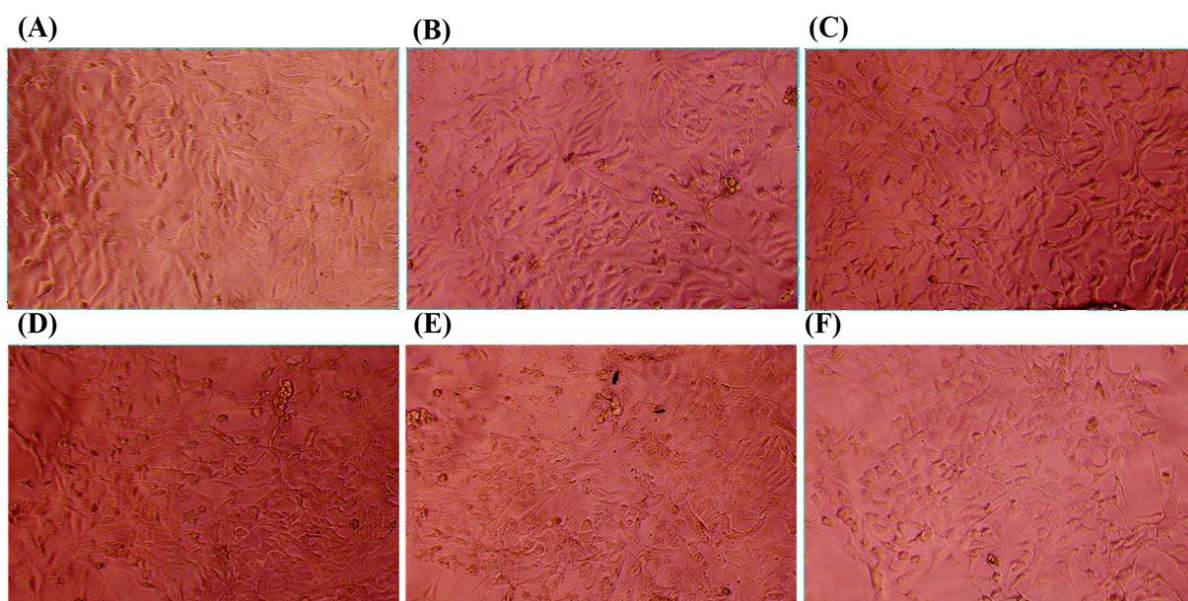


Figure 3: Photomicrographs depicting the cytotoxic effect of Losartan on cultured HUVEC cells (A) Untreated control cells (B) 6.25 (C) 12.5 (D) 25 (E) 50 and (F) 100 μg .

3.2 QUANTITATIVE CELL VIABILITY ASSESSMENT VIA MTT ASSAY

Quantitative MTT assays revealed distinct, dose-dependent cytotoxicity patterns for alpinetin, cardamonin, and losartan in HUVECs following 24 h exposure (Figures 4–6). Untreated negative control cells were defined as 100% baseline viability across all experiments. Alpinetin demonstrated a broad cytocompatibility window at lower concentrations. At 6.25 $\mu\text{g/mL}$, cell viability remained at $95.8\% \pm 1.2\%$, which was statistically non-significant compared to controls (Figure 4). Progressive reductions in viability were observed with increasing concentrations: $91.2\% \pm 1.8\%$ at 12.5 $\mu\text{g/mL}$, $86.4\% \pm 2.1\%$ at 25 $\mu\text{g/mL}$, and $80.1\% \pm 3.5\%$ at 50 $\mu\text{g/mL}$, culminating in $66.5\% \pm 2.8\%$ at 100 $\mu\text{g/mL}$ ($p < 0.0001$). Cardamonin exhibited a markedly narrower safety margin, inducing significant cytotoxicity even at the lowest tested dose. Viability declined to $81.2\% \pm 1.5\%$ at 6.25 $\mu\text{g/mL}$ and decreased sharply thereafter: $60.1\% \pm 3.2\%$ at 12.5 $\mu\text{g/mL}$, $42.1\% \pm 3.8\%$ at 25 $\mu\text{g/mL}$, and $28.3\% \pm 3.9\%$ at 50 $\mu\text{g/mL}$, with near-complete cell death at 100 $\mu\text{g/mL}$ ($17.2\% \pm 1.8\%$; $p < 0.0001$; Figure 5). Losartan displayed intermediate biocompatibility. Cell viability remained high at $98.1\% \pm 1.1\%$ (6.25 $\mu\text{g/mL}$) and $94.5\% \pm 1.3\%$ (12.5 $\mu\text{g/mL}$). A modest decline was observed at 25 $\mu\text{g/mL}$ ($91.0\% \pm 4.5\%$; $p < 0.01$), followed by further reductions to $86.2\% \pm 2.8\%$ at 50 $\mu\text{g/mL}$ and $81.1\% \pm 4.6\%$ at 100 $\mu\text{g/mL}$ ($p < 0.0001$; Figure 6). Collectively, these comparative profiles confirm that all three compounds exhibit dose-dependent cytotoxicity at elevated concentrations. However, alpinetin and losartan possess substantially wider safety margins than

cardamonin. Accordingly, optimized non-toxic sub-threshold concentrations were selected for downstream cytoprotective studies: 0.75–12.5 $\mu\text{g/mL}$ for alpinetin, 0.18–3.1 $\mu\text{g/mL}$ for cardamonin, and 1.5–25 $\mu\text{g/mL}$ for losartan.

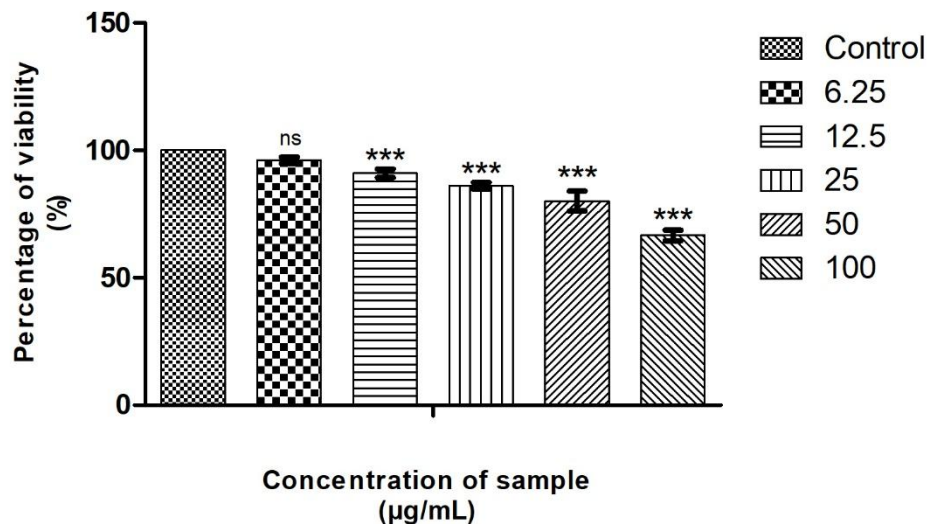


Figure 4: Graphical representation depicting the cytotoxic effect of Alpinetin by MTT assay- Along Y axis Percentage viability, Along X axis varied concentration of Alpinetin. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One-way ANOVA and Dunnets test were performed to analyse data. *** $p < 0.0001$ compared to control group, ns- non significant compared to control group.

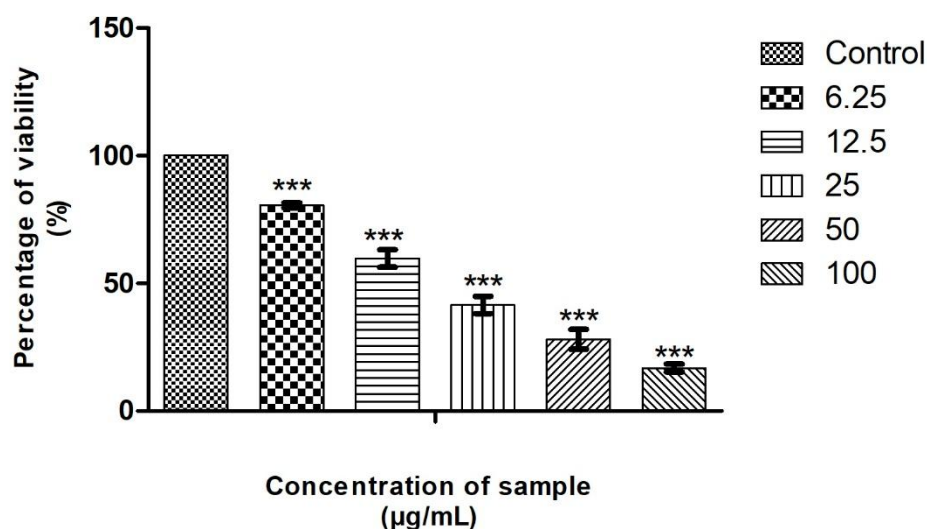


Figure 5: Graphical representation depicting the cytotoxic effect of Cardamonin by MTT assay- Along Y axis Percentage viability, Along X axis varied concentration of Cardamonin.

