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Unlocking the Antidiabetic Promise of *Acalypha indica*: Targeting NFκB and Nrf2/Keap1 Pathways in Adipocytes via Integrated Cell Line and Computational Approaches

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Abstract

Background: Diabetes mellitus, particularly type 2 diabetes mellitus (T2DM), is a chronic metabolic disorder characterised by persistent hyperglycaemia arising from inadequate insulin secretion, reduced insulin sensitivity, or both. The growing prevalence of T2DM and its close link to obesity and metabolic dysfunction highlight the need for safer and more effective therapeutic alternatives. *Acalypha indica*, a traditional medicinal herb with reported antioxidant and anti-inflammatory properties, has recently gained interest for its potential role in diabetes management. **Aim:** This study aimed to evaluate the antioxidant, antidiabetic, and anti-inflammatory effects of *A. indica* through in vitro assays, gene expression studies, and in silico analyses. **Methods:** The ethanolic extract of *A. indica* was tested for antioxidant activity using DPPH, nitric oxide, and superoxide radical scavenging assays, thereby reinforcing cellular antioxidant defence mechanisms. Enzyme inhibition assays targeted key carbohydrate-metabolising enzymes. Cytotoxicity was assessed in high-glucose-induced 3T3-L1 adipocytes. Gene expression of Nrf2, NFκB and pro-inflammatory cytokines was analysed by qRTPCR. Molecular docking was performed to evaluate binding affinities between bioactive compounds and targets related to insulin signalling and antioxidative regulation. **Results:** The extract showed strong antioxidant activity, anti-inflammatory activity and significant inhibition of carbohydrate-metabolising enzymes, suggesting reduced postprandial hyperglycaemia. Cytotoxicity testing revealed high cell viability (>80%) across tested concentrations. In 3T3-L1 adipocytes, *A. indica* upregulated Nrf2, downregulated Keap1 and suppressed NFκB and proinflammatory cytokines, indicating attenuation of oxidative stress and inflammation. Docking analyses supported strong binding of bioactive compounds to key signalling and antioxidative targets. **Conclusion:** *A. indica* exerts multimodal protective effects by modulating oxidative stress, inflammation, and glucose metabolism, supporting its promising potential as a natural therapeutic agent for insulin resistance and T2DM.

Major Findings: *Acalypha indica* ethanolic extract shows potent antioxidant, anti-inflammatory, and antidiabetic effects, mediated through modulation of the Nrf2/Keap1 and NFκB pathways and inhibition of carbohydrate-metabolising enzymes, highlighting its potential as a phytomedicine for T2DM management.

Keywords: *Acalypha indica*, Antidiabetic Activity, Inflammation, Oxidative Stress, NFκB, Nrf2/Keap1

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1. Introduction

Diabetes mellitus (DM) is a pervasive and complex metabolic disorder marked by chronically elevated blood glucose levels resulting from impaired insulin secretion, impaired insulin action and/or both. Diabetes mellitus has reached pandemic levels globally in 2025, with around 589 million were affected (20-79 years) which accounting for 11.1% of the adult group. The estimate indicates that the number could reach 853 million by 2050, with over 80% residing in low- and middle-income countries. The increasing diabetes prevalence poses substantial social, economic, and healthcare challenges worldwide[1]. The diabetes epidemic disproportionately affects developing countries, where rapid urbanisation and lifestyle transitions drive the surge. The age distribution of diabetes patients shows a majority in the 45-64 age group in developing countries and older populations in developed regions. Women tend to have a slightly higher prevalence in developed countries[2]. This surge creates substantial health, social and economic challenges, leading to substantial stress on global healthcare systems worldwide.

The disease predominantly manifests as type 2 diabetes mellitus, which accounts for approximately 90-95% of cases. Type 2 diabetes develops insidiously over years due to a dynamic interplay between genetic predisposition and lifestyle risk factors, including physical inactivity, high-calorie diets and excess adiposity. The underlying pathophysiology involves a progressive decline in insulin sensitivity in peripheral tissues, particularly skeletal muscle, liver and adipose tissue, along with a gradual deterioration of pancreatic beta cell function that impairs insulin secretion[3]. This combination results in chronic hyperglycaemia, which over time inflicts widespread cellular and tissue damage.

Persistent hyperglycaemia exerts deleterious effects on multiple organ systems. Microvascular complications include diabetic retinopathy, nephropathy and neuropathy, leading to vision impairment, kidney failure and peripheral nerve damage. Macrovascular complications involve accelerated atherosclerosis, increasing the risk of coronary artery disease, stroke and peripheral artery disease, thereby elevating overall morbidity and mortality in diabetic populations[4]. These complications are primarily driven by hyperglycaemia-triggered oxidative stress, advanced glycation end products, inflammation and endothelial dysfunction.

Obesity is a critical risk factor and a major contributor to the pathogenesis of type 2 diabetes. Excess visceral adiposity promotes secretion of pro-inflammatory cytokines such as TNFα, IL1β and IL6. This adipose tissue dysfunction fosters a sustained condition of mild, systemic inflammation, which exacerbates insulin resistance through interference with insulin receptor signaling pathways[5,6]. One of the most critical regulators of this inflammatory response is the NFκB signalling pathway, which modulates the transcriptional activity of genes participating in immune function, inflammatory cascades, and cellular homeostasis[7].

The NFκB pathway is activated in adipocytes by metabolic stressors, including lipotoxicity, oxidative stress, and excessive nutrient intake. After activation, NFκB translocates to the nucleus and stimulates the production of proinflammatory cytokine transcripts, exacerbating systemic inflammation and impairing insulin signalling[8,9]. Sustained NFκB activation promotes insulin resistance by disrupting insulin receptor–mediated signalling and inhibiting the translocation of GLUT4, thus limiting glucose uptake in adipocytes and muscle cells[10]. The interplay between inflammation and insulin resistance highlights the importance of targeting NFκB as a potential therapeutic approach for obesity-related metabolic dysfunction.

Oxidative stress is integral to the development and advancement of diabetes mellitus. Elevated blood glucose levels stimulate excess production of reactive oxygen species via mitochondrial electron transport chain dysfunction, NADPH oxidase activation, and other enzymatic pathways[11]. The resulting oxidative damage impairs pancreatic β-cell viability and insulin secretion, promotes lipid peroxidation, and damages cellular proteins and DNA. When antioxidant defence mechanisms become overwhelmed, cellular homeostasis is disrupted, intensifying insulin resistance and cellular dysfunction[12]. Excessive ROS coupled with insufficient antioxidant defence leads to oxidative stress, which is key to the origin of metabolic diseases[13].

The Nrf2/Keap1 pathway is a key regulator of redox defence mechanisms, governing the expression of several cytoprotective genes[14]. In its inactive state, Nrf2

is bound to Keap1 localised in the cytoplasm, which triggers ubiquitination and subsequent proteasomal degradation[15]. Oxidative stress induces conformational changes, releasing Nrf2, which translocates into the nucleus, binds to antioxidant response elements (AREs), and promotes the transcription of genes encoding detoxifying and antioxidant enzymes such as SOD, CAT, GPx and HO-1[16]. Disruptions in the Nrf2/Keap1 pathway are strongly associated with metabolic disorders[17]. Persistent hyperglycaemia and nutrient overload contribute to excessive ROS accumulation, which paradoxically inhibits Nrf2 activation through the involvement of pro-inflammatory mediators including NFκB and MAPKs[18]. This suppression of Nrf2 impairs the induction of endogenous antioxidants, promoting oxidative stress-induced damage, lipid peroxidation and mitochondrial dysfunction[19].

