

Anticancer Potential of Diosgenin via Modulating PARP Pathway: An in Vitro Study on A549 and HCT116 Cancer Cell Lines

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Abstract—Cancer remains the second-leading cause of mortality globally, necessitating innovative therapies that are both effective and minimally harmful. Diosgenin, a saponin derived from *Trigonella foenum graecum*, possesses anti-apoptotic and anti-proliferative properties. Though its anticancer potential remains underexplored, this study investigates Diosgenin's anticancer efficacy in A549 (lung cancer) and HCT116 (colon cancer) cell lines. The in vitro assays demonstrated that Diosgenin inhibits the cell proliferation and induces apoptosis in these cells, as shown by Caspase 3/7 related cell death. Additionally, western blot analysis revealed its modulatory effects on the PARP signaling pathway, while whole blood assay confirmed dual anticancer and anti-inflammatory roles. Collectively, these findings highlight Diosgenin's potential as a naturally occurring anticancer agent, offering promising avenues for therapeutic development against malignancies. Further investigations into its mechanism of action could solidify its role in cancer therapy.

Keywords— Diosgenin, PARP pathway, apoptosis, caspase, anti-cancer.

I. INTRODUCTION

Cancer is a foremost public health problem and the second leading reason of mortality around the world, mostly in Europe and the United States with an occurrence rate of about 2.6 million cases per year [1]. Unplanned and uncontrolled cellular growth across the range of cells is one of its defining characteristics. Currently available malignant suppression therapies include anti-cancer drugs, surgery, and radiation; however, each one carries a unique set of hazards associated, predominantly chemotherapy [1]. Despite their effectiveness in treating cancer, anti-cancer drugs have substantial adverse effects that frequently result in enormous toxicities [2]. These problems include liver function impairment, bone marrow suppression, and neurotoxicity [3]. Therefore, we still need to discover novel cancer treatments that are both efficient and permit fewer adverse effects than currently available treatments [4].

Chemo-prevention is gaining popularity as a more affordable alternative to traditional cancer therapy [5, 6]. Chemoprevention is the practice of preventing, suppressing, or reversing the beginning stages of carcinogenesis or the potential invasion of premalignant cells using natural, synthetic, or biological agents [6, 7]. Natural products have a long and rich history in the creation of bioactive compounds.

These organic components from the natural products have shown exceptional therapeutic results when used in chemotherapy for the treatment of cancer [8, 9].

Sapogenins are a group of organic compounds that are extensively present in many plant species in their glycoside form and are known for their medicinal properties[10]. The most effective bioactive substances among them are steroidal sapogenins[11]. The majority of steroidal sapogenins have shown anti-cancer action in vitro and in pre-clinical animal models. Diosgenin is the most abundant steroidal sapogenin found in fenugreek seeds [12]. It is interesting to note that large quantities of diosgenin are also present in a wide range of plant species, including *Costus speciosus*, *Smilax menispermoides*, species of *Paris*, *Alettris*, and *Trillium*, as well as species of *Dioscorea*. Diosgenin primarily exerts its effects by modulating key cell signaling pathways essential for processes such as cell cycle regulation, cell differentiation, and apoptosis. Furthermore, research has shown that Diosgenin administration induces cell cycle arrest and death in erythroleukemia cells by activating calcium-sensitive cytosolic phospholipase A2 and overexpressing cyclooxygenase 2 [13–16]. Additionally, Diosgenin was found to inhibit Skp2 that resulted in decreased cell viability and motility, prompting apoptosis in breast cancer cells. Therefore, in this study, we investigated the anti-cancerous effects of Diosgenin using in vitro approach. In vitro experiments like cell viability analysis, and apoptosis were performed on two representative cancerous cell lines, A549 and HCT116. Additionally, the caspase activity and cell cycle assessment were performed to assess the Diosgenin anticancer potentials.

II. METHODOLOGY

A. Proliferation assay

A549 and HCT116 at 1000 cells per reaction were tested for proliferation using CellTiter-Glo® (CTG) Luminescent Cell Viability Assay (G7572 Promega Corp., Madison, WI, USA). Cells were treated with Diosgenin concentrations in a 1:1 methanol: DMSO ratio, diluted with culture media (methanol concentration <0.3%). Briefly remove wasted media from treated plate, then add 20µl CTG per well to 40µl media with careful shaking. 40µl lysate was placed on a white plate for luminescence analysis and reading was taken on microplate reader.