All experiments were done in triplicates and results represented as Mean $\pm$ SD. One-way ANOVA and Dunnett's test were performed to analyse data. *** p < 0.0001 compared to control group.

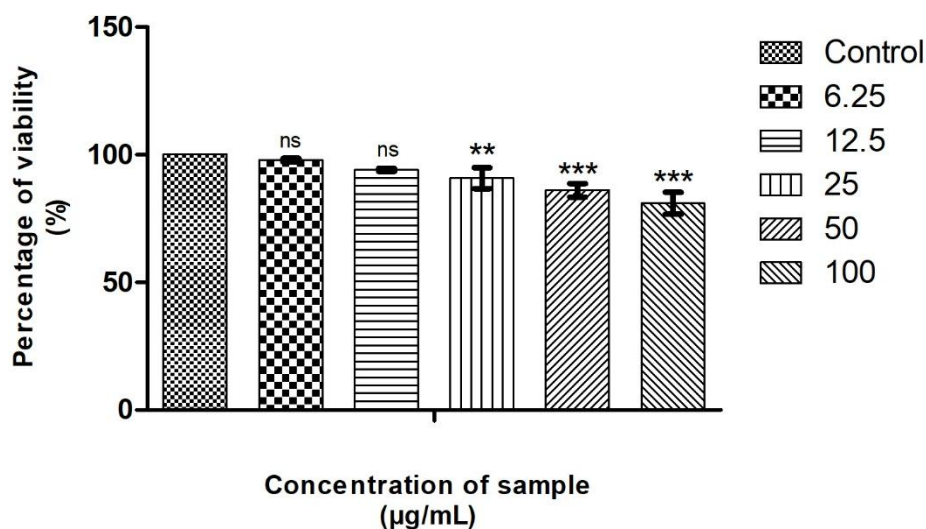


Figure 6: Graphical representation depicting the cytotoxic effect of Losartan by MTT assay- Along Y axis Percentage viability, Along X axis varied concentration of Losartan. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One-way ANOVA and Dunnett's test were performed to analyse data. *** p < 0.0001 compared to control group, ** p < 0.001 compared to control group, ns- non significant compared to control group.

2.3 CYTOPROTECTIVE EFFICACY OF COMPOUNDS AGAINST FRUCTOSE-INDUCED ENDOTHELIAL INJURY IN PROPHYLACTIC GROUP

Phase-contrast microscopy revealed that pre-conditioning with alpinetin, cardamonin, or losartan effectively protected HUVECs against fructose-induced metabolic injury in a concentration-dependent manner. Untreated negative control cells consistently exhibited a healthy, uniform, and highly confluent cobblestone-like endothelial monolayer with intact intercellular junctions (Figures 7A, 8A, 9A). In contrast, positive injury control cells exposed to 2.5 mM D-(–)-fructose alone for 24 h displayed severe structural damage, including cell rounding, cytoplasmic shrinkage, and extensive detachment, resulting in near-complete loss of monolayer integrity (Figures 7B, 8B, 9B).

Pre-conditioning with alpinetin produced a clear dose-dependent rescue effect (Figure 7). Lower concentrations (0.75–3.1 $\mu\text{g/mL}$; Figures 7C–E) provided partial protection, while higher doses (6.25–12.5 $\mu\text{g/mL}$; Figures 7F–G) nearly abolished fructose-mediated injury, restoring dense, confluent monolayers closely resembling the baseline morphology. Cardamonin displayed protective activity within a narrower sub-toxic range (Figure 8). Minimal rescue was observed at 0.18–0.37 $\mu\text{g/mL}$ (Figures 8C–D), whereas intermediate concentrations (0.75–3.1 $\mu\text{g/mL}$; Figures 8E–G) significantly reduced detachment and preserved large cellular clusters. Losartan, the clinical reference agent, also counteracted fructose toxicity in a dose-dependent manner (Figure 9). Lower concentrations (1.5–3.1 $\mu\text{g/mL}$; Figures 9C–D) provided modest relief, while higher prophylactic doses (6.25–25 $\mu\text{g/mL}$; Figures 9E–G) produced marked morphological recovery, culminating in widespread preservation of confluent monolayers with stable anchorage.

Taken together, these comparative visual observations confirm that non-toxic pre-conditioning doses of alpinetin, cardamonin, and losartan act as effective prophylactic shields against fructose-induced endothelial injury. Among them, alpinetin and losartan demonstrated superior monolayer restoration capacities, whereas cardamonin exhibited protective efficacy only within a restricted lower-dose range.

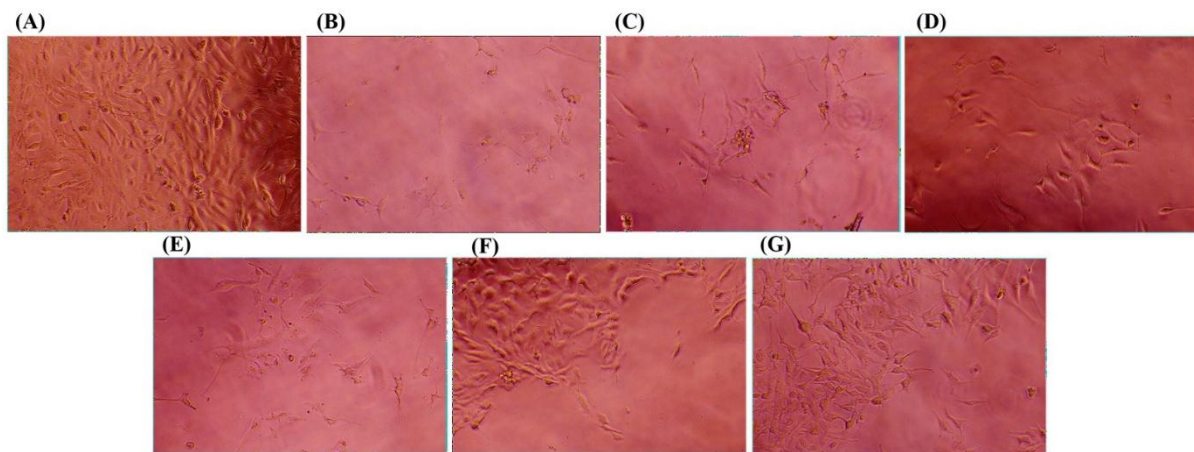


Figure 7: Photomicrographs depicting the effect of alpinetin (pre conditioning) co-administration on cultured HUVEC cells exposed to Fructose (A) Untreated control cells (B) Fructose exposed cells and Fructose exposed cells co-administrated with varied concentrations of alpinetin (C) 0.75 (D) 1.5 (E) 3.1 (F) 6.25 and (G) 12.5 μg .

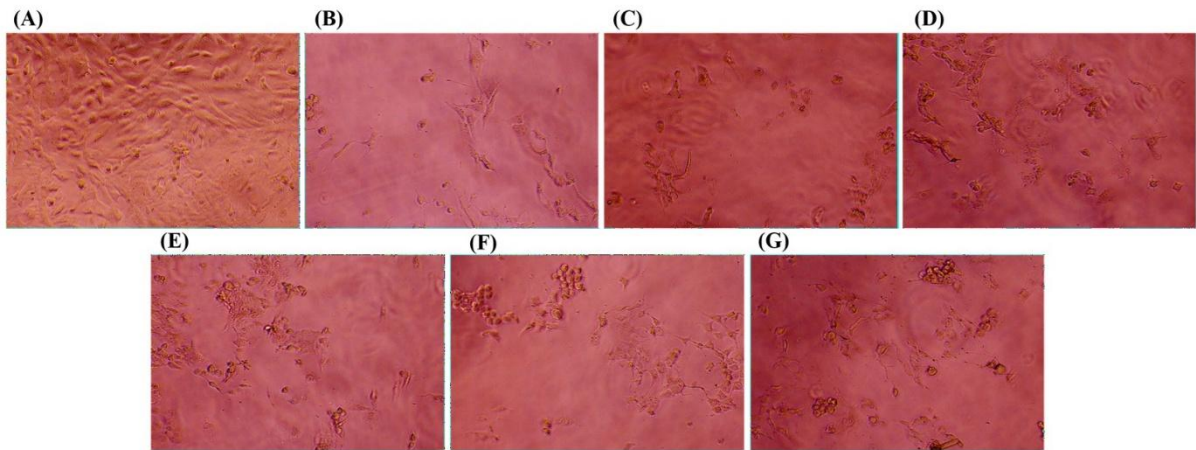


Figure 8: Photomicrographs depicting the effect of Cardamonin (pre conditioning) co-administration on cultured HUVEC cells exposed to Fructose (A) Untreated control cells (B) Fructose exposed cells and Fructose exposed cells co-administrated with varied concentrations of Cardamonin (C) 0.18 (D) 0.37 (E) 0.75 (F) 1.5 and (G) 3.1 µg.