Additionally, ROS-mediated activation of the NLRP3 inflammasome exacerbates metabolic inflammation by inducing the secretion of pro-inflammatory molecules, which further aggravate insulin resistance[20]. Notably, oxidative stress and inflammatory signalling pathways converge to impair insulin-mediated glucose uptake through inhibition of the PI3K/Akt signalling[21]. ROS-induced inhibition of Akt phosphorylation diminishes GLUT4 localisation to the plasma membrane, leading to reduced glucose uptake and metabolic dysfunction[22].

Current pharmacological management aims to control hyperglycaemia through insulin and oral hypoglycemic drugs like metformin, sulfonylureas, thiazolidinediones and DPP4 inhibitors. While these drugs effectively reduce blood glucose levels, their long-term clinical application is often restricted because of side effects including hypoglycemia, adiposity, gastrointestinal distress, and increased risk of cardiovascular events, especially in vulnerable populations[23]. Despite continued advancements, these limitations necessitate the exploration of novel therapeutic strategies targeting the underlying disease mechanisms, particularly oxidative stress, chronic inflammation and β-cell dysfunction.

Phytotherapeutic agents and natural bioactive products are increasingly recognised for their ability to modulate multiple pathophysiological pathways concurrently. Plant-derived secondary metabolites demonstrate antioxidant activity, inhibit key carbohydrate-hydrolysing enzymes, modulate pro-inflammatory signalling cascades, and enhance insulin sensitivity, providing an integrative and potentially safer therapeutic strategy. Among these, *A. indica*, a species with a longstanding ethnopharmacological background, has emerged as a promising candidate owing to its diverse phytochemical profile, encompassing alkaloids, flavonoids, tannins, and polyphenols. Emerging evidence indicates that *A. indica* exhibits significant anti-inflammatory and antioxidant properties, which may contribute mechanistically to the regulation of glucose homeostasis.

2. Materials and Methods

2.1 Plant Extraction and Phytochemical Analysis

The dried plant powder was extracted with 95% ethanol for 72 hours, filtered, concentrated, and stored at 4°C. The yield percentage was estimated. Phytochemical analysis of *A. indica* was conducted for various compounds, followed by *in vitro* antioxidant and antidiabetic assays to evaluate its pharmacological properties.

2.2 *In Vitro* Analysis

In vitro assays were performed to investigate antioxidant, anti-inflammatory and antidiabetic activities. For antioxidant activity, DPPH, NO and superoxide inhibition activities were performed, with varying concentrations of plant extract and ascorbic acid as the standard[24,25]. Protein denaturation inhibition assay was performed, with varying concentrations of plant extract and diclofenac sodium as the standard[26]. Intensity was read at 517 nm, 660 nm and 550 nm, respectively, and percentage inhibition potential was determined by applying the formula:

$$\% \text{ scavenged} = [(\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Sample}}) / \text{Absorbance}_{\text{Control}}] \times 100.$$

Antidiabetic activity was assessed by measuring α-amylase and α-glucosidase inhibition. The plant extract was incubated with the respective enzymes and substrates, and the intensity was read at 540 nm and 405 nm, respectively[27]. Acarbose served as the positive control for both enzyme inhibition assays. Inhibition was calculated as:

$$\% \text{ inhibition} = [(\text{OD Control} - \text{OD Sample}) / \text{OD Control}] \times 100.$$

2.3 Cell Culture and Viability Assay

3T3-L1 mouse adipocytes were procured from NCCS, Pune, India. Cells were cultured following established procedures: DMEM with 10% FBS, 37°C, and a humidified atmosphere containing 5% CO_2. Upon reaching confluency, cells were trypsinised, subcultured, and maintained until 80% confluency for experimentation. The MTT assay was employed to assess cell viability, where cells were treated with varying doses of *A. indica* extract for 48 h and subsequently incubated with MTT reagent. DMSO was used to dissolve the formazan crystals, and the intensity read at 570 nm was used to calculate viability:

$$\% \text{ Viability} = (\text{Sample Mean OD} / \text{Blank Mean OD}) \times 100$$

Subsequently, cells received extract treatment for ELISA and gene expression analysis. After treatments, cells were trypsinised and centrifuged to collect cell pellets. Pellets were treated with 1 ml of RIPA buffer for ELISA and 1ml of TRIzol reagent for mRNA expression analysis.

2.4 Determination of Antioxidant Enzyme Levels

Enzyme concentrations (SOD, CAT, GSH, GPx) were determined via Sandwich ELISA (Abbkine) following manufacturer instructions. Samples reacted with immobilised antibodies, then with labelled secondary antibodies and a streptavidin-enzyme complex. Colour development was measured at 450 nm, and concentrations (pg/ml) were determined using standard curves.

2.5 Quantitative RT-PCR

Gene expression was analysed using qRT-PCR. RNA was extracted with TriR Reagent (Sigma) and reverse transcribed (2 µg) using the Superscript III cDNA synthesis kit (Invitrogen, USA). PCR was carried out on an MX3000p system (Stratagene, Europe) using GoTaq Green Master Mix (Promega, USA). Specificity was confirmed by melting curve analysis. Fold change was calculated using the 2^−ΔΔCT method with CFX Manager 2.1 (Bio-Rad, USA).

2.6 *In Silico* Studies

Docking studies were performed to evaluate the binding strength between phytocompound and target proteins. The protein structure was obtained from experimental or computational sources, while the ligand was optimised for energy minimisation. Docking was carried out using AutoDock 1.5.6, and the binding poses were ranked based on binding affinity. The docked complexes were analysed, and 2D and 3D visualisations were generated using Biovia Discovery Studio.

2.7 Statistical Analysis

Results are presented as the mean ± standard deviation derived from three independent experiments, each conducted in triplicate. Statistical significance was determined by one-way ANOVA with a significance threshold of $p < 0.05$.

3. Results

3.1 Phytochemical Analysis

Preliminary phytochemical screening revealed the presence of alkaloids, glycosides, saponins, flavonoids, phenolic compounds, terpenoids, steroids and tannins in the ethanolic extract of *A. indica*.

3.2 Antioxidant Activity

Oxidative stress contributes to diabetes mellitus pathogenesis, and antioxidant therapies have gained significant attention in managing diabetes-related complications. The antioxidant effect of *A. indica* extract was assessed through the DPPH and NO radical neutralising assays.

3.2.1 DPPH Radical Scavenging Activity

Free radical scavenging activity increased in a dose-dependent manner. The extract showed 47.65% inhibition at 100 µg/ml, rising to 74.12% at 500 µg/ml, compared with the reference standard (93.81%) (Figure 1).

3.2.2 Nitric Oxide Radical Scavenging Activity

The method for assessing NO radical scavenging showed that *A. indica* extract exhibited an inhibition rate of 53.85% at the lowest tested concentration, which increased to 84.09% at the highest concentration.

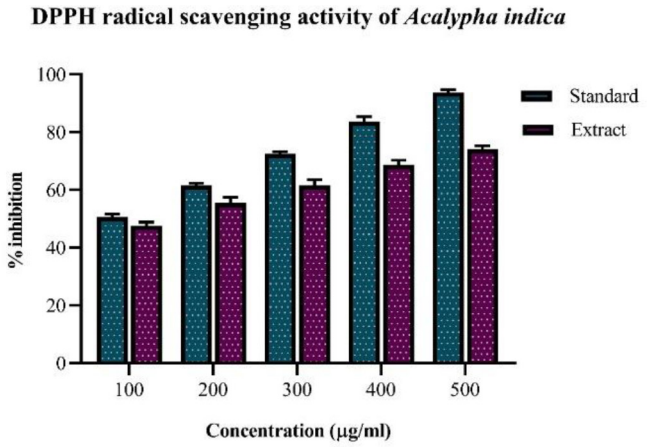


Figure 1. DPPH free radical neutralisation potential of *A. indica* extract at various concentrations compared to ascorbic acid. Results indicate a dose-dependent increase in antioxidant potential.

