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Original Article

Anacardic Acid Ameliorates Anti-Inflammatory and Antioxidant Signaling Pathways in Human Hepatocellular Carcinoma HepG2 ARE Cells

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ABSTRACT

Background: Anacardic Acid (AA) is derived from cashew kernel shells and cashew apples and recognized for its extensive pharmacological properties, which include significant antitumor activity. Previous studies have established the anticancer efficacy of AA across other malignancies; however, its specific function and the underlying mechanisms in hepatic cancer are still to be thoroughly investigated. **Materials and Methods:** HepG2-ARE hepatocellular carcinoma cells were treated with different concentrations of AA and subjected to luciferase, MTS, glucose uptake, cell migration, Western blot and immunofluorescence assays to assess the effect of AA on NRF2 activation, cell viability, glucose uptake, cell migration and protein expression respectively. **Results:** We found that AA significantly lowered the vitality of hepatic cancer cells and their ability to take up glucose. Furthermore, AA inhibited the migratory ability of cancer cells and activated the NRF2 signaling pathway, resulting in the upregulation of antioxidant enzymes, including SOD1, NQO1, and HO1. It also regulated the PI3K/AKT/mTOR and Wnt/β-catenin pathways essential to the growth and survival of cancer cells. AA reduced phosphorylated NF-κB and proinflammatory cytokines, attenuating NF-κB signaling in inflammation, and activated MAPK/JNK signaling by increasing phosphorylation of p38 and JNK. Immunofluorescence examination substantiated reduced phosphorylated NF-κB levels in AA-treated cells relative to untreated controls, corroborating its function in NF-κB pathway suppression. **Conclusion:** These results indicate that AA has potential as a therapeutic agent by modulating multiple signaling pathways, which may result in potent anticancer effects against hepatic carcinoma. Nonetheless, additional *in vivo* investigations are required to confirm its effectiveness and clinical relevance.

Keywords: Anacardic acid, Cell signaling pathways, Hepatic cancer cells, Immunofluorescence, Oxidative stress.

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INTRODUCTION

Anacardic Acid (AA), which belongs to the *Anacardiaceae* family, is a combination of 2-hydroxy-6-alkylbenzoic acid analogs. It is widely recognized for its many therapeutic benefits, including acting as a mitochondrial uncoupler of oxidative phosphorylation. It is mostly present in cashew apples (*Anacardium occidentale*), *Ginkgo* leaves, and other fruits (Mattison *et al.*, 2018). AA has shown strong anti-tumor effects, stopping the growth of breast, lung, and prostate cancers, among others (Park *et al.*, 2018). Mechanistically, AA is identified as an inhibitor of eukaryotic histone acetyltransferases and also attenuates NF-κB and metalloproteinase activities (Gomes Júnior *et al.*, 2020; Gutiérrez-Paz *et al.*, 2024; Hollands *et al.*, 2016). Moreover, AA exhibits a wide array of biological activities, including antioxidant, antibacterial, and antimutagenic effects (Hamad and Mubofu, 2015; Oiram Filho *et al.*, 2023; Runjala and Kella, 2017).

Oxidative stress significantly contributes to the onset of several malignancies by inducing the overproduction of Reactive Oxygen Species (ROS), disrupting redox homeostasis, and impairing cellular activities. These occurrences are significantly correlated with the onset and advancement of several cancer types (Iqbal *et al.*, 2024). In cancer cells, high amounts of ROS change the expression of important regulatory proteins like MAPK phosphatase and PTEN, which then affect many signaling pathways in a cell-specific way, including NRF2, MAPK/ERK, PI3K/AKT/mTOR, and NF-κB. Moreover, tumor cells utilize oxidative stress to initiate anti-apoptotic and pro-survival signaling pathways (Hayes *et al.*, 2020; Takahashi *et al.*, 2018). Restoring redox balance is a promising way to fight the bad

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effects of oxidative stress and improve the results of treatments. These methods include medicines that use antioxidants and redox-modulating drugs, which help restore normal cellular functioning and signaling pathway regulation (Azmanova and Pitto-Barry, 2022).

Natural plant bioactives provide several activities in cancer including cell proliferation inhibition, antioxidant and anti-inflammatory activities (Maiuolo *et al.*, 2021). AA has been reported for suppression or stimulation of various metabolic pathways in different types of cancers. AA induced pituitary adenoma cells apoptosis (Sukumari-Ramesh *et al.*, 2011), suppressed estrogen receptor α proliferation, anti-proliferative and pro-apoptotic activity in MCF-7 cells (Schultz *et al.*, 2018). AA were reported to suppress NF-κB expression (Sung *et al.*, 2008), triggered apoptosis in prostate cancer cells (Tan *et al.*, 2012) and promoted osteoblast differentiation in MG63 osteosarcoma cells (Venugopal *et al.*, 2021). Moreover, AA stimulated adaptive immune system *via* activating the phosphorylation of MAPK along with NF-κB pathway (Gnanaprakasam *et al.*, 2015). Interestingly, AA induced cell apoptosis related to ATF-4 dependent ER stress (Huang *et al.*, 2014), stimulated cell apoptosis in prostate cells *via* ER stress/DAPK3/ Akt signaling cascade (Tan *et al.*, 2017).

Although numerous studies have illustrated the anticancer potential of Anacardic Acid (AA) in various cancer types, its mechanistic role in hepatic cancer, particularly in the HepG2-ARE cell line, remains largely unexplored. To address this gap, the current investigation examines the dose-dependent effects of AA on cell viability, migration, and glucose uptake in HepG2-ARE cells. Furthermore, both intrinsic and extrinsic *in vitro* pathways associated with oxidative stress, inflammation, and proliferation were investigated. Moreover, immunofluorescence analysis was performed to confirm the inhibition of NF-κB transcriptional activity in AA-treated hepatic cancer cells.

MATERIALS AND METHODS

Chemicals and Reagents

Anacardic acid (98% pure) was procured from Sigma-Aldrich, USA. Primary antibodies were sourced from Abcam (USA) and Cell Signaling Technology (USA). All solvents and reagents used were molecular biology grade.

Cell Culture and Cell Viability

Hepatocellular carcinoma luciferase reporter stable cell line (HepG2-ARE) was obtained from Signosis, USA. The culturing of the cells was performed under standard conditions utilizing DMEM high glucose (DMEM HG, HiMedia, India) was added with Fetal Bovine Serum (FBS, 10% v/v) (HiMedia, India), antibiotic solution i.e., penicillin (100 U/mL) and streptomycin (100 µg/mL) (HiMedia, India). The cells were placed at 37°C in a humidified incubator with 5% CO_2.

NRF2-ARE Activation Luciferase Reporter Assay

Luciferase assay was performed through plating the cells in 96-well plates ($1x10^4$ cells/well) for 24 hr in humidified conditions at 37°C containing 5% CO_2. The cells were starved for 12 hr followed by AA treatment at varying concentrations (1, 5, 10, 20, 25, 40, 80, and 100 µM) in triplicates. Further, the cells were kept at 37°C for 24 hr with similar CO_2 conditions. Following incubation, washing of cells in each well was done with 100 µL 1XPBS buffer followed by addition of lysis buffer (20 µL) and kept at room temperature for 15 min. Further, firefly luciferase substrate (50 µL) was added, and luminescence was taken within 10 sec after substrate addition using multiwell microplate reader. NRF2 activation of treated cells was calculated relative to control.

MTS Assay

The cell viability of HepG2-ARE was evaluated *via* MTS assay using MTS reagent (Promega, USA, G5421). The cell viability percentage was estimated with CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay Kit (Promega, USA, G5421). The seeding of the cells ($1x10^4$ cells/well) was performed into 96-well plates in triplicates with different AA concentrations ranging from 1-100 µM. The group treated with DMSO (0.1% v/v) was taken as control. The cells were kept for duration of 24, 48, and 72 hr at 37°C in humidified conditions with 5% CO_2 after 12 hr starvation. After incubation, MTS solution was added to each well (20 µL) and kept at 37°C for another 3 hr. The cell viability percentage was measured at 490 nm in Cytation5 multiwell plate reader (BioTek, USA).