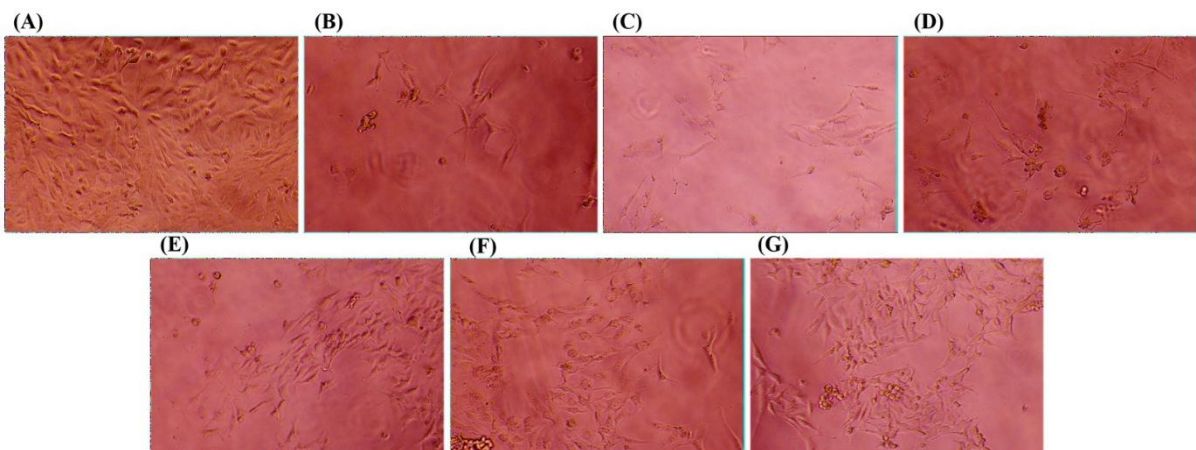


Figure 9: Photomicrographs depicting the effect of Losartan (pre conditioning) co-administration on cultured HUVEC cells exposed to Fructose (A) Untreated control cells (B) Fructose exposed cells and Fructose exposed cells co-administrated with varied concentrations of Losartan (C) 1.5 (D) 3.1 (E) 6.25 (F) 12.5 and (G) 25 µg.

2.4 CYTOPROTECTIVE EFFICACY OF COMPOUNDS AGAINST FRUCTOSE-INDUCED ENDOTHELIAL INJURY IN THERAPEUTIC GROUP

Phase-contrast microscopy demonstrated that post-injury co-administration of alpinetin, cardamonin, or losartan effectively mitigated fructose-induced endothelial damage in a concentration-dependent manner (Figures 10–12). Untreated control groups consistently

maintained intact, confluent cobblestone-like endothelial monolayers (Figures 10–12A), whereas positive injury controls exposed to 2.5 mM D-(–)-fructose alone exhibited severe cytotoxicity, including cell shrinkage, rounding, and widespread detachment (Figures 10–12B).

Among the test compounds, cardamonin displayed the highest therapeutic potency, initiating protection at sub-microgram concentrations and achieving substantial restoration of cell spreading and cluster integrity at 3.1 $\mu\text{g/mL}$ (Figure 11G). Alpinetin exhibited intermediate efficacy, requiring 12.5 $\mu\text{g/mL}$ to fully reverse fructose-induced cytotoxicity and restore cell density to baseline levels (Figure 10G). Losartan required the highest dose range, with negligible effects at ≤ 3.1 $\mu\text{g/mL}$ but progressive recovery at higher concentrations, culminating in marked monolayer restoration at 25 $\mu\text{g/mL}$ (Figure 12G).

Comparison with the prophylactic (pre-conditioning) model (Figures 7–9) revealed a reversal in efficacy hierarchy. Cardamonin, while most effective in post-injury rescue, was limited prophylactically by its narrow sub-toxic window (≤ 3.1 $\mu\text{g/mL}$), which preserved clusters but failed to re-establish a seamless monolayer. In contrast, alpinetin and losartan provided superior preventive protection, safely scaling to higher concentrations (12.5 and 25 $\mu\text{g/mL}$, respectively) and completely neutralizing the metabolic insult before injury cascades were initiated.

These findings highlight distinct mechanistic roles: cardamonin excels in mitigating active post-injury pathways, whereas alpinetin and losartan act as broader metabolic stabilizers, offering robust prophylactic preservation of endothelial monolayer integrity.

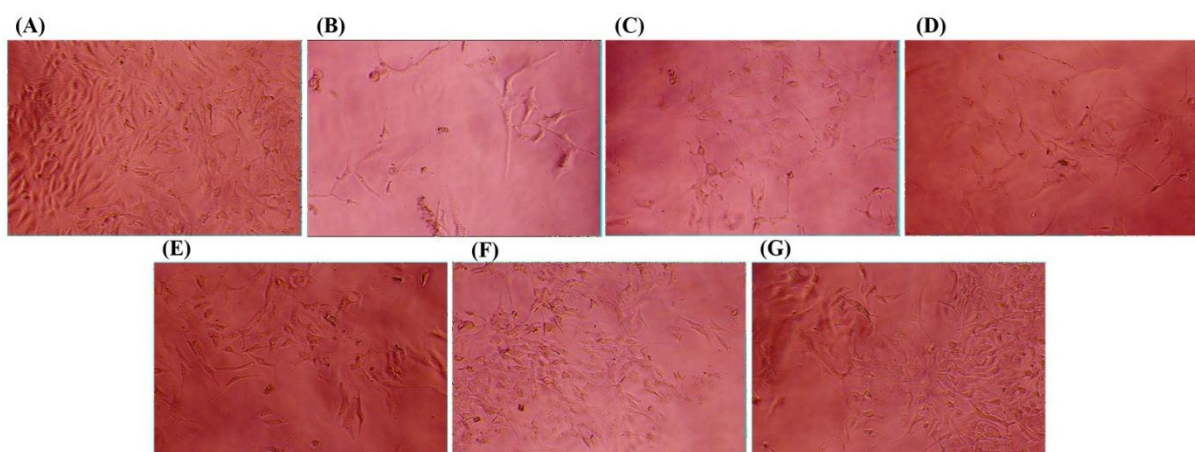


Figure 10: Photomicrographs depicting the effect of alpinetin (post conditioning) co-administration on cultured HUVEC cells exposed to Fructose (A) Untreated control cells (B)

Fructose exposed cells and Fructose exposed cells co-administrated with varied concentrations of alpinetin (C) 0.75 (D) 1.5 (E) 3.1 (F) 6.25 and (G) 12.5 μ g.