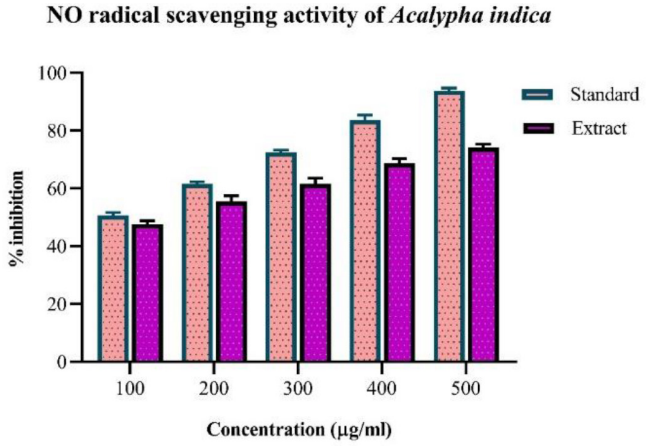


Figure 2. Antioxidant effect of *Acalypha indica* at varying concentrations. The extract exhibited significant free radical scavenging ability.

Comparatively, ascorbic acid demonstrated 94.20% inhibition at 500 µg/mL (Figure 2).

3.2.3 Superoxide Radical Scavenging Activity

The antioxidant efficacy of *A. indica* extract was evaluated at different concentrations and compared with a reference standard. The extract exhibited significant antioxidant effect in a dose-dependent manner. At 100 µg/ml, the extract demonstrated 45.65% inhibition, which progressively increased to 75.56% at 500 µg/ml. Although the extract showed slightly lower inhibition percentages compared to the reference standard, the results indicate a significant antioxidant potential of

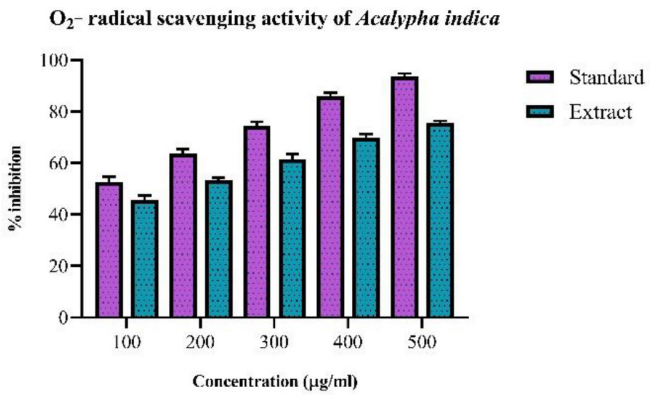


Figure 3. Inhibition effect of *Acalypha indica* extract on superoxide anion at different concentrations compared with the reference standard. Data are depicted as Mean % inhibition ± SD.

A. indica (Figure 3). These results imply that the extract possesses antioxidant ability, which could be attributed to its bioactive constituents.

3.3 Anti-inflammatory Potential

Chronic inflammation is a key factor in obesity-associated metabolic disorders, especially T2DM. To assess the therapeutic efficacy of *A. indica*, the expression levels of key molecules in proinflammatory (IL1β, IL6, TNFα) and NFκB signalling (NFκB, IKKβ) were analysed in high glucose (HG)-induced 3T3-L1 adipocytes.

3.3.1 Protein Denaturation Inhibition Assay

Protein denaturation is a well-established marker of inflammation, as denatured proteins can trigger immune responses and exacerbate inflammatory conditions. The ability of *A. indica* extract to prevent protein denaturation was assessed in comparison with a standard anti-inflammatory agent (diclofenac sodium). A concentration-dependent suppression of protein denaturation was observed, with the extract showing 46.57% inhibition at 100 µg/ml, which increased to 82.93% at 500 µg/ml (Figure 4). The reference standard (diclofenac sodium) exhibited a maximum inhibition of 90.53% at the highest concentration tested. These results suggest that *A. indica* possesses strong anti-inflammatory properties by stabilising proteins and preventing inflammatory damage.

3.4 Antidiabetic Activity

The extract exhibited a concentration-dependent inhibition of these enzymes, with maximum inhibition

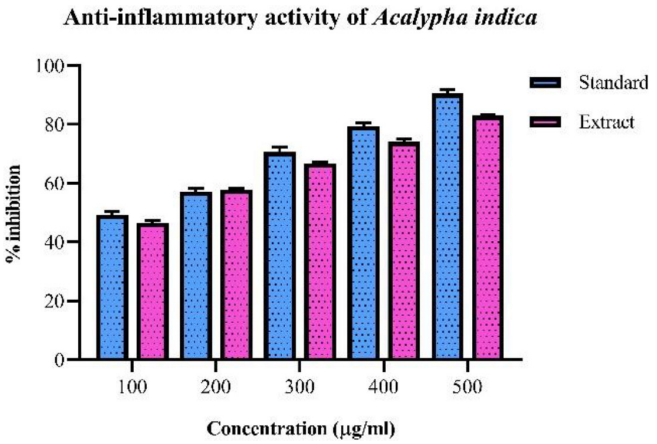


Figure 4. Inhibition effect of *Acalypha indica* extract on protein denaturation inhibition at different concentrations compared with the reference standard (diclofenac sodium). Data are depicted as Mean % inhibition ± SD.

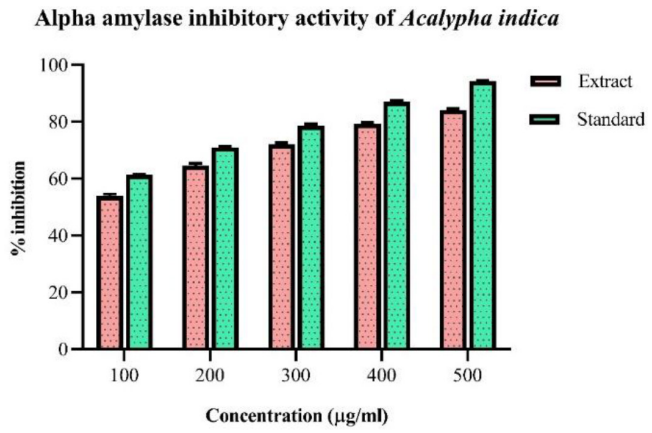


Figure 5. Inhibition effect of *Acalypha indica* extract on alpha-amylase at different concentrations, compared with the reference standard. Data are depicted as Mean % inhibition ± SD.

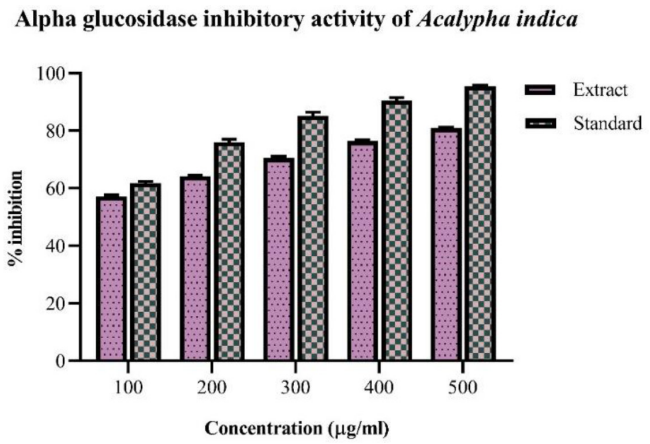


Figure 6. Alpha-glucosidase enzyme inhibition of *Acalypha indica* extract at varying concentrations, compared to the standard. Results were presented as Mean % inhibition ± SD.