Antioxidant Activity of AA

The antioxidant activity of AA in HepG2-ARE cell line was measured with EZAssay™ antioxidant activity estimation kit (CUPRAC, HiMedia, India). The cells ($2x10^7$ cells per well) were seeded on 6-well plate, cultured and starved for 24 hr and 12 hr, respectively. The cells were treated at three AA concentrations (5, 10 and 15 µM) and placed for 24 hr at 37°C in a humidified CO_2 environment. After this, medium was removed followed by cell washing using 1X PBS and sonicated thrice at pulse of 10 sec on/off on ice. Further, deionized water (100 µL) was added to each well and 10 µL of respective standards and samples. The chromogenic substrate (100 µL) was added and incubated at room temperature for 10 min. The absorbance was read at 450 nm. The results were expressed as Trolox equivalent.

Glucose Uptake Assay

HepG2-ARE cells were grown ($1x10^5$ cells per well) in 96 well plate at 37°C for 24 hr in a humidified CO_2 (5%) atmosphere. Cells were treated with varying concentration of AA (1-20 µM) for 48 hr. Glucose uptake assay was executed using Glucose assay kit (GAGO, Sigma-Aldrich, India) according to manufacturer protocol. Spent culture medium was taken out and swapped with incubation medium i.e., DMEM with 0.5 mM glucose (50 µL),

0.1% BSA for 4 hr. Following this, incubation medium (20 µL) was transferred to another fresh 96-well plate and absorbance was taken at 540 nm. The glucose uptake by the cells was determined *via* subtraction of cell-containing wells from cell-free wells.

Cell Migration Assay

The ability of HepG2-ARE cells migration was evaluated using a cell scratch migration assay. Cells were grown in 6-well plates ($1x10^6$ cells per well). The cells underwent 12 hr serum deprivation followed by 36 hr exposure at AA's two concentrations *viz.* 5 and 15 µM. The cell monolayer was consistently scraped using sterile pipette tip to replicate a wound. The floating cells were cleaned thrice with PBS in each well. The adherent cells were maintained using serum-free medium. The images were taken using an inverted microscope (Olympus, Japan). The cell migration distance was determined by calculating the area of the cell-free gap at specific time points using ImageJ software. For each sample, the images were subjected to quantitative analysis with ImageJ. The distance between the opposing edges of the scratch was measured for each image. By comparing these measurements from the initial (time 0) to the final time point, the extent of scratch closure was considered as follows:

Wound-healing rate (%) =(Area of original wound - Area of actual wound at different times)/Area of original wound ×100.

Protein Extraction

HepG2-ARE cells ($2 x10^5$) were grown in 6-well plate which was followed by starvation for 12 hr. Different concentrations of AA i.e., 1, 5, 7.5, 10, 15 and 20 µM were given to cells for 24 hr. After this, the washing of cells was completed using 1X PBS and protease inhibitor in RIPA buffer (HiMedia, India). was used for protein extraction. The cells were lyzed using a sonicator with pulse 10 sec on/off and placed in ice for 30 min. The centrifugation of cell lysate was completed at 16,000 x *g* for 20 min at 4°C followed by supernatant collection to obtain a full cell protein extract. To measure the protein concentration, BCA assay kit (ThermoFisher, USA) was used as per the manufacturer's directions. The absorbance was taken at 540 nm. Bovine Serum Albumin (BSA) was taken as reference standard for BCA assay.

Western Blot Analysis

For Western blotting, protein per lane (20 µg) was electrophoresed on SDS polyacrylamide gels (10%, Bio-Rad Laboratories, USA). The proteins were transferred to a PVDF membrane employing the Trans-Blot Turbo method (Bio-Rad, USA). Blocking of the membranes was completed in 5% BSA for 1 hr at room temperature. Subsequent to three washes using 1X TBST, blots were placed at 4°C overnight with corresponding primary antibodies for AP-1 (1:2000), APC (EP701Y), Axin2, c-Fos, CK1, c-Myc, Frizzled5, HO-1, NQO1, P-p38 MAPK (T180/Y182), p-GSK3 Beta (S9), Phospho-SAPK/JNK (Thr183/Tyr185), Phospho-β-Catenin (Ser45), P-LRP6 (Ser1490), p-mTOR (Ser2481), p-NF-κB p65 (Ser536), p-NRF2, p-PDK1 (Ser241), p-S6 Ribosomal Protein, SOD1, Wnt-5a, β-actin at (1:1000) dilutions whereas KEAP1, p-PI3K p110 Alpha at 1:800 and IL18, IL1α, IL1β, IL-2, p-AKT (Ser473) at 1:750 dilution. After the incubation with primary antibodies, the membranes were exposed to secondary antibodies (anti-mouse, anti-rabbit, 1:5000) coupled with horseradish peroxidase (Cell Signaling Technology, USA) for 1 h at room temperature. The washing of blots was accomplished with 1X TBST for 10 min each with gentle rocking. Protein expression was observed using the SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher, USA). Images were taken using Bio-Rad ChemiDoc™ MP system and densitometry analysis of each band was executed using ImageJ software. β-actin was taken as loading control.

Immunofluorescence Analysis

The immunocytochemical technique was used following the protocol of Dutta *et al.*, with slight adaptations to assess the impact of AA on HepG2-ARE cells, particularly emphasizing on the expression of phosphorylated NF-κB in the cytoplasm (Dutta *et al.*, 2020). The cells were grown in 6-well plates containing coverslips at 37°C for 24 hr in an incubator with 5% CO_2. The starvation of cells was done for a duration of 12 hr followed by 15 µM AA treatment for 24 hr. Afterwards, the cells were fixed with ice-cold methanol as well as 0.3-0.5% Triton X-100 followed by blocking using 5% BSA. The cells were maintained at 4°C for the whole night with primary anti-phospho-NF-κB antibody (dilution 1:200). After 1x PBS buffer wash, the cells were kept in secondary antibodies at room temperature for 1 hr with Alexa Fluor 555 diluted at 1:400. Further, the cells were again washed with 1x PBS and counterstained with DAPI to visualize the cell nuclei. Images were taken using a fluorescence microscope (Olympus, Japan) at 20x and 100x magnification.

Fluorescence images were analyzed using ImageJ software. Images were opened in ImageJ and channels were split using Image > Color > Split Channels. The red channel (Alexa Fluor 555) and blue channel (DAPI) were selected for analysis. Using the Rectangle Tool or Freehand Tool, regions of interest (ROIs) were drawn around red fluorescent cells. DAPI channel was used to confirm the presence of nucleus and to guide ROI selection. After marking the ROI, Analyze> Measure was used to obtain raw values. The Mean Fluorescence Intensity (MFI) was determined as:

MFI = Integrated Density - (Area of selected cell × Mean background fluorescence)

To get the background fluorescence, an empty (non-stained) area in the same image was selected and measured. The same ROI size was used across all samples for consistency. MFI values

were calculated for each image, and average MFI was plotted for treated and control groups.

Statistical Analysis

All experiments were performed in triplicates. Results were stated as Mean±SD. For the comparisons between groups, unpaired student's *t*-test and one-way ANOVA were employed with $p \leq 0.05$ (*$p \leq 0.03$, **$p \leq 0.002$, ***$p \leq 0.0002$, ****$p \leq 0.0001$) represents statistical significance.

RESULTS

Anacardic Acid Activates NRF2 Signaling

To assess the activation of NRF2 by AA, luciferase assay was performed at varying concentrations of AA ranging from 1-100 µM after 24 hr treatment in a concentration-dependent manner (Figure 1). The activity of NRF2 increased in a concentration-dependent manner, reaching its zenith at 10 µM, and then experiencing a significant decline at higher doses. At 40 µM, a significant decrease was observed, which is likely due to the cytotoxic effects that occur at high concentrations. Notably, the significant increase in Relative Fluorescence Units (%RFU) at 10 µM was an unambiguous indication of the maximum activation of NRF2 by AA.

Anacardic Acid Inhibits HepG2-ARE Cell Survival

The cell viability of HepG2-ARE cell line was evaluated using MTS assay after treating a range of AA concentration from 1-100 µM for 24, 48 and 72 hr. The HepG2-ARE cells were sensitive to AA treatment in dose- and time-dependent manner. The cell viability subsided at 5 µM, increased at 10 µM and sharply declined afterwards after 24 and 72 hr AA treatment (Figure 2). However, after 48 hr, the cell viability declined gradually with the increase in AA concentration depicting the inhibitory effect of AA on HepG2-ARE cells (Figure 2).