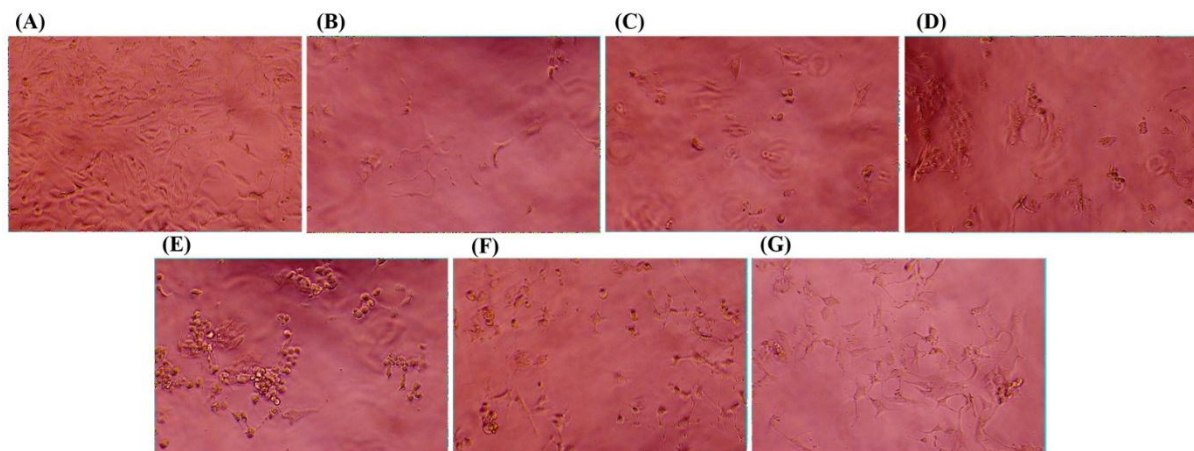


Figure 11: Photomicrographs depicting the effect of Cardamonin (post conditioning) co-administration on cultured HUVEC cells exposed to Fructose (A) Untreated control cells (B) Fructose exposed cells and Fructose exposed cells co-administrated with varied concentrations of Cardamonin (C) 0.18 (D) 0.37 (E) 0.75 (F) 1.5 and (G) 3.1 μ g.

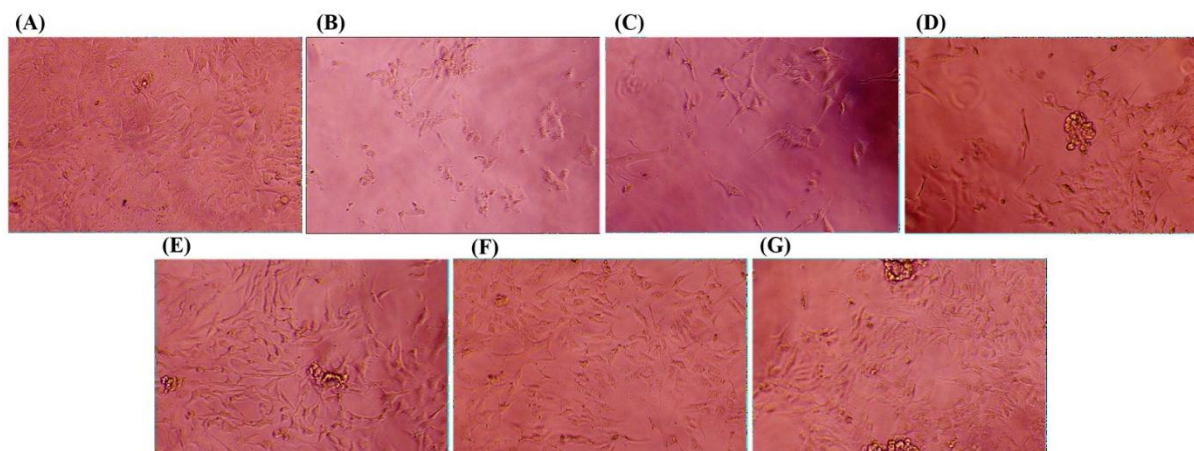


Figure 12: Photomicrographs depicting the effect of Losartan (post conditioning) co-administration on cultured HUVEC cells exposed to Fructose (A) Untreated control cells (B) Fructose exposed cells and Fructose exposed cells co-administrated with varied concentrations of Losartan (C) 1.5 (D) 3.1 (E) 6.25 (F) 12.5 and (G) 25 μ g.

2.5 QUANTITATIVE CYTOPROTECTIVE EVALUATION OF ALPINETIN, CARDAMONIN, AND LOSARTAN VIA MTT ANALYSIS

Colorimetric MTT assays were employed to quantitatively assess the cytoprotective efficiencies of alpinetin, cardamonin, and losartan against fructose-induced endothelial stress under both prophylactic pre-conditioning (Figures 13–15) and therapeutic post-conditioning (Figures 16–18) regimens. A uniform 24-h challenge with 2.5 mM D-(–)-fructose consistently suppressed HUVEC viability to ~50% relative to untreated controls (100%; *** $p < 0.0001$), thereby establishing a reproducible model of metabolic cytotoxicity.

Under pre-conditioning parameters, cardamonin demonstrated significant protection beginning at 0.75 $\mu\text{g/mL}$ (** $p < 0.001$; ~54% viability) and achieved maximal recovery (~65%) at 3.1 $\mu\text{g/mL}$ (Figure 14; ** $p < 0.0001$). Alpinetin required a higher threshold of 3.1 $\mu\text{g/mL}$ ($p < 0.05$; ~55% viability) but scaled safely to 12.5 $\mu\text{g/mL}$, restoring viability to ~75% (Figure 13; $p < 0.0001$). Losartan exhibited immediate preventive efficacy at 1.5 $\mu\text{g/mL}$ ($p < 0.0001$; ~58% viability) and progressively enhanced survival, reaching ~81% at 25 $\mu\text{g/mL}$ (Figure 15; *** $p < 0.0001$).

Cross-regimen comparison revealed a distinct statistical advantage in the therapeutic post-conditioning model, with lower effective thresholds and higher survival ceilings. Cardamonin's therapeutic onset shifted to 0.37 $\mu\text{g/mL}$ (* $p < 0.05$), peaking at ~66% viability at 3.1 $\mu\text{g/mL}$ (Figure 17; ** $p < 0.0001$). Alpinetin initiated rescue at 1.5 $\mu\text{g/mL}$ ($p < 0.01$) and achieved ~79% recovery at 12.5 $\mu\text{g/mL}$ (Figure 16; $p < 0.0001$). Losartan maintained consistent superiority, protecting cells at 1.5 $\mu\text{g/mL}$ ($p < 0.0001$; ~60% viability) and culminating in ~83% survival at 25 $\mu\text{g/mL}$ (Figure 18; *** $p < 0.0001$).

These quantitative findings corroborate the morphological observations recorded by phase-contrast microscopy, confirming that cardamonin functions as a highly sensitive low-dose responder, while alpinetin and losartan provide enhanced post-injury recovery profiles. Collectively, the data demonstrate that therapeutic intervention yields superior viability restoration compared to prophylactic stabilization, with alpinetin and losartan maximizing survival ceilings and preserving endothelial integrity more effectively in the post-conditioning setting.

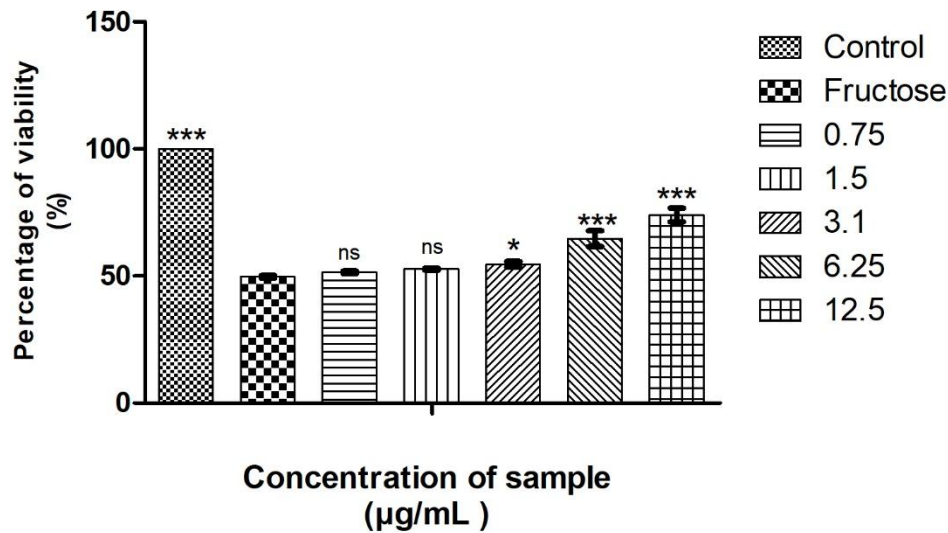


Figure 13: Graphical representation depicting the cytoprotective effect of alpinetin on HUVEC cell line by MTT assay. Along Y axis Percentage viability, Along X axis varied concentration of alpinetin. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One- way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.0001$ compared to fructose induced groups, * $p < 0.05$ compared to fructose induced groups, ns- non significant compared to fructose induced groups.