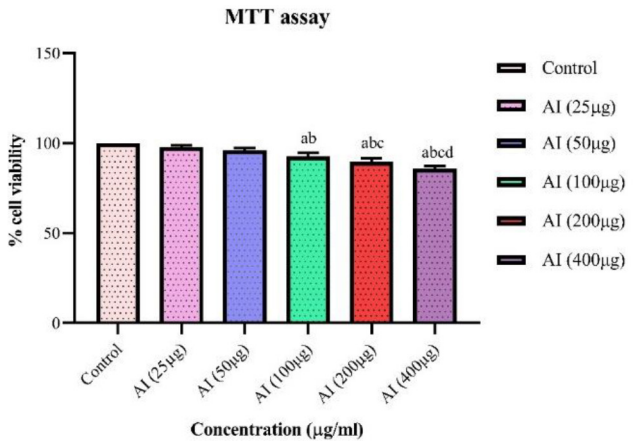


Figure 7. Cell viability (MTT assay) of 3T3-L1 adipocytes treated with *Acalypha indica* extract. The results show minimal cytotoxicity across different concentrations.

of 80.99% for α-amylase and 76.41% for α-glucosidase at 500 µg/mL (Figures 5 and 6).

3.5 Cell Viability Assay

The cytotoxicity of *A. indica* extract was assessed in 3T3-L1 adipocytes using the MTT assay. These findings confirm that cell viability remained above 80% at all tested concentrations (Figure 7).

3.6 Regulatory Effect of *A. indica* on Endogenous Antioxidant Enzymes

To analyse the antioxidant potential of *A. indica*, we evaluated its influence on key endogenous antioxidant enzymes (SOD, CAT, GSH, GPx) in high-glucose-induced adipocyte cells. These enzymes are essential to combat oxidative stress by neutralising ROS and regulating cellular redox balance. The results demonstrated a significant dose-response enhancement in the activities of these enzymatic antioxidants upon treatment with *A. indica* extract (Figure 8a-d).

These findings suggest that *A. indica* extract effectively enhances the enzymatic antioxidants, providing a robust defence against oxidative stress in adipocytes. The upregulation of these enzymes reinforces the antioxidant potential of *A. indica*, which may be due to the presence of bioactive

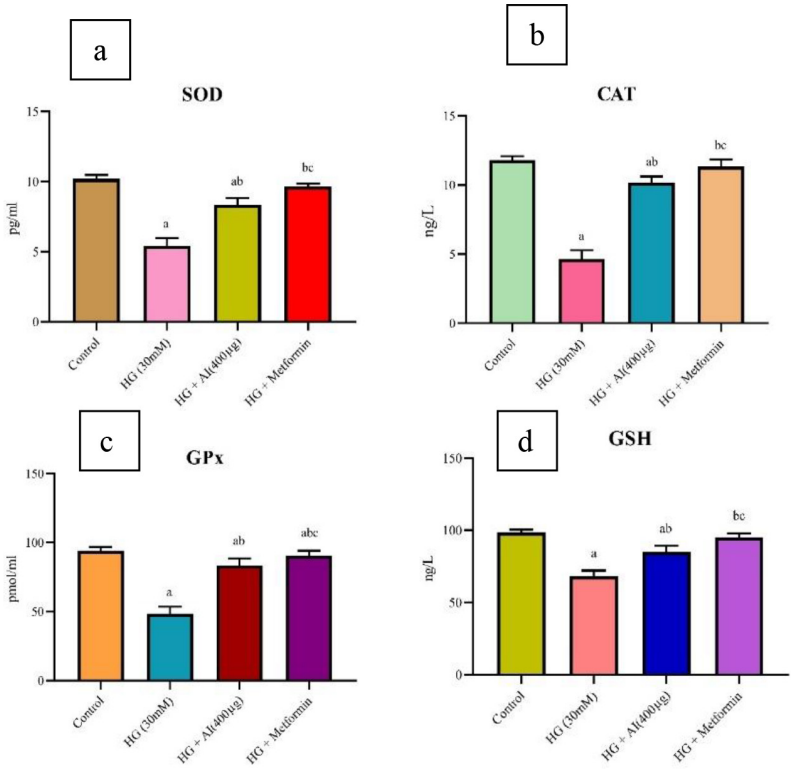


Figure 8 **(a-d).** Effect of *Acalypha indica* extract on endogenous antioxidant enzymes such as a)SOD, b)CAT, c)GSH and d)GPx. Data are depicted as Mean % inhibition ± SD.

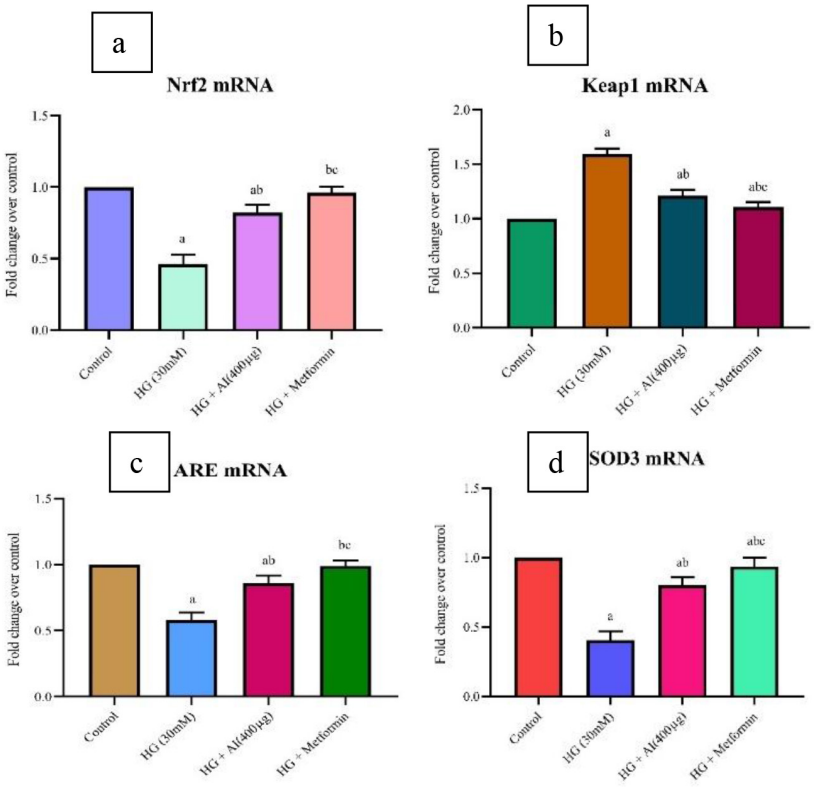


Figure 9 **(a-d).** Effects of *Acalypha indica* on a)Nrf2, b)Keap1, c)ARE, and d)SOD3 mRNA in high-glucose-induced adipocyte cells.

phytochemicals such as flavonoids and polyphenols. These results align with the molecular data demonstrating the activation of Nrf2 signalling and downregulation of Keap1, further supporting the hypothesis that *A. indica* potentiates antioxidant responses through the Nrf2/Keap1 pathway. These data underscore the pharmacological approach of *A. indica* in combating oxidative stress-associated metabolic dysfunctions.

3.7 Regulation of Nrf2/Keap1 Mediated Antioxidant Signaling in Adipocytes

To explore the molecular basis of the antioxidant potential of *A. indica*, key Nrf2/Keap1 pathway gene expression was assessed in high glucose-induced adipocyte cells. The mRNA expression of Nrf2 was significantly upregulated in adipocytes treated with *A. indica* extract compared to the untreated high glucose-induced control cells (Figure 9a). This suggests that *A. indica* enhances the stimulation of Nrf2, a central regulator of antioxidant defence mechanisms. Conversely, Keap1 mRNA levels were markedly reduced upon treatment with *A. indica* (Figure 9b). Keap1 modulates Nrf2 function by degradation, and its downregulation further supports antioxidant defence mechanisms by activating Nrf2 signalling. The expression levels of ARE and SOD3 increased significantly with *A. indica* treatment (Figures 9c and 9d). The activation of the ARE-driven transcriptional program leads to the upregulation of antioxidant and cytoprotective genes, reinforcing the role of *A. indica* in combating oxidative stress in adipocytes.