Anacardic Acid Exhibits Strong Free Radical Scavenging Capacity

The antioxidant potential of AA on HepG2-ARE cells was assessed using CUPRAC assay. In comparison to untreated controls, AA significantly increased antioxidant activity in a concentration-dependent manner after 24 hr of treatment ($p \leq 0.05$). At 5 µM, the free radical scavenging activity increased from 438.74 Trolox equivalents to 760.68 Trolox equivalents at 15 µM AA ($p \leq 0.05$, Figure 3). These results indicate that AA possesses a robust antioxidant capacity, which may be crucial in the enhancement of cellular defense mechanisms.

Anacardic Acid Inhibits Glucose Uptake in Hepatic Cancer Cells

Cancer cells show characteristic alterations in metabolic processes such as increased uptake of glucose along with enhanced glutaminolysis and fatty acid synthesis (Pliszka and Szablewski, 2021). Glucose uptake was significantly reduced with the escalating concentrations of AA in HepG2-ARE cells (Figure 4). AA-treated cells at highest concentration of AA i.e., 20 µM (18.44 % 4.45) showed a significant reduction in glucose uptake than in untreated cells (Figure 4). A significant sharp decline in glucose uptake was noticed from 5 to 15 µM of AA in HepG2-ARE cells (Figure 4). These findings indicated that AA treatment suppressed the glucose uptake in hepatic carcinoma cells, thereby inhibiting proliferation of cancer cells.

Anacardic Acid Suppresses Cell Migration in Human HepG2-ARE Cancer Cells

The effect of AA treatment on cell migration was estimated *via* treating hepatocarcinoma cells at two different concentrations, *viz*. 5 and 15 µM for 36 hr. Untreated HepG2-ARE cells showed faster migration than AA-treated cells (Figure 5). In untreated cells, the reduction in the gap indicated the faster migration of cells however, in case of AA-treated cells, the cell migration was significantly suppressed at 15 µM (10.26 % 7.99) in comparison to untreated HepG2-ARE cells (***$p \leq 0.001$, Figure 5). AA exhibited a suppressive effect on the migration of HepG2-ARE cells which could be further exploited as a plausible role of AA in cancer prevention *via* suppression of cell migration.

Anacardic Acid Modulates PI3K/AKT/mTOR Pathway in HepG2-ARE cells

In eukaryotic cells, PI3K/AKT/mTOR pathway is highly conserved signal transduction network promoting cell growth, survival and cell cycle progression (Glaviano *et al*., 2023). Treatment of AA to HepG2-ARE cells at various concentrations led to significant

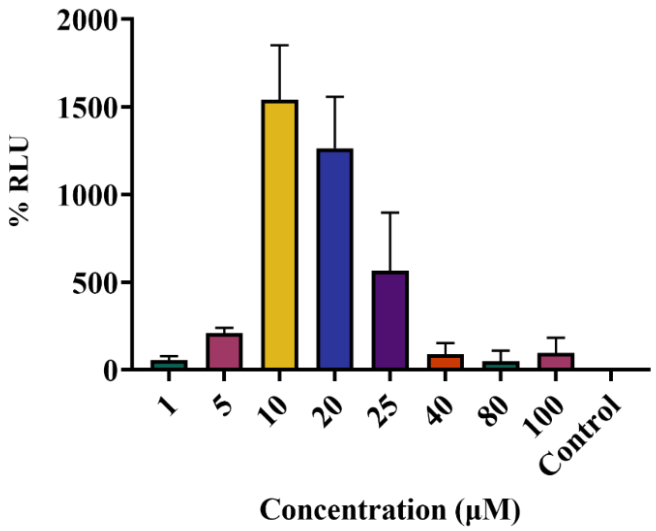

Figure 1: Effect of AA on NRF2 gene activation in HepG2 cells via NRF2/ARE luciferase reporter assay. Results are presented as Mean±SEM from three replicates.

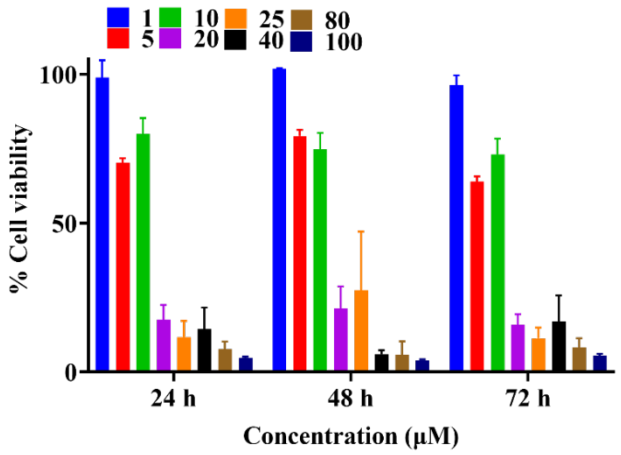

Figure 2: Effect of AA on cell viability in HepG2-ARE cells for 24, 48, and 72 hr. Results are presented as mean±SEM from three replicates.

changes in protein expressions involved in PI3K/AKT/mTOR pathway (Figure 6). The expressions of phosphorylated mTOR (Ser2481) (Figures 6a, b), p-PI3K p110α (Figures 6a, c), p-AKT(Ser473) (Figure 6a, d), p-PDK-1 (Figure 6a, e) were significantly downregulated in AA-treated cells when compared to control group (Figure 6). p-mTOR expression was significantly reduced at 7.5 (0.530.15), 10 (0.480.16), 15 µM (0.410.14) and 20 µM (0.160.12) in AA-treated cells in comparison to untreated HepG2-ARE cells (Figure 6a, b). A significant reduction in p-PI3K p110α was observed at 15 µM (0.200.10) and 20 µM (0.170.15) AA treatment in HepG2-ARE cells compared to control (Figure 6a, c). Further, in protein expression of p-AKT(Ser473), cells treated with AA showed significant decline at 10 (0.650.07), 15 µM (0.550.08) and 20 µM (0.250.08) (Figure 6a, d). The protein expression of p-PDK-1 in AA-treated hepatocarcinoma cells was significantly downregulated at 10 (0.350.01), 15 µM (0.360.03) and 20 µM (0.1830.06) (Figure 6a, e). However, Western blot analysis further revealed the treatment with AA caused a substantial enhancement in protein expression of phosphorylated GSK3β (S9) at 10 µM (1.610.24), 15 µM (1.790.11) and 20 µM (2.310.53) (Figure 6a, f); and in p-S6 ribosomal protein at 7.5 (1.620.24), 10 (1.960.12), 15 µM (2.080.42) and 20 µM (1.530.19) (Figure 6a, g). *In vitro* results demonstrated a significant role of AA in modulation of protein expressions involved in PI3K/AKT/mTOR pathway.

Anacardic Acid Alters Wnt/β-Catenin Pathway in Hepatic Cancer Cells

Wnt/β-Catenin pathway is crucial in cancer and its abnormal activation leads to many attributes of cancer including commencement, progression and consequently, into malignant transformation. Dysregulation of this pathway ultimately leads to non-negligible effects on cancer-linked death (Yu *et al.*, 2021). HepG2-ARE cells were given AA treatment for 24 hr to assess AA impact on proteins related to Wnt/β-catenin signaling pathway. The significant downregulated expression of key proteins including p-LRP6, APC, axin2, c-Myc, frizzled 5, β-catenin, Wnt5a and CK1 were observed in AA-treated cells as compared to control group in Western blot analysis (Figure 7). Interestingly, expression of phosphorylated-LRP6 was significantly decreased on AA treatment at 15 µM (0.260.21) and 20 µM (0.080.07) as that in untreated HepG2-ARE cells (Figure 7a, b). In APC protein expression, the downregulation was found to be significant at 1 (0.840.12), 5 (0.870.28), 7.5 (0.870.20), 10 (0.520.27), 15 (0.260.21), and 20 µM (0.080.07) in comparison to untreated hepatocarcinoma cells (Figure 7a, c). Further, Axin2 was observed to be upregulated at 7.5 (1.850.11), 10 (1.750.15), 15 (1.750.11) and 20 µM (1.590.31) in AA-treated HepG2-ARE cells (Figure 7a, d). Moreover, in c-Myc, a significant decline in protein expression was noticed at higher AA concentrations *viz.* 10 (0.540.16), 15 (0.290.12) and 20 µM (0.260.14) (Figure 7a, e). Frizzled-5 was significantly downregulated at 15 (0.360.06) and 20 µM (0.210.08) in comparison to untreated cells (Figure 7a, f). The expression of phosphorylated β-catenin was found to be decreased depicting important role of AA in modulation of Wnt/β-catenin signaling in HepG2-ARE cells at 5 (0.560.07), 7.5 (0.380.18), 10 (0.170.06), 15 (0.100.07), and 20 µM (0.0980.09) (Figure 7a, g). Further, the decrease in Wnt5a protein expression suggested alteration by AA at concentration i.e., 1 (0.790.03), 5 (0.720.32), 7.5 (0.560.35), 10 (0.270.10) 15 (0.150.04), and 20 µM (0.120.003) in comparison to untreated cells (Figure 7a, h). Additionally, in CK1, an enhanced expression was noticed in AA-treated HepG2-ARE cells at 1 (1.460.35), 5 (2.050.47), 7.5 (2.200.48) and 10 (2.460.23), 15 (2.141.31) and 20 µM (2.551.31) (Figure 7a, i).