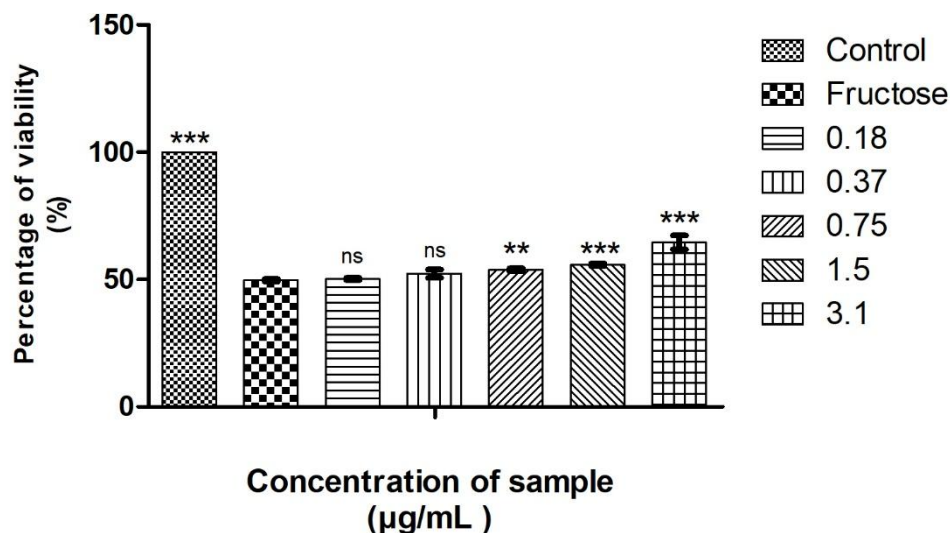


Figure 14: Graphical representation depicting the cytoprotective effect of cardamomin on HUVEC cell line by MTT assay. Along Y axis Percentage viability, Along X axis varied concentration of cardamomin. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One- way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.0001$ compared to fructose induced groups, ** $p < 0.01$ compared to fructose induced groups, ns- non significant compared to fructose induced groups.

0.0001 compared to fructose induced groups, ** $p < 0.001$ compared to fructose induced groups, ns- non significant compared to fructose induced groups.

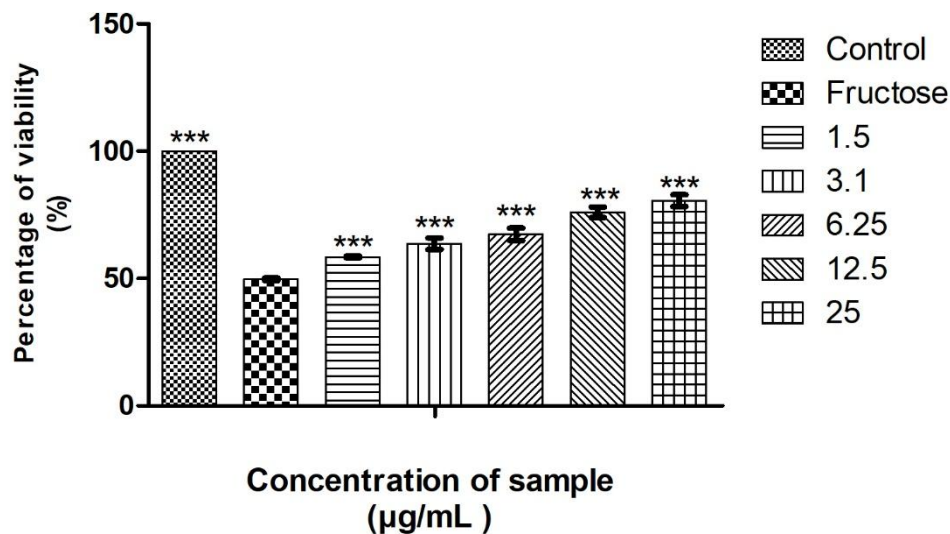


Figure 15: Graphical representation depicting the cytoprotective effect of losartan on HUVEC cell line by MTT assay. Along Y axis Percentage viability, Along X axis varied concentration of losartan. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One- way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.0001$ compared to fructose induced groups.

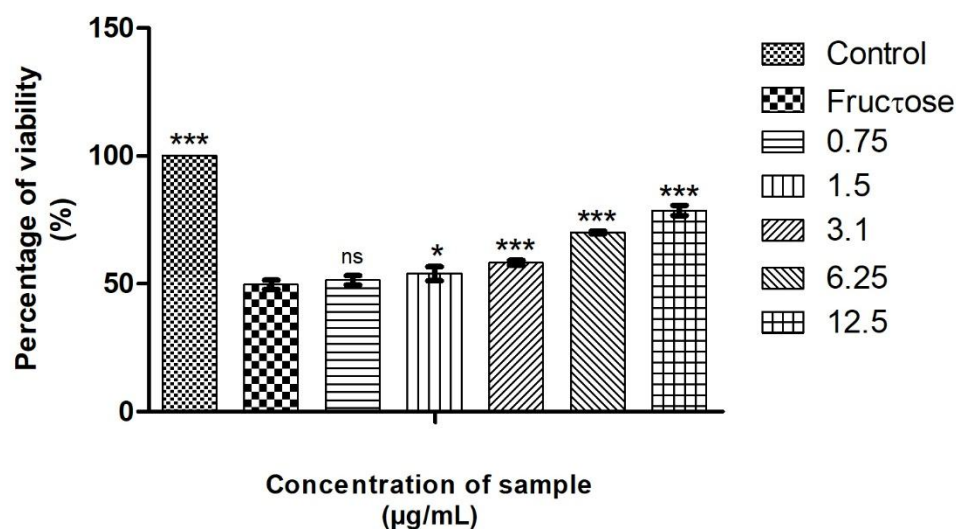


Figure 16: Graphical representation depicting the cytoprotective effect of alpinetin on HUVEC cell line by MTT assay. Along Y axis Percentage viability, Along X axis varied concentration of alpinetin. All experiments were done in triplicates and results represented as Mean $\pm$ SD.

SD. One- way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.0001$ compared to Fructose induced groups, * $p < 0.01$ compared to Fructose induced groups, ns- non significant compared to Fructose induced groups.

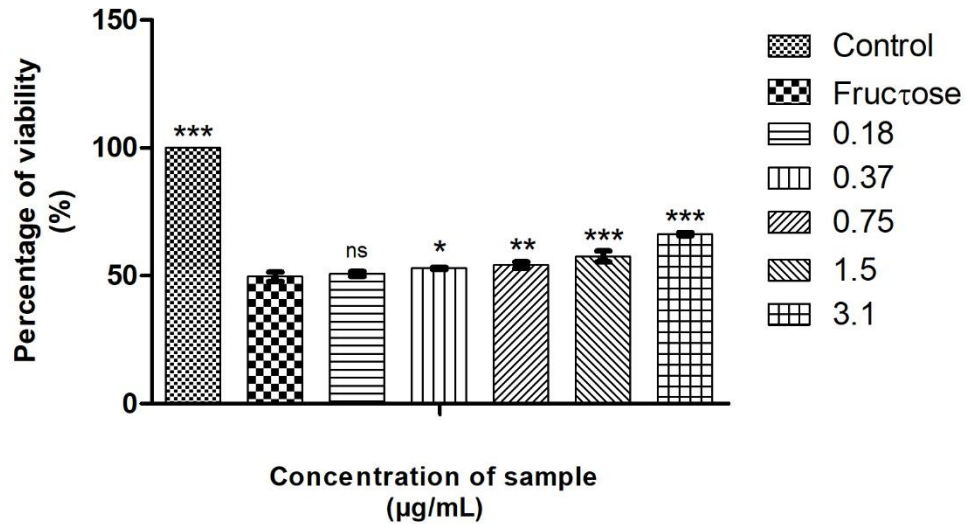


Figure 17: Graphical representation depicting the cytoprotective effect of cardamomin on HUVEC cell line by MTT assay. Along Y axis Percentage viability, Along X axis varied concentration of cardamomin. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One- way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.0001$ compared to Fructose induced groups, ** $p < 0.001$ compared to Fructose induced groups, * $p < 0.05$ compared to Fructose induced groups, ns- non significant compared to Fructose induced groups.