3.8 Effect of *Acalypha indica* Extract on Proinflammatory Signalling Molecules in HG-induced Adipocytes

To further investigate the anti-inflammatory potential, the expression levels of key proinflammatory molecules and NFκB pathway components were analysed in high glucose (HG)-induced 3T3-L1 adipocytes. A significant downregulation of IL1β, IL6, and TNFα mRNA

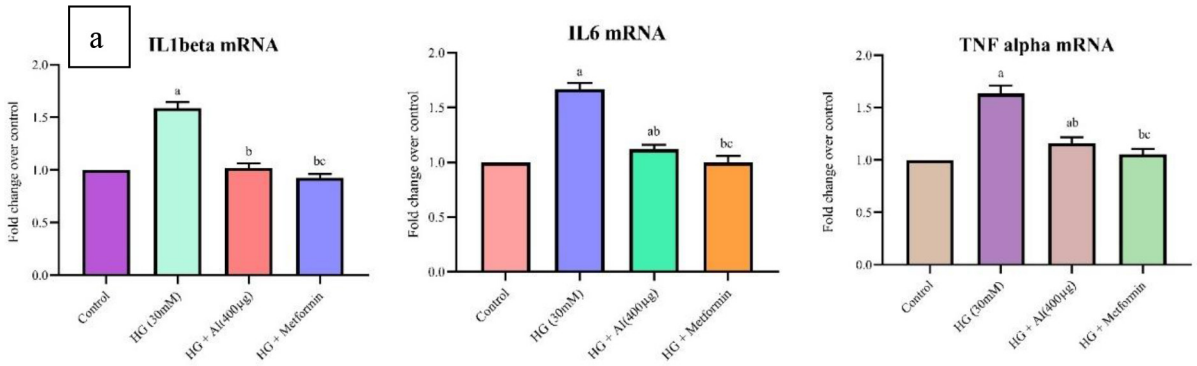


Figure 10 **(a-c).** Modulation of IL1β, IL6, TNFα mRNA levels by *Acalypha indica* in high-glucose-treated adipocytes.

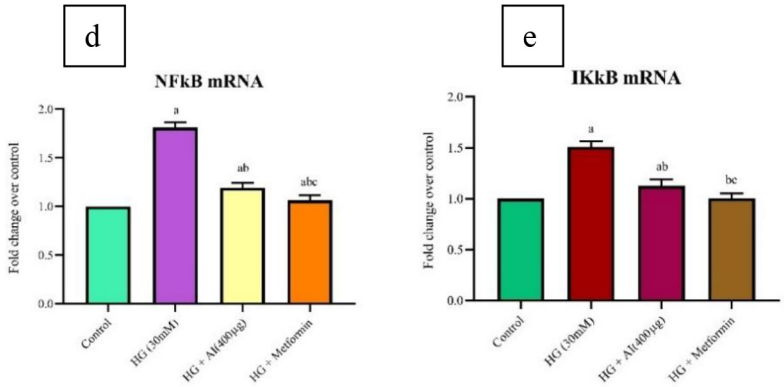


Figure 10 **(d-e).** Influence of *Acalypha indica* on d)NFκB and e) IKκB mRNA in adipocytes cultured in high glucose.

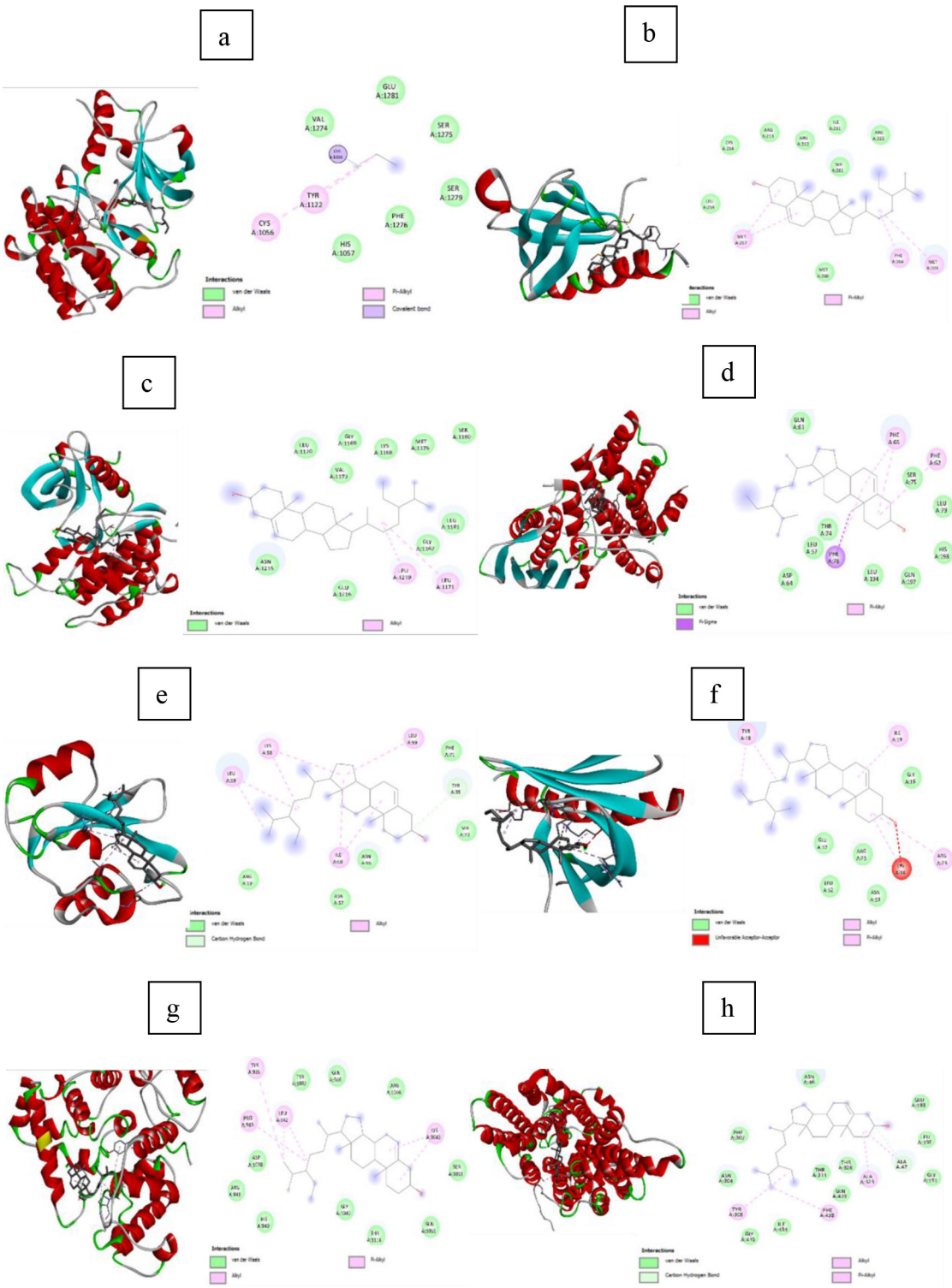


Figure 11 **(a-h).** Depicts the 3D interaction of β-sitosterol with target proteins including a)IR, b)IRS1, c)IRS2, d)PDK, e)PI3K, f)Akt, g)AS160, h)GLUT4. Molecular docking analysis was carried out using PyRx software and visualised using BioVIVA 3D Discovery Studio.

expression was observed upon treatment with *A. indica* extract, indicating a reduction in inflammatory signalling. Additionally, the extract inhibited NFKB and IKKβ expression, suggesting suppression of NFκB activation and downstream inflammatory cascades (Figure 10 (a-e)).