Anacardic Acid Activates NRF2/ARE Signaling in HepG2-ARE Cells

The NRF2 pathway is involved in regulation of oxidative stress, redox balance, cytoprotection, survival, proliferation, proteosome degradation and other physiological processes. NRF2 activation serves as a cellular response to oxidative stress, leads to upregulation of antioxidant system which comprises enzymes such as glutathione, Superoxide Dismutase (SOD) and catalase (Lin *et al.*, 2023). The expression of phosphorylated NRF2 and antioxidant enzymes were markedly changed on AA treatment in a dose-dependent manner (Figure 8). A significant increase in expression of p-NRF2 was observed in AA-treated HepG2-ARE cells, particularly at 7.5 (2.030.41), 10 (2.180.43), 15 (2.210.21) and 20 µM (1.850.20) than that of control (Figure 8a, b). Conversely, KEAP-1 expression was significantly declined upon AA treatment at 10 (0.660.11), 15 (0.610.10) and 20 µM (0.380.04) when compared with untreated HepG2-ARE cells (Figure 8a, c). The expression of NRF2 targeted key antioxidant enzymes was significantly upregulated in AA-treated cells including enzymes NQO1 at 5 (1.590.09), 7.5 (1.900.14) 10 (2.100.25), 15 (2.080.26), and 20 µM (1.840.18) (Figure 8a, d);

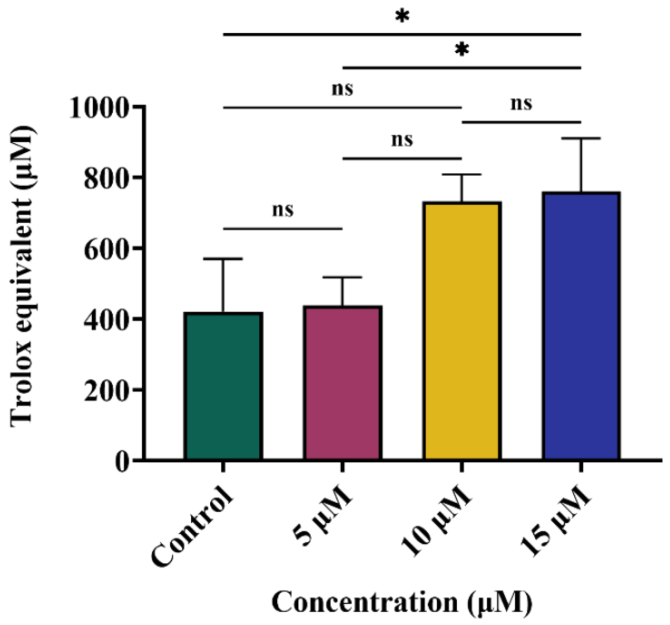

Figure 3: Antioxidant potential of AA in HepG2 Cells using CUPRAC assay. Results are expressed as mean±Standard Deviation (SD) relative to untreated control cells ($p \leq 0.05$ versus control).

HO-1 at 5 (1.590.35) 7.5 (1.900.14), 10 (2.100.25), 15 (2.080.26) and 20 µM (1.840.18) (Figure 8a, e); SOD1 at 5 (1.720.15), 7.5 (2.050.25), 10 (1.790.62), 15 (2.130.38), 20 µM (1.790.27) (Figure 8a, f) in comparison to control, thereby signifying increased cellular antioxidant response against oxidative stress upon AA treatment in hepatocarcinoma cells.

Anacardic Acid Alters MAPK/JNK Pathways in Hepatic Cancer Cells

In tumor cells, MAPK/JNK pathway is mainly related to cellular stress and apoptosis and has a crucial role in signal transduction (Guo *et al.*, 2020). To investigate the AA treatment induced modulation in MAPK/JNK pathway, HepG2-ARE cells were treated with various concentrations of AA and protein expression was examined *via* Western blotting. A substantial alteration in expression of crucial proteins in MAPK/JNK pathway including c-FOS, p-JNK1/2, p-p38 and AP-1 was observed (Figure 9). The protein expression of c-FOS was decreased in a dose-dependent manner in AA-treated cells at 7.5 (0.360.13), 10 (0.280.08), 15 (0.2450.19) and 20 µM (0.070.03) when compared with untreated HepG2-ARE cells (Figure 9a, b). Conversely, the protein expression of p-JNK1/2 was significantly enhanced upon AA treatment at 7.5 (5.470.14), 10 (5.850.21), 15 (6.530.72), and 20 µM (6.180.32) in comparison to control group (Figure 9a, c). In addition, AA treatment resulted in a significant enhanced expression of p-p38 at 7.5 (1.790.25), 10 (2.340.27), 15 (2.530.39) and 20 µM (1.830.73) compared to untreated HepG2-ARE cells. Moreover, AA treatment significantly suppressed the expression of AP-1 at 7.5 (0.340.11), 10 (0.300.10), 15 (0.310.10) and 20 µM (0.150.01) in hepatic cancer cells as compared to untreated cells (Figure 9a, d).

Anacardic Acid Modulates NF-κB Pathway in Hepatic Cancer Cells

NF-κB pathway mediates the pleiotropic function in innate and adaptive immune responses. NF-κB activation enhances inflammation in tumor microenvironment *via* uprising the secretion by pro-inflammatory cytokine eventually, leading to rapid proliferation of the tumor cells (Zinatizadeh *et al.*, 2021). The effect of AA treatment on the expression of critical proteins linked with NF-κB pathway as well as inflammatory response in NRF2/ARE Luciferase Reporter HepG2 cells was assessed *via* Western blot analysis. The results showed that higher concentration of AA significantly altered the NF-κB pathway along with significant decline in cytokines protein expressions *viz.* IL-18, -2, IL-1α and IL-1β in HepG2-ARE cells in comparison to control group (Figure 10). The expression of NF-κB p65 phosphorylation was decreased significantly at 10 (0.400.04), 15 (0.370.07) and 20 µM (0.190.06) in HepG2-ARE AA treated cells compared to control cells (Figure 10a, b). Further, AA treatment modulated IL-1α at 10 (0.460.13), 15 (0.370.06), 20 µM (0.380.06) (Figure 10a, c) and IL-1β at 7.5 (0.560.07), 10 (0.400.03), 15 (0.310.06), 20 µM (0.200.12) (Figure 10a, d) in AA treated HepG2-ARE cells in comparison to untreated cells. The downregulated expression of IL-18 was noticed at 7.5 (0.640.09), 10 (0.390.07), 15 (0.340.12), and 20 µM (0.240.10) in AA-treated cells when compared with untreated cells (Figure 10a, e). Moreover, IL-2, the protein expression was significantly declined at 10 (0.560.09), 15 (0.370.07), and 20 µM (0.280.06) upon AA treatment as compared to control group.

Immunofluorescence analysis demonstrated the effect of AA treatment (15 µM) on phosphorylated NF-κB expression in HepG2-ARE cells. The fluorescence intensity was significantly reduced in the AA-treated group compared to the untreated control (Figure 11a). Specifically, both DAPI (used for nuclear staining) and p-NF-κB signals were markedly decreased in AA-treated cells (****$p \leq 0.0001$, Figure 11b). The reduction in p-NF-κB fluorescence suggested that AA strongly suppressed NF-κB activation, which is a key regulator of inflammatory and survival pathways in hepatocellular carcinoma cells.