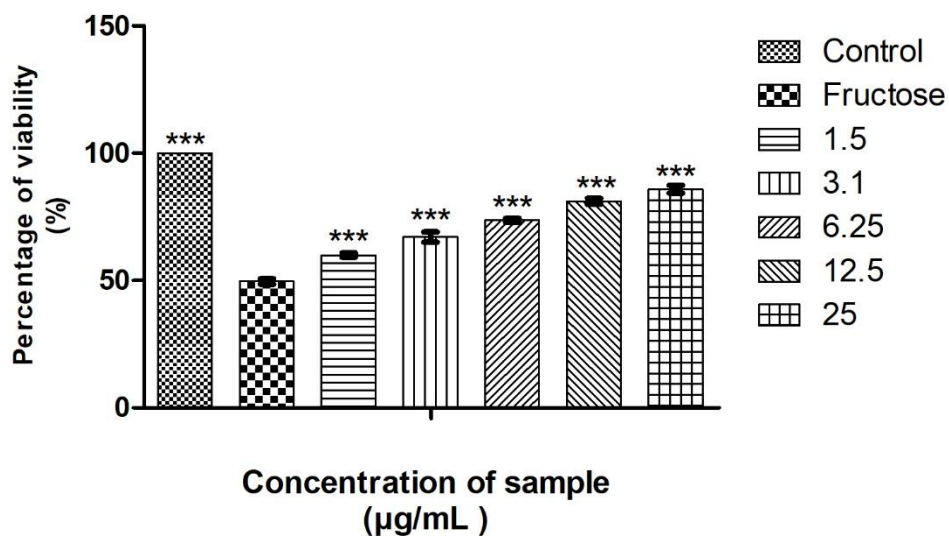


Figure 18: Graphical representation depicting the cytoprotective effect of losartan on HUVEC cell line by MTT assay. Along Y axis Percentage viability, Along X axis varied concentration of losartan. All experiments were done in triplicates and results represented as Mean $\pm$ SD. One- way ANOVA and Dunnett's test were performed to analyse data. *** $p < 0.0001$ compared to Fructose induced groups.

2.6 QUANTITATIVE REAL-TIME PCR ANALYSIS OF ENDOTHELIAL TARGET GENES

To investigate the molecular pathways underlying the cytoprotective effects of alpinetin, cardamonin, and losartan, RT-qPCR analysis was performed for key endothelial markers: endothelial nitric oxide synthase (eNOS; Figure 19), receptor for advanced glycation end-products (RAGE; Figure 20), Endothelin-1 (Figure 21), and intercellular adhesion molecule-1 (ICAM-1; Figure 22). A 24-h exposure to 2.5 mM D-(–)-fructose induced pronounced transcriptional stress, characterized by downregulation of eNOS (~0.84-fold; Figure 19) and significant upregulation of RAGE (~1.26-fold; Figure 20), Endothelin-1 (~1.31-fold; Figure 21), and ICAM-1 (~1.28-fold; Figure 22) relative to untreated controls (defined as 1.0-fold).

For vascular tone and metabolic markers (eNOS, RAGE, Endothelin-1), therapeutic post-conditioning demonstrated superior efficacy compared to prophylactic pre-conditioning. Pre-treatment with losartan maintained eNOS transcripts near baseline (~1.0-fold; Figure 19), while cardamonin and alpinetin moderately enhanced eNOS expression (1.26-fold and 1.31-fold, respectively) and normalized RAGE (~0.95-fold; Figure 20) and Endothelin-1 (~0.93–1.0-fold; Figure 21). In contrast, post-conditioning interventions produced a more pronounced transcriptional recovery. Cardamonin and losartan elevated eNOS to 1.50-fold and 1.65-fold, respectively, while suppressing RAGE (0.66–0.71-fold; Figure 20) and Endothelin-1 (0.77–0.82-fold; Figure 21). Alpinetin post-conditioning yielded the most robust effect, markedly upregulating eNOS to ~2.96-fold (Figure 19) and reducing RAGE and Endothelin-1 to 0.59-fold and 0.71-fold, respectively.

In contrast, ICAM-1 regulation exhibited an opposite trend. Prophylactic pre-conditioning provided stronger transcriptional suppression than post-conditioning (Figure 22). While post-conditioning reduced ICAM-1 to 0.72–0.93-fold, pre-treatment with cardamonin and alpinetin lowered expression to 0.80-fold and 0.63-fold, respectively. Losartan

pre-conditioning achieved the greatest suppression, reducing ICAM-1 transcripts to ~0.58-fold relative to controls.

Collectively, these data confirm that all three agents re-establish molecular homeostasis under fructose-induced stress. Therapeutic post-conditioning excels in restoring metabolic stability and eNOS-mediated pathways, whereas prophylactic pre-conditioning provides superior frontline defense against leukocyte adhesion signaling via ICAM-1 suppression.