Table 1. Shows the binding affinity of the target proteins with β-sitosterol

Compound	Target Protein	Binding Energy (kcal/mol)
β-sitosterol	IR	-8.5
	IRS1	-6.1
	IRS2	-6.9
	PDK	-8.4
	PI3K	-6.3
	Akt	-6.8
	AS160	-7.2
	GLUT4	-7.6

These findings collectively indicate that *A. indica* shows significant anti-inflammatory potential, likely through NFκB inhibition and cytokine suppression,

Table 2. Shows the binding affinity of the target proteins with quinine

Compound	Target Protein	Binding Energy (kcal/mol)
Quinine	IL1β	-6.3
	IL6	-6.6
	TNFα	-6.2
	NFκB	-5.8
	IKκB	-6.1

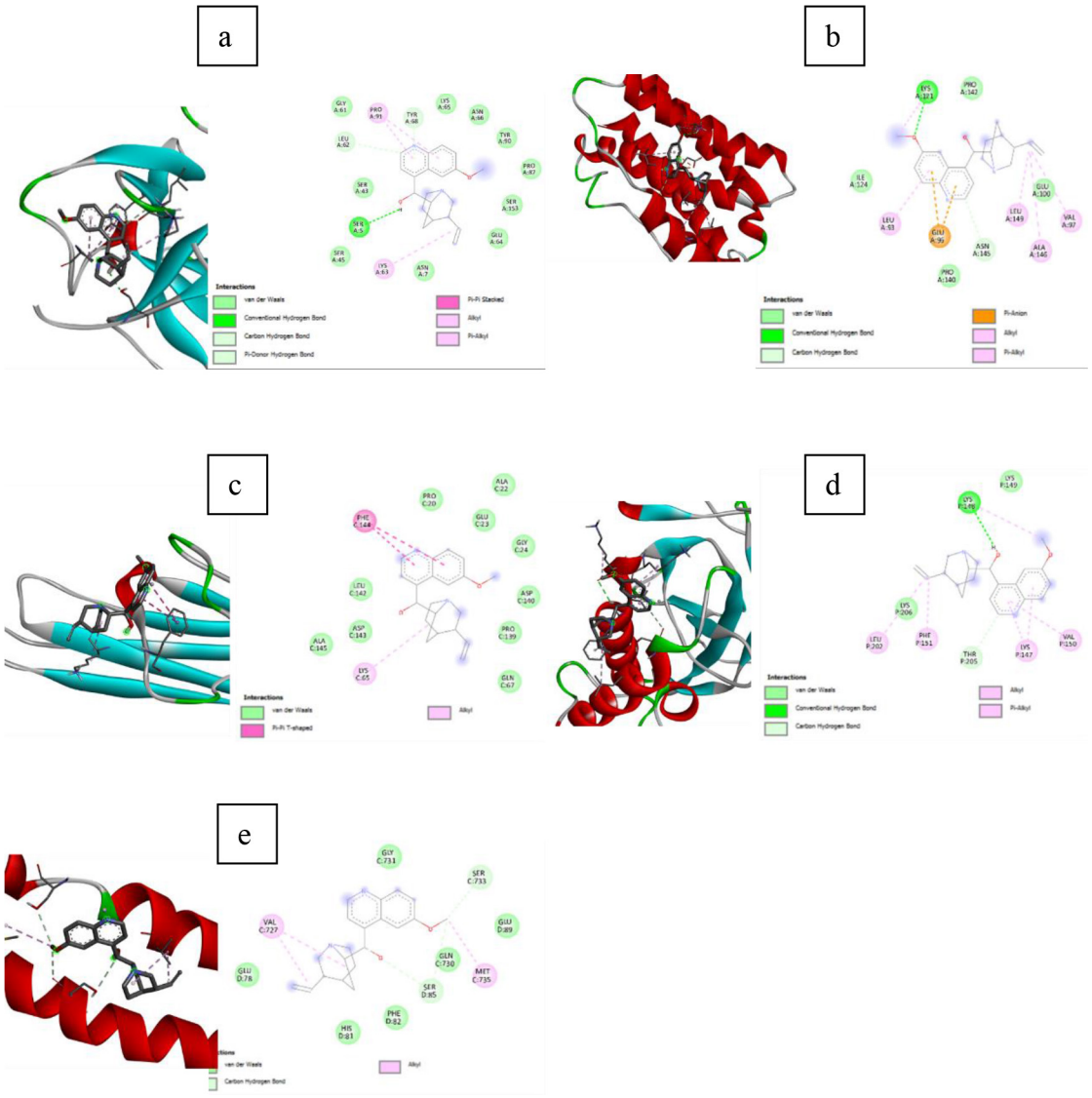


Figure 12 **(a-e).** Depicts the 3D interaction of quinine with target proteins, including a)IL1β, b)IL6, c)TNFα, d)NFκB and e)IKκB. Docking analyses were performed using PyRx, and BioVIA Discovery Studio was used for visualisation.

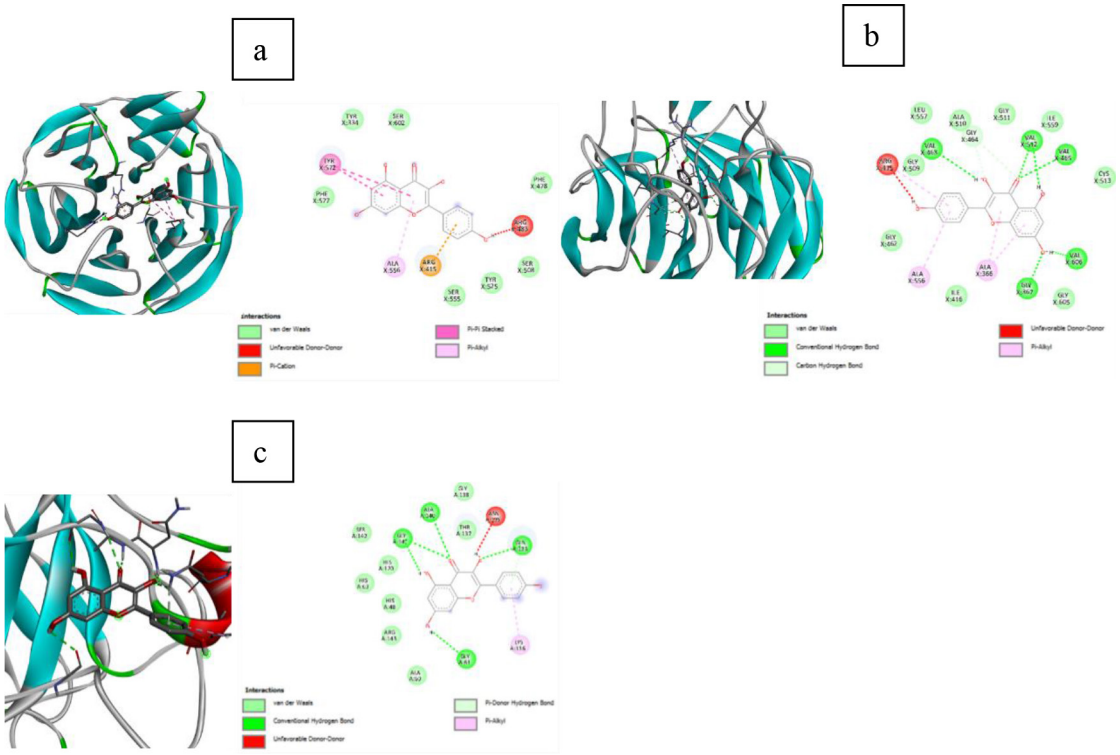


Figure 13 **(a-c).** Depicts the 3D interaction of kaempferol with target proteins, including a)Nrf2, b)Keap1 and c) SOD. Molecular docking analysis was carried out using PyRx software and visualised using BioVIVA 3D Discovery Studio.

supporting its therapeutic application in metabolic inflammation and insulin resistance.