DISCUSSION

Liver cancer is a highly prevalent cancer worldwide and is associated with a high mortality rate, with regional variations in both incidence and fatality rates (Oh and Jun, 2023). Anacardic Acid (AA), a natural chemical derived from cashew nut shells, has been documented to exhibit many bioactivities, including significant anticancer properties (Gomes Júnior *et al.*, 2020; Hollands *et al.*, 2016; Morais *et al.*, 2017; Zafar *et al.*, 2020). In our prior investigation, *in silico* analysis demonstrated that AA targets critical proteins associated with inflammatory and

antioxidant responses, subsequently corroborated by Western blotting, which indicated the activation of p-NRF2 and the downregulation of p-NF-κB (Panda *et al.*, 2025). In this study, AA inhibited cell proliferation, migration and glucose uptake in dose- and time-dependent manner. AA showed enhanced antioxidant capacity in hepatic cancer cells. Based on that, we demonstrated the anti-hepatic cancer effects of AA *via* multiple signaling pathways. Antioxidant NRF2 pathway was induced by AA-treatment along with key antioxidant enzymes. Activation of PI3K/AKT/mTOR pathway was inhibited in AA-treated cells. Intriguingly, AA markedly blocked the NF-κB signaling pathway in HepG2-ARE cells in addition to various pro-inflammatory cytokines inhibition. Hence, our hypothesis included AA as a potential candidate in preventing stress and inflammation in hepatic cancer cells in future.

A critical role in the development and proliferation of tumor cells, the PI3K/AKT/mTOR pathway is a critical intracellular signaling cascade that responds to nutrient availability, hormonal stimulation, and growth factors. PI3K heterodimers are central regulators within this pathway; upon activation, they induce phosphorylation of AKT, thereby promoting cell survival and proliferation (Peng *et al.*, 2022; Yang *et al.*, 2019). Further, mTOR, serine/threonine protein kinase, is a downstream P13K and AKT, contributing to cancer cell metabolism and cell growth. Inhibition of P13K/AKT/mTOR pathway results in inhibition of cancer cell growth, proliferation along with cell metabolism (He *et al.*, 2021). In the current study, AA markedly inhibited the expression of phosphorylated mTOR, P13 kinases, AKT, and PDK-1 highlighting the suppression of P13K/AKT/mTOR signaling pathway by AA in hepatic cancer cells.

Excessive production of Reactive Oxygen Species (ROS) contributes to the pathogenesis of numerous diseases, despite the fact that physiological levels of ROS are essential for numerous cellular processes. NRF2 is a critical transcription factor that regulates the cellular antioxidant defense system during oxidative stress by regulating the expression of antioxidant enzymes, thereby safeguarding cells from oxidative injury (Glorieux *et al.*, 2024). NRF2 target genes are essential for sustaining redox homeostasis, survival, autophagy, DNA repair, and mitochondrial function (Rojo de la Vega *et al.*, 2018). The rapid induction of antioxidant enzymes to neutralize ROS or remediate oxidative damage through transcriptional regulation of antioxidant genes is a critical function of NRF2 in the prevention of carcinogenesis. Cytoprotective mechanisms are initiated in response to oxidative stress, with NRF2 signaling serving as a critical component. NRF2 dissociates from KEAP1 in the cytoplasm, translocates to the nucleus, binds to the Antioxidant Response Element (ARE), and promotes transcription of cytoprotective proteins and antioxidant enzymes, including Superoxide Dismutases (SODs) and Catalase (CAT), after being phosphorylated (Hammad *et al.*, 2023; Ndlovu *et al.*, 2021). In accordance with this mechanism, our research revealed an increase in phosphorylated NRF2 and an increase in the expression of antioxidant enzymes, such as SOD1, NQO1, and HO-1, which underscored the activation of NRF2 signaling. NRF2 and its antioxidant targets are significantly upregulated, which strongly implies that AA exerts its effects

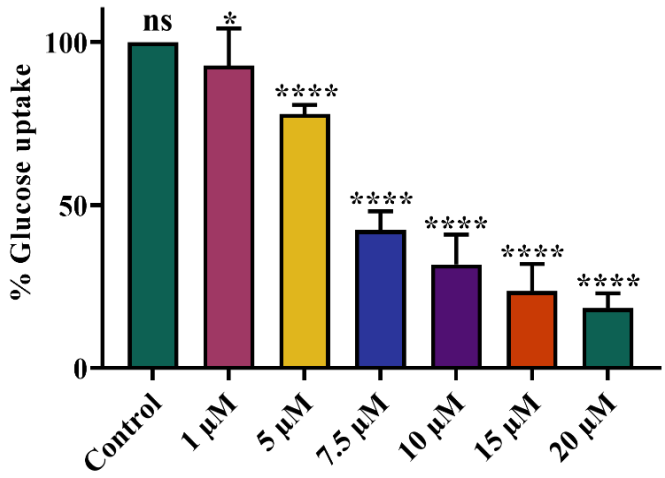

Figure 4: Assessment of glucose uptake capacity of AA in HepG2-ARE cells. Results are expressed as mean±Standard Deviation (SD) of glucose uptake relative to untreated control cells (**$p \leq 0.01$ versus control; *** $p \leq 0.001$ versus control).

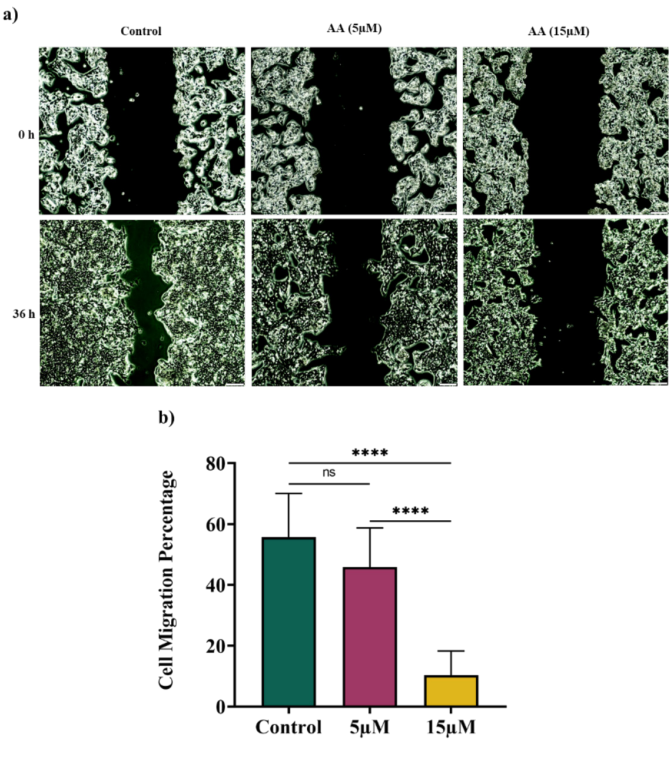

Figure 5: AA inhibits the migration of HepG2-ARE cells. a): The scratch assay demonstrated the effects of AA on HepG2-ARE migration. Untreated HepG2 cells exhibited a significantly faster migration rate compared to those treated with 5 and 15 µM AA. b) indicates the percentage of migrated cells upon AA treatment. Results are expressed as mean±SD of glucose uptake relative to control.