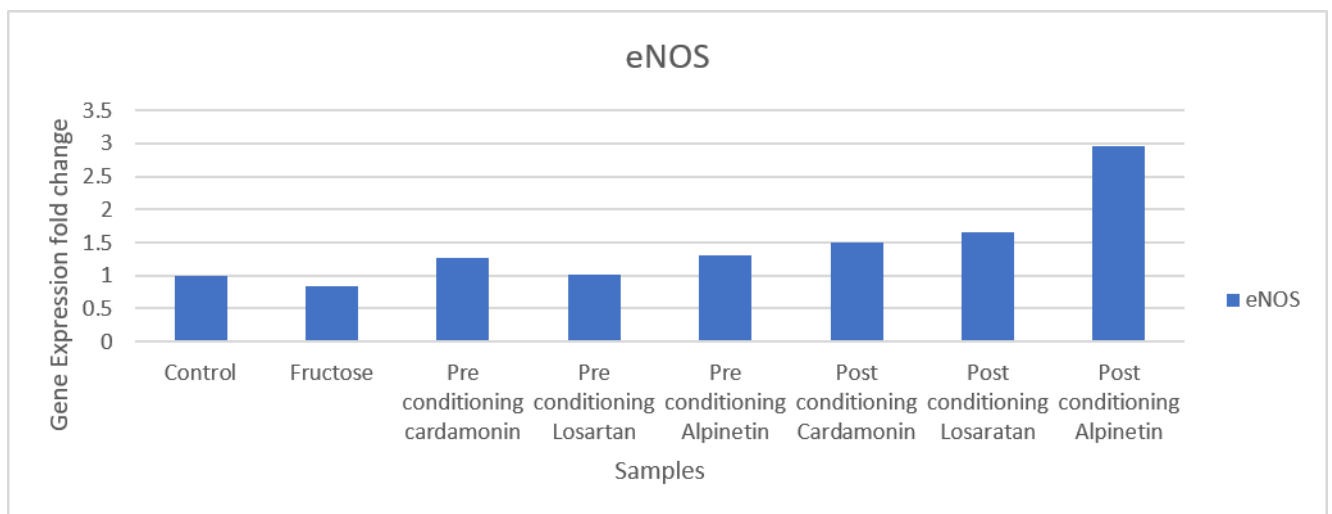


Figure19: Graphical representation depicting the expression pattern of eNOS

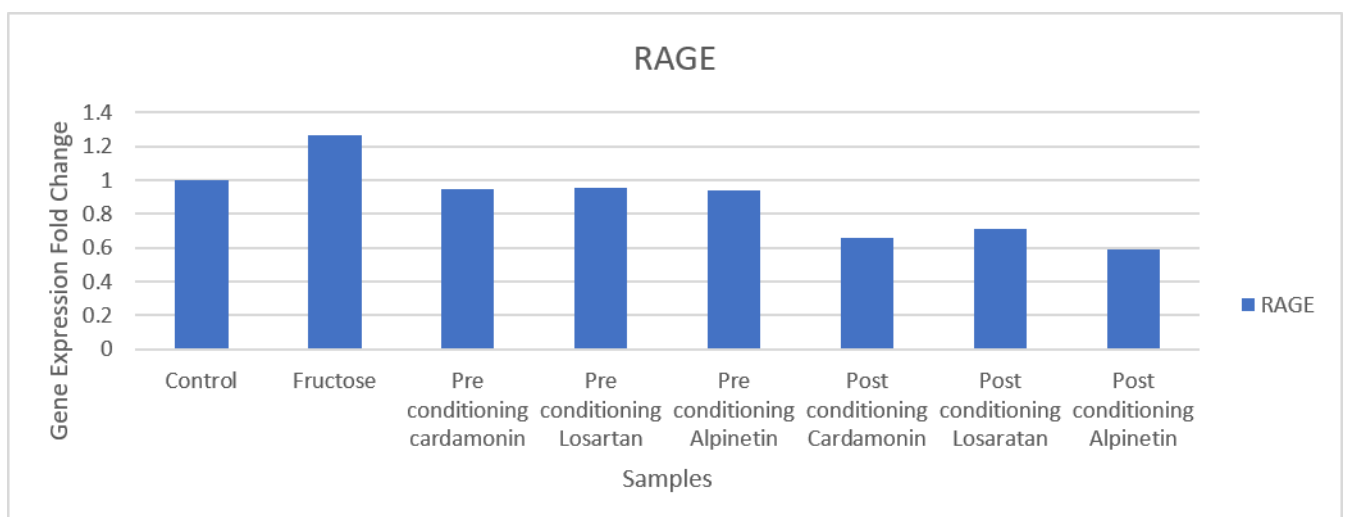


Figure 20: Graphical representation depicting the expression pattern of RAGE

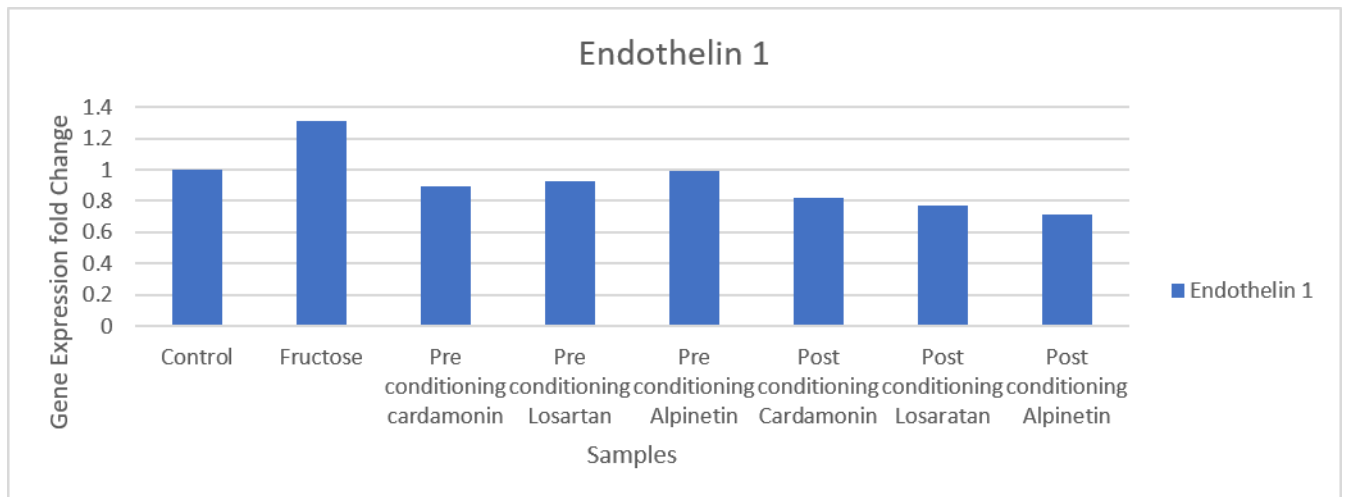


Figure 21: Graphical representation depicting the expression pattern of Endothelin1

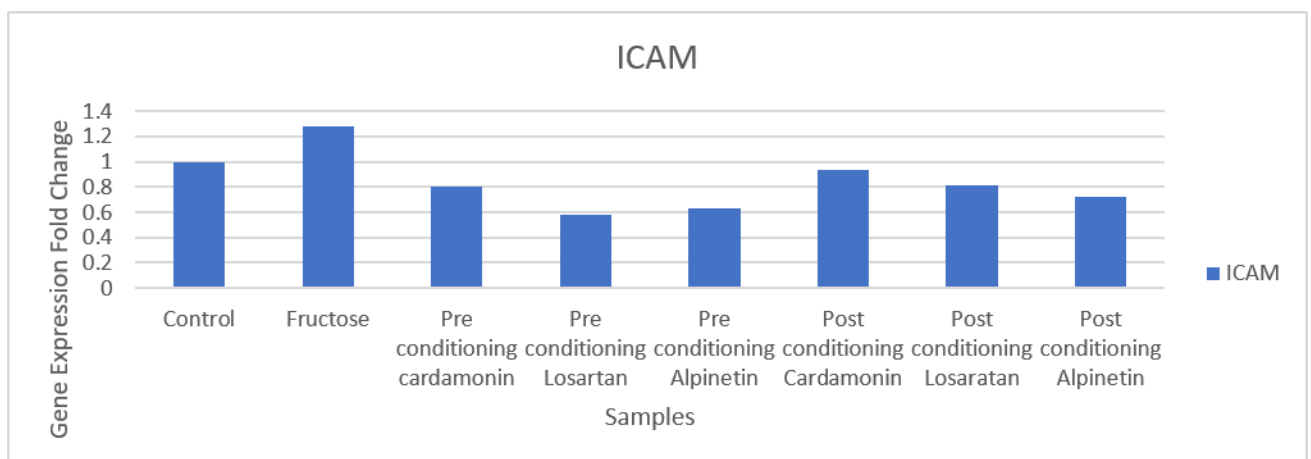


Figure 22: Graphical representation depicting the expression pattern of ICAM

2.7 AGAROSE GEL ELECTROPHORESIS ANALYSIS OF AMPLIFIED TRANSCRIPTS

Agarose gel electrophoresis (Figure 23) revealed distinct transcriptional changes in HUVECs following a 24-h exposure to 2.5 mM D-(–)-fructose. GAPDH, used as the internal housekeeping control, displayed uniform band intensities across all lanes (Figure 23, lanes i–viii), confirming equal cDNA loading and assay reliability. Untreated control cells exhibited strong baseline eNOS amplicon bands (Figure 23, lane i), whereas fructose exposure markedly attenuated eNOS expression (Figure 23, lane ii) and simultaneously intensified RAGE, Endothelin-1, and ICAM-1 bands, consistent with inflammatory activation.

Both prophylactic pre-conditioning and therapeutic post-conditioning interventions counteracted these fructose-induced transcriptional shifts. For metabolic and vascular tone markers (eNOS, RAGE, Endothelin-1), post-conditioning treatments produced more pronounced recovery than pre-conditioning. Preventive treatments moderately restored eNOS band density and reduced RAGE and Endothelin-1 signals (Figure 23, lanes iii–v), whereas post-conditioning interventions (lanes vi–viii) drove a more comprehensive reversal. Alpinetin post-conditioning (lane viii) was particularly effective, strongly enhancing eNOS band intensity while reducing RAGE and Endothelin-1 signals to minimal levels.

In contrast, ICAM-1 regulation followed an opposite trend. Pre-conditioning provided stronger suppression than post-conditioning, with losartan pre-treatment (lane iv) nearly abolishing the fructose-induced ICAM-1 band. Post-conditioning therapies reduced ICAM-1 expression relative to fructose controls but did not achieve the same degree of suppression as preventive regimens.

Overall, electrophoretic band patterns corroborated RT-qPCR data, confirming that alpinetin post-conditioning maximizes eNOS transcript recovery, whereas losartan pre-conditioning provides the most effective barrier against ICAM-1-mediated leukocyte adhesion signaling.

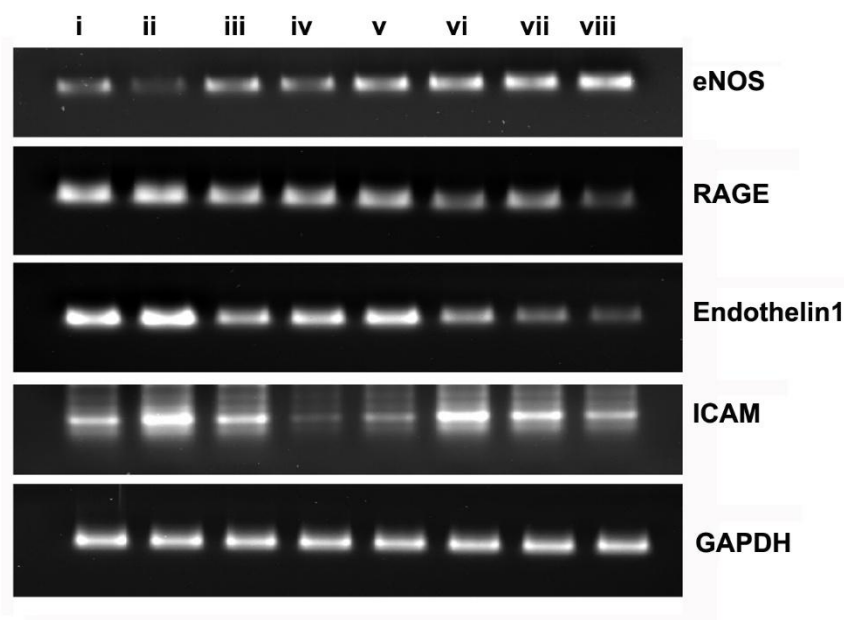


Figure 23: Photomicrograph of a typical 1.5 % agarose gel depicting the gene expression pattern of eNOS, RAGE, Endothelin1, ICAM and housekeeping gene GAPDH, in HUVEC cells (i) control (ii) Fructose Treated (iii) Pre- Fructose+Cardamonin (iv) Pre- Fructose+Losartan (v) Pre- Fructose+Alpinetin (vi) Post- Fructose+Cardamonin (vii) Pre- Fructose+Losartan (viii) Pre- Fructose+Alpinetin. The gel image was captured by ChemiDoc imaging system, Biorad.