3.9 Molecular Docking Analysis

Docking was performed to understand the interaction of bioactive compounds from *A. indica* with key proteins involved in diabetes management, such as IR, IRS-1/2, PI3K, AKT, and GLUT4. Results demonstrated significant binding interactions between the phytoconstituents and the target proteins, with binding energies ranging from -6.1 to -8.5 kcal/mol (Table 1). To further demonstrate the mechanism of anti-inflammatory activity, molecular docking studies were performed to examine interactions between bioactive compounds from *A. indica* and proinflammatory signalling proteins. Quinine, a key phytoconstituent, demonstrated strong binding affinities with IL1β, IL6, TNFα, NFκB, and IKKβ, with binding energies ranging from -5.5 to -7.0 kcal/mol (Table 2). These interactions suggest that *A. indica* may exert its anti-inflammatory effects by directly altering key regulatory proteins involved in inflammation. Kaempferol

Table 3. Shows the binding affinity of the target proteins with kaempferol

Compound	Target Protein	Binding Energy (kcal/mol)
Kaempferol	Nrf2	-7.5
	Keap1	-9.6
	SOD	-6.6

exhibited strong binding affinities with Nrf2 (-7.5 kcal/mol), Keap1 (-9.6 kcal/mol), and SOD (-6.6 kcal/mol) (Table 3). The strongest interaction was observed with Keap1, suggesting that kaempferol may facilitate Nrf2 activation by disrupting Keap1-mediated inhibition. Additionally, the favourable binding energy with SOD implies potential direct stabilisation and activation of this crucial antioxidant enzyme (Figures 11 (a-h), 12 (a-e) and 13 (a-c)).

The cumulative findings indicate that *A. indica* enhances antioxidant defences by activating Nrf2 signalling and suppressing Keap1 expression. This regulatory effect leads to the enhanced production of antioxidant enzymes via ARE activation, including

SOD3, which has a crucial function in mitigating oxidative stress in adipocytes. Furthermore, the molecular docking analysis suggests that kaempferol contributes to these effects by interacting with key regulatory proteins. Collectively, the current observations underscore the efficacy of *A. indica* as a novel treatment candidate for oxidative stress-related metabolic disorders, particularly those linked to inflammation and insulin resistance.

4. Discussion

The present study provides comprehensive insight into the multifaceted pharmacological potential of *A. indica* by systematically evaluating its antioxidant, antidiabetic, and anti-inflammatory activities through a combination of biochemical assays, cell-based experiments and molecular docking analyses. These findings collectively highlight its therapeutic significance in managing type 2 diabetes mellitus (T2DM) and obesity-associated metabolic dysfunctions. The observed bioactivities are attributed to the plant's diverse phytoconstituents, including tannins, flavonoids, polyphenols, quinine, and kaempferol, which act synergistically through modulation of oxidative, inflammatory, and insulin signalling pathways. Phytochemical richness of this nature has been widely associated with pleiotropic bioactivity in ethnomedicinally relevant plants, and the results of the present investigation are consistent with this premise[28].

The antioxidant capacity of *A. indica* was confirmed through DPPH and NO scavenging assays, which revealed significant radical neutralisation activity (Figures 1, 2). These activities are primarily attributable to the presence of polyphenolic and flavonoid compounds that possess strong electron-donating and hydrogen-atom transfer capacities, enabling them to stabilise reactive oxygen species and interrupt the oxidative chain reactions that underlie cellular damage. Comparable antioxidant properties have been documented in *Ocimum sanctum* and *Azadirachta indica*, both recognised for their ability to neutralise free radicals and defend cells from tissue injury[29]. The DPPH assay measures the ability of antioxidants to donate a hydrogen atom or electron to a stable free radical, while the NO scavenging assay reflects the extract's capacity to inhibit nitric oxide-mediated oxidative damage, which is particularly relevant in inflammatory microenvironments. Superoxide scavenging (Figure 3) further confirmed the broad-spectrum antioxidant profile of the extract, underscoring its ability to neutralise the most abundant and reactive physiological radical, O_2^-, which serves as a precursor to more damaging species such as hydrogen peroxide and hydroxyl radicals.

Oxidative stress is a critical determinant in the progression of T2DM, where persistent ROS accumulation induces pancreatic β-cell dysfunction, impairs insulin resistance, and perpetuates chronic low-grade inflammation[30]. The antioxidant effect of *A. indica* indicates its capability to counteract ROS-induced damage and preserve cellular homeostasis, which is relevant to metabolic disease. To validate this further at the cellular level, enzymatic antioxidant assays were performed in high-glucose-induced 3T3-L1 adipocytes. Treatment with *A. indica* extract significantly elevated the activities of superoxide dismutase, catalase, glutathione and glutathione peroxidase, essential enzymes responsible for the first line of intracellular antioxidant defense[31] (Figure 8a-d). The observed upregulation of these enzymes suggests that the extract does not merely scavenge free radicals directly but also enhances the cell's endogenous antioxidant defenses. This is particularly relevant given that chronic hyperglycaemia is known to suppress these enzymatic pathways, contributing to the progressive oxidative burden that characterises T2DM.

Mechanistically, treatment with *A. indica* extract significantly activated the Nrf2/Keap1 signalling pathway in high glucose-induced adipocytes, a central regulatory axis in redox homeostasis (Figure 9a-d). Under basal conditions, Nrf2 is sequestered in the cytoplasm by its repressor Keap1, which targets it for ubiquitin-mediated proteasomal degradation[32]. Upon oxidative challenge, or in the presence of phytochemical activators, this interaction is disrupted, allowing Nrf2 to translocate to the nucleus where it binds to antioxidant response elements (AREs) within the promoter regions of cytoprotective genes[33]. Consistent with this mechanism, the extract increased nuclear Nrf2 translocation and downregulated Keap1 expression in a concentration-dependent manner, thereby facilitating ARE-dependent activation of antioxidant defence genes. This upregulation was

further evidenced by elevated SOD3 expression, an extracellular enzyme that plays a critical role in protecting extracellular matrix and tissue interfaces from oxidative injury[34]. The coordinated induction of nuclear Nrf2, suppression of Keap1 and upregulation of SOD3 constitute robust activation of the Nrf2/Keap1/ARE axis, providing a mechanistic explanation for the enhanced enzymatic antioxidant activities observed in the biochemical assays.

Molecular docking studies provided structural validation of this pathway. Kaempferol, one of the dominant phytochemicals, exhibited strong binding affinities with Nrf2 (−7.5 kcal/mol), Keap1 (−9.6 kcal/mol), and SOD (−6.6 kcal/mol) (Figure 13a-c). The exceptionally high affinity of kaempferol for Keap1 is particularly noteworthy, as it suggests competitive displacement of Nrf2 from its repressor complex, which disrupts Keap1-mediated suppression and promotes sustained nuclear Nrf2 activity. Simultaneously, its interaction with Nrf2 itself may stabilise the active transcriptional conformation, while binding to SOD may enhance the enzyme's catalytic stability. These molecular interactions substantiate the functional activation of the Nrf2/Keap1 axis observed in vitro and highlight a precise molecular basis for the extract's antioxidant defence capabilities.