B. Caspase 3 Activity Assay

Post 5 hours of seeding HCT116 and A549 at 1000 cells per reaction, they were treated with different concentrations of Diosgenin and one concentration of Staurosporine was added as an assay control. Cells were concurrently spiked with DMSO: Methanol (1:1). The plates were further incubated for a total of 24, 48, 72, and 96 hours to study time course-based caspase and cell viability assessments. Caspase-Glo® 3/7 and CellTiter-Glo® were added to the treated wells and incubated at room temperature in dark with gentle shaking based on manufacturer's instructions. Cell lysate was transferred to the half area of 96 well-white plates, and Luminescence was recorded.

C. NAD⁺ assessment

HCT116 and A549 cells were seeded in 24 well plates at a density of 0.1 million cells per well, per ml and left to incubate overnight period at 37°C with 5% CO₂. Cells were treated with different concentrations of Diosgenin and Pemetrexed was used as assay control. A parallel set of treated conditions were supplemented with Nicotinamide (NAM) to restore the depleted NAD⁺ levels and enhance cell viability and metabolic activity. The cells were simultaneously spiked with methanol: DMSO (1:1) in control wells and incubated for 24 & 48h at 37°C, 5 % CO₂ incubator. Following the full removal of the incubation medium, manufacturer's protocol was followed to detect NAD⁺ levels in standard and test samples and luminescence was recorded.

D. Western blotting analysis

A549 and HCT116 cells were seeded at a density of 0.5 million cells per well in a 6-well plate and incubated overnight before treatment. After overnight incubation, fresh medium containing 50µM and 100µM of diosgenin and 1µM of staurosporine was added and further incubated for 1, 2, 4, 6, 24 and 48 hours for A549 cells and 2, 4, 24, 48 & 72 hours for HCT116 cells. Samples were processed in RIPA buffer and protein estimation was performed. Normalized lysate concentrations were run on SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 1% BSA for 1 hr at room temperature followed by the incubation with primary antibodies such as PARP antibody (#9532), cleaved PARP (#9541) and GAPDH (#2118L) procured from CST and incubated overnight at 4°C. The blots were washed with 1X PBST and probed with secondary antibody and developed on image analyzer.

E. Blood Cytokine assay

Human whole blood was drawn from healthy volunteers in Na-Heparin vacutainers. Briefly, 180µl of whole blood was added to U-bottom 96 well plate. Diosgenin was added to the whole blood starting from 100µM, 9-point dose response curve with semi-log dilution scheme and cultured for 1h at 37°C, 5 % CO₂. Whole blood was stimulated with LPS at 100ng/ml and further incubated for 5h. After indicated time points cytokines TNFα (#555212 BD Biosciences) and IL-2 (#555220 BD Biosciences) were measured in cell supernatant by following the manufacturer's instructions.

III. RESULTS

A. Diosgenin abrogates the proliferation and growth of lung carcinoma and colon cancer cells.

Anticancer effect of Diosgenin was assessed using CellTiter-Glo® Luminescent Cell Viability assay. Cell Viability Assay was performed on lung carcinoma cell line A549 and colon cancer cell line HCT116 following 96 hours treatment with Diosgenin at 9-point dose response curve to determine the effect of Diosgenin treatment on cell proliferation. The results from this assay indicated that the treatment of A549 and HCT116 cells with Diosgenin resulted in an IC₅₀ of 13.96µM and 1.68 µM respectively at the end of 96h. Therefore, the inhibitory effect of Diosgenin on the growth of A549 and HCT116 cell lines was dose-dependent, as demonstrated by the IC₅₀ values of Diosgenin (Figure 1A and 1B).

B. Caspase 3/7 related cell death activity assessment

Family of cytosolic cysteine proteases such as caspase-3 and caspase-7 are known to serve as primary effectors in mediating the progress of apoptosis. Cells exposed to apoptotic stimuli Diosgenin at various concentrations in a time course dependent manner showed higher cytotoxicity. Caspase 3/7 levels were found elevated in A549 cells treated with 100, 50, 25, and 12.5 µM of Diosgenin at 24 and 48 hours when compared to control. However, ~2-fold increase in caspase activity was seen at 48 hours in Staurosporine treated cells. At 6.25 µM of Diosgenin treatment, the levels of caspase 3/7 activity were almost similar to Staurosporine treated cells. Moreover, in Diosgenin treated cells at 72, and 96 hours the caspase activity was seen to be negligible (Figure 2A). HCT116 cells sensitized with Diosgenin showed greater than 3-fold increase in caspase 3/7 activity at highest tested concentration, followed by dose dependent response at 24 hrs when compared to control (Figure 2B). To further confirm the correlation between the caspase 3/7 activity and cell viability, the proliferation assay was performed in parallel to caspase activity with same assay conditions and only terminations being assay specific. The data indicated that with progressive time point the cell viability reduced in both the cell lines which can be co-related to cells going through apoptosis through caspase 3/ 7 activity (Figure 2C and 2D).