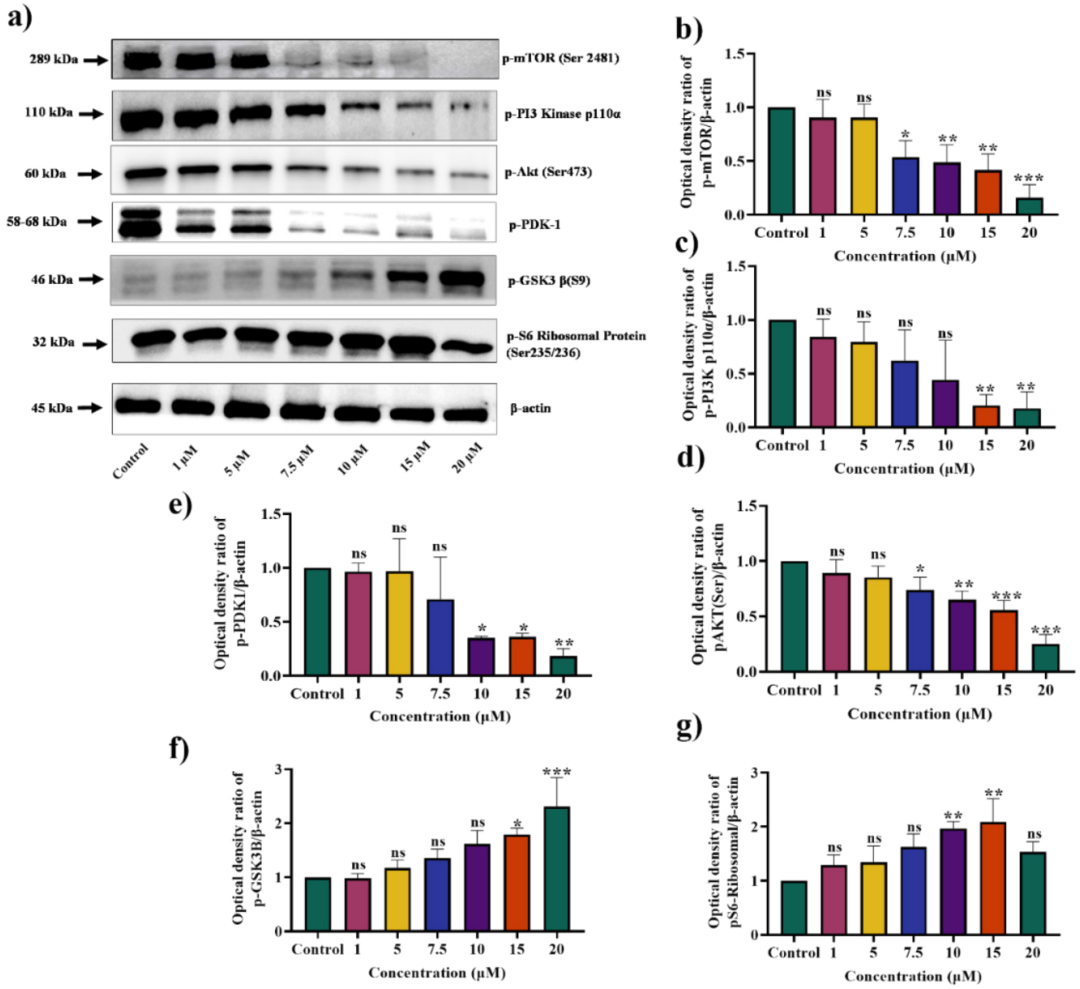

Figure 6: Anacardic acid modulates the PI3K/Akt/mTOR signaling pathway in HepG2-ARE cells. a) AA treatment resulted in the downregulation of p-mTOR, p-PI3K110α, p-AKT (Ser), and p-PDK-1 along with the upregulation of p-GSK3β and p-S6 ribosomal protein. b-g) Histogram of p-mTOR, p-PI3K110α, p-AKT (Ser), p-PDK-1, p-GSK3β and p-S6 ribosomal protein.

through the mitochondrial pathway, as SODs are mitochondrial proteins that protect against oxidative damage.

NF-κB proteins control the inflammation process by targeting the body's complex mechanisms under inflammation conditions. The process involves up- and down-regulation of crucial genes including chemokines and pro-inflammatory cytokines (Zinatizadeh *et al.*, 2021). Experimental evidence has indicated that cellular damage resulted from oxidative stress triggered inflammatory response *via* mediating the triggering of transcription factors such as NF-κB which further induced the production of several cytokines (Augusto *et al.*, 2020; Sung *et al.*, 2008). In our study, the inhibition of phosphorylated NF-κB p65 was accompanied by concomitant reduction in expressions of pro-inflammatory cytokines i.e., IL-1α, -1β, -18, and IL-2 which was further confirmed by immunofluorescence analysis *via* lowered expression of phosphorylated NF-κB in the cytoplasm. Additionally, NF-κB transcriptional factors also participate in cell proliferation as well as cell death.

Wnt/β-catenin signaling pathway is a promising target in cancer therapy and plays a critical role in tumorigenesis. The transcription of oncogenic genes, such as c-Myc, is subsequently activated by the nuclear accumulation of β-catenin, which is the result of aberrant regulation of this pathway (Shang *et al.*, 2017; Yu *et al.*, 2021; Zhang and Wang, 2020). In our investigation, the expression of critical components of the Wnt/β-catenin pathway, such as c-Myc, Frizzled5, β-catenin, Wnt5a, p-LRP6, APC, Axin, and CK1, was repressed by AA treatment in a dose-dependent manner. This downregulation underscores the modulatory influence of AA on Wnt/β-catenin signaling.

In addition, prior research indicates that the JNK pathway is linked to apoptosis and the stress response (Guo *et al.*, 2020; Papa *et al.*, 2019). This is consistent with our observation of an increase in phosphorylated JNK1/2 in response to AA treatment, which emphasizes its role in the mediation of mitogenic and stress-related signal transduction in hepatic cancer cells. In summary, these results indicate that hepatocarcinoma is

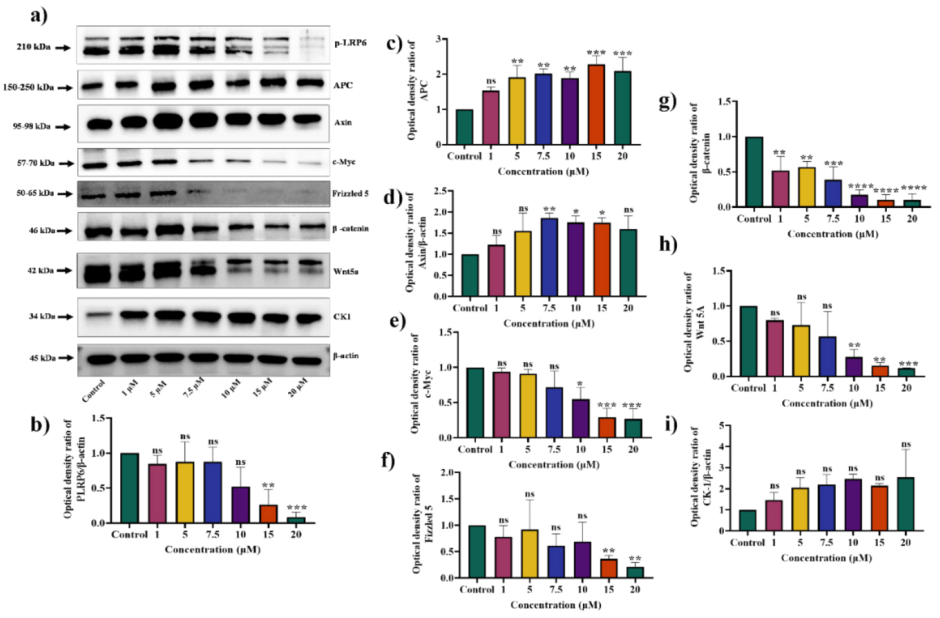

Figure 7: AA modulates the Wnt/β-catenin signaling pathway in HepG2-ARE cells. a) Representative Western blot image of AA treatment downregulated β-catenin, Wnt5a, PLRP6, c-Myc, and Frizzled-5, indicating suppression of Wnt pathway activity. Conversely, increased levels of AXIN, CK1, and APC suggest enhanced β-catenin degradation via negative regulators. b-i) Histogram represents densitometric analysis of proteins (PLRP6, APC, Auxin, c-Myc, Frizzled 5, -catenin, Wnt5A, and CK-1.