Additionally, cytotoxicity was assessed using the MTT assay in 3T3-L1 adipocytes (Figure 7). The extract maintained cell viability above 80% across all tested concentrations, demonstrating minimal cytotoxic potential and confirming its safety profile for further mechanistic pharmacological investigations. Importantly, this favourable cytotoxicity profile confirms that the observed antioxidant and anti-inflammatory effects reflect specific pharmacological activity rather than non-specific cytotoxicity. This also validates the biological relevance of the concentration range employed in downstream assays. Similar cytotoxic safety profiles have been reported for other medicinal plant extracts used in antidiabetic research, reinforcing the general tolerability of polyphenol-rich herbal preparations at pharmacologically active doses[35].

The enzyme inhibition analysis demonstrated that *A. indica* effectively inhibited digestive carbohydrases including α-amylase and α-glucosidase in a concentration-dependent manner (Figures 5 and 6). This inhibitory activity indicates the extract's capability to reduce the rate of polysaccharide hydrolysis and glucose absorption in the intestinal lumen, thereby moderating postprandial hyperglycaemia – a major contributor to glycemic variability and oxidative stress in T2DM. The mechanism resembles that of acarbose, a clinically used α-glucosidase inhibitor that acts at the primary site of nutrient absorption to delay carbohydrate digestion. However, unlike acarbose, which is associated with gastrointestinal side effects due to fermentation of undigested carbohydrates in the colon, phytochemical inhibitors may offer a comparably efficacious but potentially better tolerated alternative. Similar findings have been reported in *Trigonella foenum-graecum* and *Morus alba*, which also exhibit inhibitory effects on carbohydrate-hydrolyzing enzymes and have been validated as complementary antidiabetic agents[36,37].

Beyond carbohydrate enzyme inhibition, molecular docking analysis revealed strong binding affinities between *A. indica* bioactives and key components of the insulin signalling cascade, including IR, IRS-1/2, PI3K, AKT, and GLUT4 (Figure 11a-h). These molecular targets play critical and sequential roles in glucose uptake and insulin sensitivity. These results suggest that the extract upregulates the insulin signalling cascade by facilitating insulin receptor binding and downstream activation of PI3K/AKT, ultimately enhancing GLUT4 translocation into the plasma membrane. Previous molecular docking studies on *Syzygium cumini* and *Andrographis paniculata* reported similar interactions, further supporting the potential of phytoconstituents to modulate glucose homeostasis through direct binding with the insulin signaling pathway[38].

Persistent low-grade inflammation is a characteristic feature of T2DM and obesity associated metabolic disorders. The NFκB signalling pathway serves as a crucial mediator linking inflammatory cytokines with insulin resistance, as NFκB activation drives the transcription of proinflammatory genes that directly interfere with IRS phosphorylation and downstream glucose metabolism[39]. In high glucose-induced 3T3-L1 adipocytes, treatment with *A. indica* produced a marked reduction in the mRNA expression of NFκB and its upstream regulator IKKβ, leading to decreased transcription of proinflammatory cytokines including TNFα, IL6, and IL1β (Figure 10a-e). This pattern of

gene expression suppression is consistent with the inhibition of the canonical NFκB activation cascade, in which IKKβ phosphorylates the inhibitory IκB protein, triggering its degradation and permitting NFκB to translocate to the nucleus. By reducing IKKβ expression, the extract likely interrupts this cascade upstream, preventing NFκB nuclear entry and its subsequent transcriptional activity. The downstream consequence of reduced TNFα, IL6, and IL1β is of considerable metabolic significance, as these cytokines are known inducers of serine phosphorylation of IRS1, which is a key mechanism of obesity induced insulin resistance[6]. Furthermore, the extract also exhibited notable inhibition of protein denaturation, an established *in vitro* indicator of anti-inflammatory potential, with inhibitory activity comparable to diclofenac sodium, a standard nonsteroidal anti-inflammatory drug. This result bridges biochemical and cellular anti-inflammatory findings, reinforcing the extract's broad-spectrum anti-inflammatory efficacy.

Molecular docking studies of quinine, a predominant alkaloid in *A. indica*, revealed strong binding affinities (ranging from −5.5 to −7.0 kcal/mol) with central proinflammatory mediators such as NFκB, IKKβ, TNFα, IL6, and IL1β (Figure 12a- e). These binding energies are indicative of thermodynamically favourable ligand-protein interactions and suggest that quinine may directly interfere with proinflammatory signalling at multiple nodes of the NFκB pathway. The interaction with IKKβ is particularly significant, as direct IKKβ inhibition would prevent IκB phosphorylation and degradation, thereby retaining NFκB in the cytoplasm in an inactive state[40]. Concomitant interactions with cytokine molecules (TNFα, IL6, and IL1β) may additionally reduce receptor binding or downstream signalling, further amplifying the anti-inflammatory effect. Previous research has demonstrated that suppression of the NFκB pathway leads to enhanced insulin receptor sensitivity and enhanced glucose transport in adipocytes, providing a direct molecular link between the observed anti-inflammatory activity and the extract's antidiabetic potential[10]. These gene expression results (Figure 10a-e) and the docking results (Figure 12a-e) for the NFκB axis strengthen the mechanistic interpretation and support quinine as a key anti-inflammatory bioactive constituent.

The integrated modulation of redox homeostasis and inflammatory signalling pathways observed in this study suggests that *A. indica* exerts its bioactivity through a pleiotropic, multi-targeted mechanism. Activation of the Keap1-Nrf2 axis leads to elevated antioxidant gene transcription, whereas simultaneous inhibition of NFκB signalling attenuates proinflammatory cytokine production. These two pathways are not independent: the crosstalk between Nrf2 and NFκB drives inflammatory gene transcription through competition for shared coactivators such as CBP and through direct inhibition of IKK activity[32,41]. This reciprocal relationship implies that the antioxidant action of *A. indica* may synergistically amplify its anti-inflammatory effect via this crosstalk and vice versa. Furthermore, the inhibition of α-amylase and α-glucosidase coupled with the modulation of insulin signalling intermediates reinforces the hypoglycemic efficacy of the extract through complementary peripheral and intestinal mechanisms[42]. Collectively, these findings establish *A. indica* as a potent phytotherapeutic candidate for diabetes management, acting through the integrated modulation of oxidative stress, inflammation, and glucose metabolism.

While this study demonstrates robust pharmacological potential of *A. indica,* certain limitations warrant acknowledgement for appropriate interpretation. The *in vitro* experimental design using 3T3-L1 adipocytes and biochemical assays offers valuable mechanistic insights but would benefit from complementary *in vivo* validation to capture systemic T2DM physiology. The crude ethanolic extract reflects traditional preparation methods; future fractionation studies could elucidate specific phytoconstituent contributions. Pharmacokinetic profiling and long-term safety assessment represent clinical validation. However, future studies involving *in vivo* modelling, clinical validation, and pharmacokinetic analysis are essential to substantiate its therapeutic efficacy, biological safety, and clinical applicability.

5. Conclusion

This study highlights that *A. indica* targets cellular redox and inflammatory signalling, with particularly strong modulation of the Nrf2/Keap1 and NFκB pathways in insulin-resistant adipocytes. These data suggest that *A.*

indica could serve as a multitargeted phytotherapeutic candidate for oxidative stress-linked insulin resistance in type 2 diabetes. Future studies should focus on validating these effects in preclinical animal models of diabetes and on identifying the specific bioactive constituents responsible for this dual pharmacological efficacy to support rational development of standardised herbal formulations.

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Data curation, writing – review and editing; Ponnulakshmi Rajagopal: Methodology, validation, formal analysis, and investigation; Vishnu Priya Veeraghavan: Formal analysis, software, Visualisation, writing–review and editing; Selvaraj Jayaraman: Conceptualisation, supervision, project administration, funding acquisition, writing – review and editing, corresponding author. All authors have read and agreed to the published version of the manuscript.