4. DISCUSSION

The vascular endothelium functions as a highly dynamic, semi-permeable boundary layer that precisely regulates leukocyte rolling, fluid–electrolyte balance, and localized inflammatory parameters[53]. Disruptions to this homeostatic barrier constitute the initial pathogenetic events underlying metabolic syndrome, modern atherogenesis, and complex macrovascular disorders[42]. Chronic intake of D(–)-fructose triggers endothelial dysfunction through a multi-pathway network rather than a single isolated cascade. Structurally, excess fructose accelerates intracellular non-enzymatic glycation, leading to the accumulation of advanced glycation end-products (AGEs). These products bind to the cell-surface Receptor for Advanced Glycation End-products (RAGE), triggering a pro-inflammatory cascade mediated by NF-κB and a massive influx of reactive oxygen species (ROS). This oxidative environment induces eNOS uncoupling and ATP depletion, which drastically limits bioactive nitric oxide (NO) pools and impairs endothelium-dependent vasodilation [64]. Concurrently, the upregulation of Endothelin-1 (ET-1) worsens vasoconstriction, while increased cell adhesion molecules like ICAM-1 initiate leukocyte recruitment and structural monolayer breakdown[65, 66]. Because fructose-induced endothelial damage occurs across a complex web of independent networks rather than a single isolated cascade, discovering agents with multi-target potential is crucial for comprehensive vascular protection [67, 68].

Conventional antihypertensive drugs, such as ACE inhibitors and ARBs, follow a single-target model that fails to address the complex metabolic pathways of fructose-induced endothelial damage, including the AGE–RAGE inflammatory cascade. Additionally, these drugs cannot prevent fructose-mediated PP2A activation, which leads to eNOS uncoupling, or manage the resulting intracellular uric acid overproduction, necessitating higher, less tolerable doses for management [69, 70]. In this context, the natural chalcone cardamonin and the flavanone alpinetin—both prominent bioactive agents isolated from the medicinal plant genus *Alpinia*

(Zingiberaceae)—were selected for this study due to their distinct, documented capacities to regulate cellular survival, vascular tone, and oxidative stress pathways [71, 72, 73].

Baseline cytotoxicity of alpinetin, cardamonin, and losartan was first determined using the MTT assay [74]. To understand how alpinetin, cardamonin, and losartan halt fructose-induced endothelial damage, their effects must be mapped directly onto the specific vascular pathologies controlled by the four target genes: eNOS, RAGE, Endothelin-1, and ICAM-1 [75].

The present findings demonstrate that fructose exposure markedly suppresses eNOS transcription, reflecting enzyme uncoupling and nitric oxide depletion. This aligns with previous reports that PP2A-mediated dephosphorylation at Ser1177 destabilizes eNOS activity, shifting its output from protective NO to harmful superoxide radicals. High vascular concentrations of fructose trigger the PP2A pathway, which strips a crucial phosphate group from eNOS at the Ser1177 site. This deactivation uncouples the enzyme, causing it to produce toxic superoxide radicals instead of protective NO, leading to rapid cell death and severe suppression of eNOS gene transcription [76, 77, 78]. Under therapeutic post-conditioning parameters, alpinetin demonstrated extraordinary effectiveness by actively overriding this ongoing post-injury degradation complex, triggering a massive over-compensatory transcription surge that tripled eNOS expression. This profound recovery indicates that alpinetin can rapidly restore NO bioavailability following acute metabolic insults, outperforming cardamonin and losartan therapeutic groups which nevertheless showed valuable multi-target utility [79, 80].

To explore the impact of the treatments on fructose-induced advanced glycation signaling pathways, the transcriptional adjustments of the test compounds mapped directly onto the RAGE pathway. When excess fructose enters endothelial cells, it bypasses normal metabolic checkpoints and rapidly forms fructose-derived AGEs. These Fruc-AGEs ligate cell-surface RAGE receptors, creating a vicious autocrine feedback loop that activates NF- κ B inflammatory signaling [81, 82]. This ligation drives chronic oxidative stress, causes severe mitochondrial damage, and ultimately forces cellular detachment. Prophylactic pre-conditioning with cardamonin and losartan effectively normalized RAGE transcript levels, while alpinetin pre-treatment suppressed expression further. Therapeutic post-conditioning interventions executed a significantly more profound transcriptional suppression, with alpinetin achieving the most robust attenuation [83, 84].

To assess the impact of the experimental treatments on potent endothelium-derived vasoconstrictor pathways, transcriptional adjustments were mapped onto the ET-1 gene pathway. Under healthy physiological conditions, the constricting force exerted by ET-1 is balanced by the dilating actions of NO. However, fructose-induced oxidative stress and downstream RAGE activation disrupt this equilibrium, prompting severe overproduction of ET-1 that drives vascular wall inflammation, vessel remodeling, and metabolic hypertension [85, 86, 87, 88]. Prophylactic pre-conditioning with alpinetin maintained transcription at baseline levels, while losartan and cardamonin normalized ET-1 transcript levels. Therapeutic post-conditioning interventions executed a significantly more profound suppression, with alpinetin achieving the strongest attenuation [89, 90].

Finally, ICAM-1 expression was examined as a marker of leukocyte adhesion. Fructose stress markedly elevated ICAM-1 transcription, consistent with earlier reports of endothelial adhesion signaling [91]. Prophylactic pre-conditioning interventions executed a more profound suppression of ICAM-1 than post-conditioning. Cardamonin reduced transcripts significantly, alpinetin compressed ICAM-1 expression further, and losartan achieved the most complete prophylactic neutralization. Post-conditioning interventions also reversed fructose-induced inflammatory spikes, with alpinetin again showing superior therapeutic efficacy [92, 93].

Collectively, these findings highlight that while all three agents—alpinetin, cardamonin, and losartan—exert multi-target protective effects, their strengths differ by regimen. Alpinetin excels in therapeutic post-conditioning rescue, cardamonin demonstrates potent low-dose recovery, and losartan provides robust prophylactic stabilization. This dichotomy underscores the translational potential of phytochemicals as adjuncts to conventional therapies, offering complementary strategies for mitigating metabolic endothelial dysfunction.

5. CONCLUSION

In conclusion, this study demonstrates that while alpinetin, cardamonin, and losartan function as effective, dose-dependent cytoprotective agents against fructose-induced endothelial stress, they utilize distinct, timing-dependent strategies to hit multiple targets and preserve HUVEC integrity. The small molecule cardamonin acts as a sensitive, low-dose therapeutic responder that excels in acute, post-injury rescue at minimal concentrations. Conversely, the clinical reference drug losartan operates as a premier frontline prophylactic shield, combining high cellular safety boundaries with excellent efficiency in pre-blocking inflammatory *ICAM-1* leukocyte adhesion targets. Most notably, the natural chalcone alpinetin demonstrates great

potential as a dynamic, multi-target therapeutic stabilizer; its ability to drive an extensive over compensatory up-regulation of protective *eNOS* amplicons while simultaneously forcing the active suppression of *RAGE* and *Endothelin-1* loops outlines its strong clinical utility for reversing established metabolic vascular injury across several separate physiological pathways. These complementary profiles provide valuable pharmacological insights for developing optimized, multi-targeted natural therapies to treat cardiovascular-metabolic diseases.

6. CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

VS Arya: Writing– original draft, Project administration, Investigation, Data curation, Conceptualization. **Kavitha R:** Writing – review & editing, Project administration, Investigation.

7. DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

8. AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this article and its supplementary information files

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12. DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work the authors used Grammarly (Premium version) to correct errors in spelling and grammar and while Quillbot assisted in rephrasing selected sections. After using this service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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