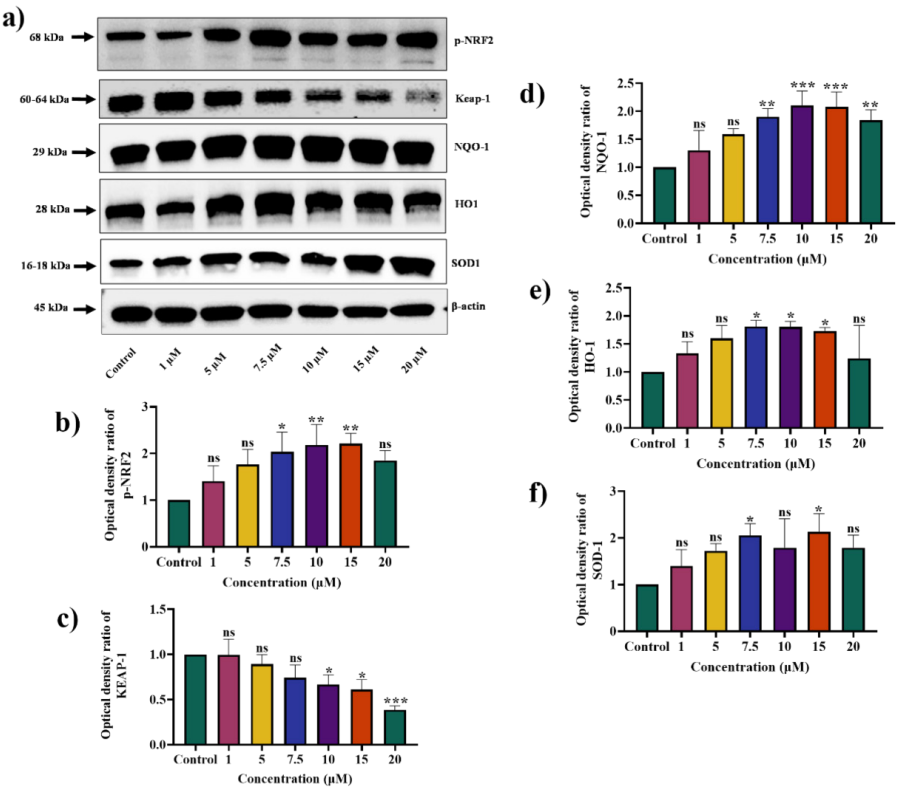

Figure 8: AA enhances NRF2-mediated antioxidant response in HepG2-ARE cells. a) Representative Western blot images of protein regulated upon AA treatment. Treatment with AA led to upregulation of HO1, SOD1, p-NRF2, and NQO1, along with downregulation of KEAP-1 indicating activation of the NRF2 signaling pathway and enhanced antioxidant defense. b-f) Histogram represents densitometric analysis of proteins involved: p-NRF2, KEAP-1, NQO1, HO1 and SOD1.

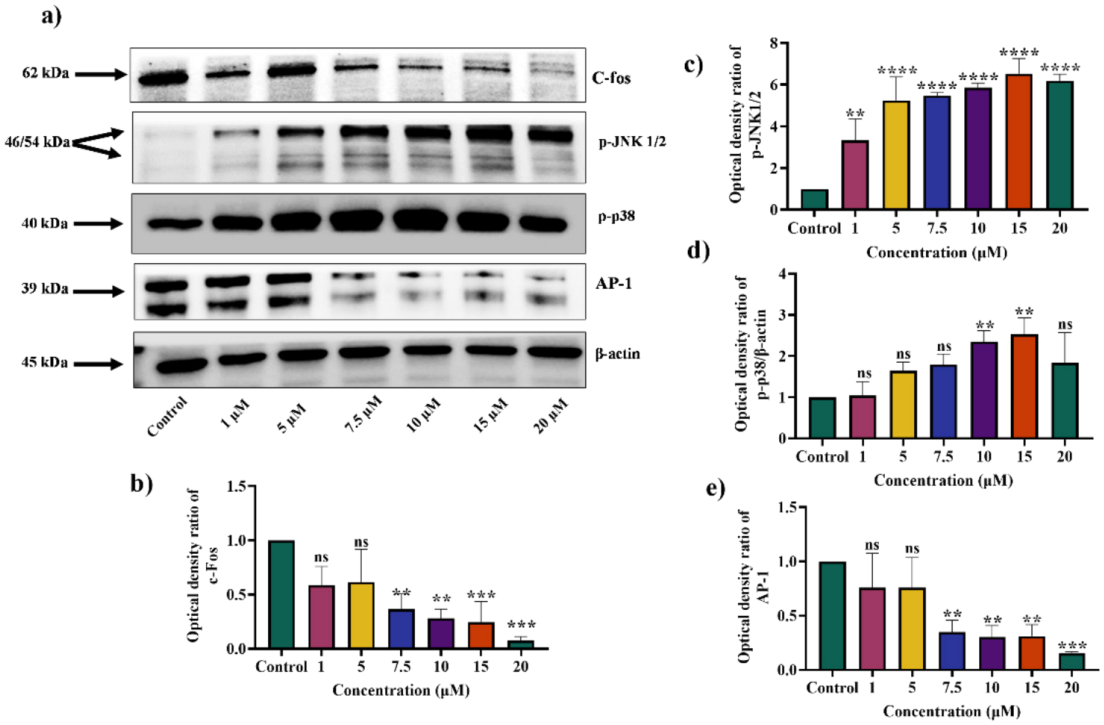

Figure 9: AA modulates the MAPK/JNK signaling pathway. a) Representative Western blot image of proteins upon AA treatment. b-e) Histogram represents densitometric analysis of proteins c-Fos, p-JNK1/2, p-P38, and AP-1.

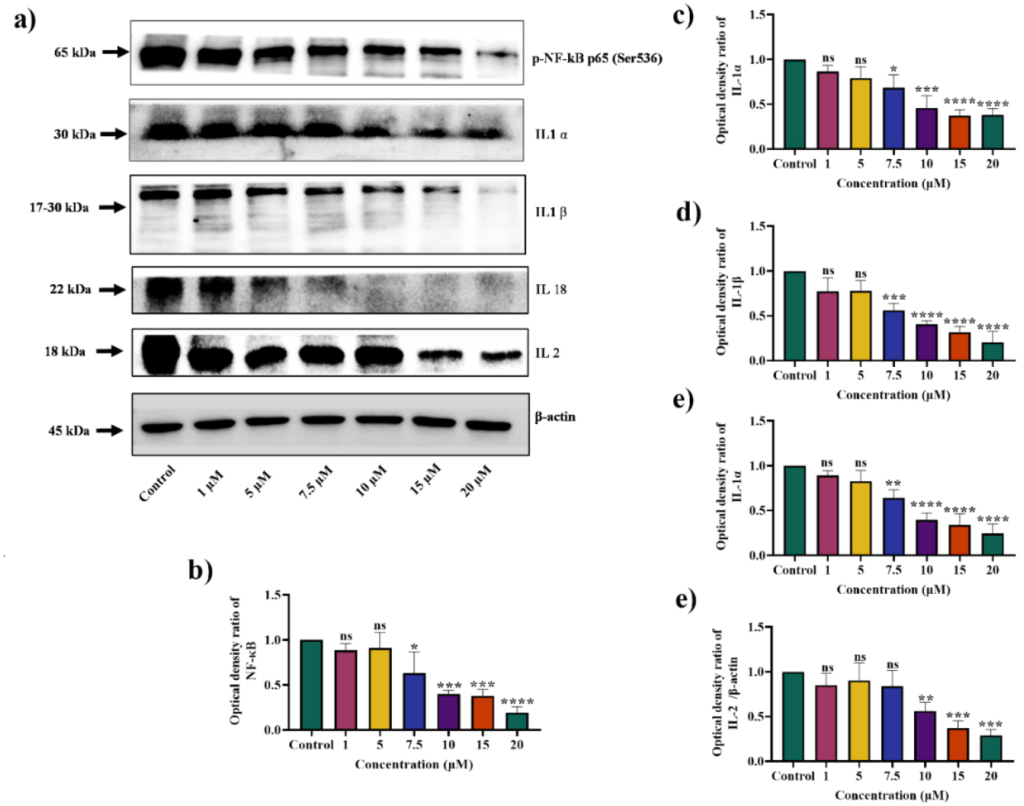

Figure 10: AA modulates the NF-κB signaling pathway and inflammatory cytokine expression. a) Representative Western blot images of protein regulated upon treatment with AA. b-e) Histogram represents densitometric analysis of proteins such as NF-κB, IL-1, IL-1, IL-18 and IL-2.

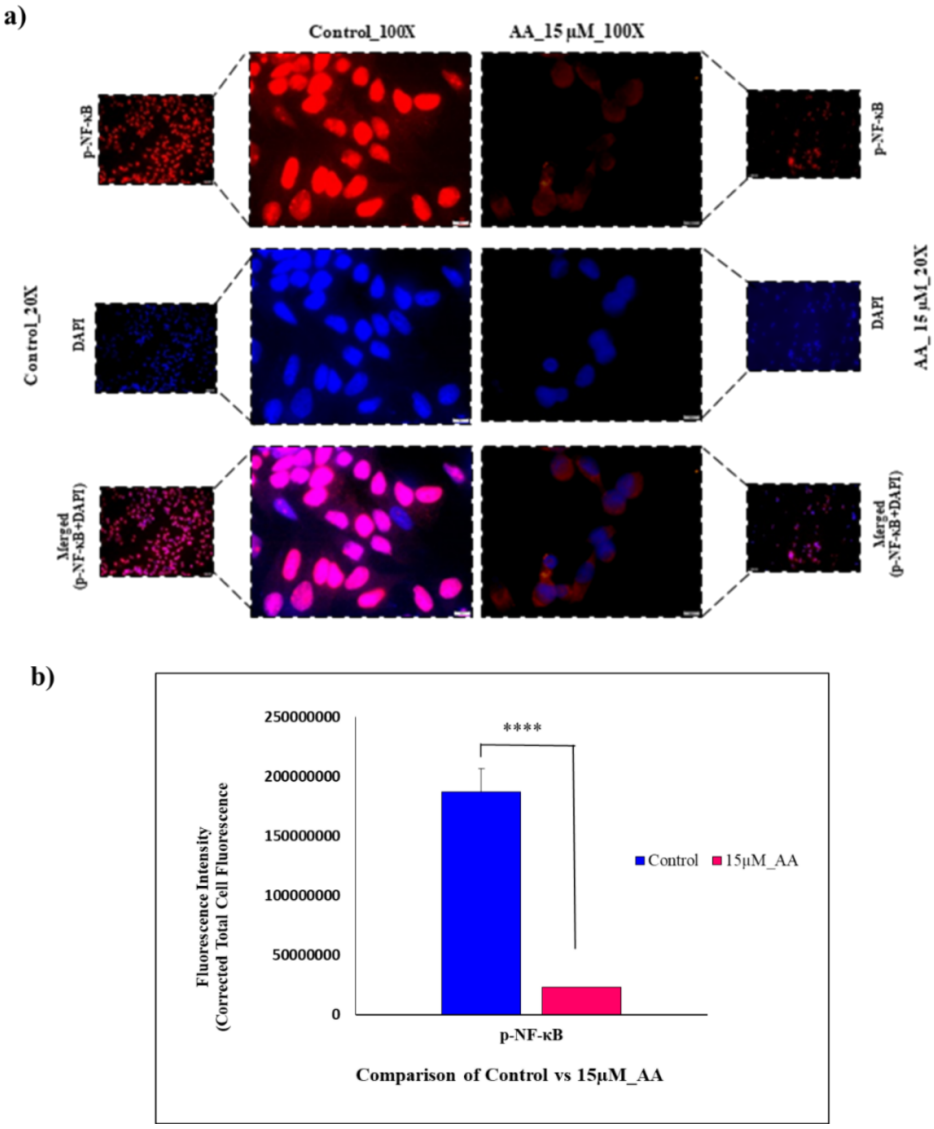

Figure 11: AA significantly downregulated phosphorylated NF-κB expression in HepG2-ARE cells. a) Immunofluorescence analysis of phosphorylated NF-κB in HepG2-ARE cells following AA treatment. Decreased p-NF-κB fluorescence intensity (red) was observed in cells treated with 15 µM AA compared to untreated controls. White arrows indicate reduced signal. Nuclei are stained with DAPI (blue). b) Quantification of fluorescence intensity shows a significant reduction in p-NF-κB levels (****p<0.0001).

significantly associated with chronic inflammation and increased oxidative stress, which are mediated by critical signaling proteins. Consequently, regulating oxidative stress with natural substances possessing ROS-scavenging characteristics, such as AA, may constitute an efficacious treatment approach for progressing hepatocarcinoma.

CONCLUSION

Our results show that AA prevents hepatic cancer cells from proliferating and migrating. Mechanistically, AA increased the expression of antioxidant enzymes by activating the NRF2 signaling pathway through mitochondrial regulation. Furthermore, AA strongly inhibited the PI3K/AKT/mTOR pathway and dose-dependently decreased the Wnt/β-catenin pathway. In addition to controlling oxidative stress, AA also lowered NF-κB activation and the levels of pro-inflammatory cytokines. Immunofluorescence analysis corroborated diminished cytoplasmic levels of phosphorylated NF-κB, indicating potential nuclear translocation. In summary, our findings suggest that AA could be a promising option for preventing and treating liver cancer. Nonetheless, additional *in vivo* studies and clinical investigations are imperative to comprehensively unravel the mechanisms of AA and evaluate its therapeutic potential in patients with hepatic cancer.

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None.

ABBREVIATIONS

AA: Anacardic Acid; **AP-1:** Activator Protein 1; **APC (EP701Y):** Adenomatous Polyposis Coli; **Axin2:** Axin2; **BCA:** Bicinchoninic Acid Assay; **BSA:** Bovine Serum Albumin; **β-actin:** Beta-actin, **c-Fos:** c-Fos Proto-oncogene; **CK1:** Casein Kinase 1; **c-Myc:** c-Myc Proto-oncogene; **DAPI:** 4′,6-Diamidino-2-Phenylindole; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **Frizzled5:** Frizzled Class Receptor 5; **HO-1:** Heme Oxygenase 1; **IL-2:** Interleukin-2; **IL18:** Interleukin-18; **IL1α:** Interleukin-1 Alpha; **IL1β:** Interleukin-1 Beta; **KEAP1:** Kelch-like ECH-associated Protein 1; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide; **NQO1:** NAD(P)H Quinone Dehydrogenase 1; **p-Akt (Ser473):** Phospho-Akt (Serine 473); **p-GSK3β (Ser9):** Phospho-Glycogen Synthase Kinase 3 Beta (Serine 9); **P-LRP6 (Ser1490):** Phospho-Low-Density Lipoprotein Receptor-Related Protein 6; **p-mTOR (Ser2481):** Phospho-Mammalian Target of Rapamycin (Serine 2481); **p-NF-κB p65 (Ser536):** Phospho-Nuclear Factor Kappa B p65 (Serine 536); **p-NRF2:** Phospho-Nuclear Factor Erythroid 2-Related Factor 2; **p-PDK1 (Ser241):** Phospho-3-Phosphoinositide-Dependent Kinase-1; **p-PI3K p110α:** Phospho-Phosphoinositide 3-Kinase p110 Alpha; **p-S6 Ribosomal Protein:** Phospho-S6 Ribosomal Protein; **P-p38 MAPK (T180/Y182):** Phospho-p38 Mitogen-Activated Protein Kinase; **Phospho-β-Catenin (Ser45):** Phosphorylated Beta-Catenin (Serine 45); **Phospho-SAPK/JNK (Thr183/Tyr185):** Phosphorylated Stress-Activated Protein Kinase/c-Jun N-terminal Kinase; **RIPA:** Radioimmunoprecipitation Assay Buffer, **SD:** Standard Deviation; **SOD1:** Superoxide Dismutase 1; **TBST:** Tris-Buffered Saline with Tween; **Wnt-5a:** Wnt Family Member 5A.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Sangita Panda: Investigation, Experimentation, Formal analysis, Software, Writing - original draft, Writing - review and editing, Visualization; **Enketeswara Subudhi:** Project administration; **Anand Bhaskar:** Writing - original draft, Writing - review and editing; **Siddharth Modi:** Project administration; **Dilip Mehta:** Project Administration; **Sujit Nair:** Conceptualization, Supervision, Formal analysis, Review and editing, Project administration.

ETHICAL STATEMENT

This study utilized commercially available HepG2 ARE cell lines. No human participants or animal subjects were involved and therefore, specific ethical approval from an institutional review board was not required.

SUMMARY

This research demonstrates that Anacardic Acid (AA) effectively suppresses the progression of hepatocellular carcinoma in HepG2 ARE cells by inhibiting vital survival mechanisms, including glucose uptake and cell migration. Mechanistically, AA induces the NRF2 antioxidant pathway to combat oxidative stress while simultaneously downregulating pro-inflammatory NF-κB signaling and disrupting the oncogenic PI3K/AKT/mTOR and Wnt/β-catenin pathways. These findings position AA as a multi-target bioactive candidate for liver cancer prevention.

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