C. NAD⁺ assessments

Nicotinamide adenine dinucleotide (NAD), a redox cofactor, is essential for cell metabolism. The levels of NAD⁺ in Diosgenin-sensitized A549 and HCT116 cells were measured using the Promega NAD/NADH Glo assay kit in the presence and absence of a precursor which plays a pivotal role in the synthesis of NAD, nicotinamide (NAM). The results obtained showed that in absence of the NAM Diosgenin-treated cells at different concentrations showed similar levels of NAD⁺ at 24 hours and 48 hours. Whereas, NAM supplementation elevated the levels of NAD⁺ in 50 and 100 µM of Diosgenin-treated cells at 24 hours and 48 hours when compared to the respected controls [24]. In HCT116 cell lines, the levels of NAD⁺ in absence of NAM were considerably low in 50 and 100 µM of Diosgenin-treated cells at 24 hours and 48 hours. Besides the levels of NAD⁺ in absence of NAM in 12.5 and 6.25 µM Diosgenin-treated cells presented near normal levels of NAD⁺. A similar pattern of the result was seen in Diosgenin treated cells in presence of NAM (Figure 3A and 3B).

D. Caspase mediated PARP cleavage inducing cell death

In the current study, the expression pattern of PARP1 and cleaved PARP1 because of enhanced caspase activity through Diosgenin 50, and 100 µM sensitization in A549 cells at different time intervals such as 2h, 4h, 6h, 24h and 48h using western blot analysis was investigated. In parallel, staurosporine a well-known apoptosis inducer was assessed as assay control at 1µM concentration. Western blot analysis using PARP1 antibody and cleaved PARP antibody was able to detect both full-length PARP1 of 116 kDa and cleaved PARP1 at 86 kDa protein. Basal PARP1 levels at 113 kDa appeared unchanged upto 24h at all tested conditions in controls (media and media with DMSO) and treated lysates Diosgenin 50 and 100 µM and staurosporine 1µM. At 48h PARP1 intensity in controls remain unchanged but the treated lysates showed decrease in PARP1 band intensity. In

staurosporine sensitized cells cleaved PARP activity appears to peak at 2h indicating early response of apoptotic events in A549 cells [25]. Post 2 hour the overall band intensities at 89 kDa appeared faint when compared to respective time points indicating cells progressing towards apoptosis with reduction in cell viability. In Diosgenin treated cells at both tested concentrations of Diosgenin cleaved PARP band intensities have peaked at 2-hour post which there was no enhancing effect seen. Similar to staurosporine the metabolic events appear to happen at early time (Figure 4A). HCT116 cells at 2 hours has not shown any change in basal PARP1 levels, whereas faint band intensities were seen in control and Diosgenin treated lysates especially at 100 μ M. At 4h increase in cleaved PARP was detected in staurosporine but Diosgenin at both tested concentrations showed faded band intensities. At 24 staurosporine 1 μ M and diosgenin 50 μ M treated lysates showed reduction in PARP1 levels and increase in cleaved PARP levels indicating the total PARP being cleaved by caspases. After 24 hours of treatment with Diosgenin at a concentration of 100 μ M, there was an absence of cleaved PARP, suggesting that cell metabolism extends beyond the apoptosis pathway. Similar results seen in staurosporine and Diosgenin 50 μ M at 48 and 72h. GAPDH was used as housekeeping protein. (Figure 4B).

E. POC study with Diosgenin to evaluate the anti-inflammatory properties in human whole blood assay

The preliminary pilot studies performed in whole blood of five donors stimulated with 10, 100 and 1000ng/ml of LPS for 5h indicated that the pro-inflammatory cytokines TNF α showed and IL-6 to be consistent across donors (Figure 5A). The cytokine release from the pilot study indicated LPS at 100ng/ml to be ideal concentration to stimulate the TNF α and IL-6 blood cytokines. Further, the whole blood cells from different donors exposed to Diosgenin at various concentrations (100 μ M downwards; 9-point dose response curve with semi log dilutions) and stimulated with LPS at 100ng/ml. Diosgenin inhibited TNF α with an IC₅₀ of 27 nM and 29nM for donor 1 and donor 2 respectively (Figure 5B). Also, similar response was seen for IL-6 cytokine with IC₅₀ of 147 nM and 63nM for donor 1 and donor 2 respectively (Figure 5C).

IV. DISCUSSION

Understanding of cancer and its treatments are advancing day by day; but cancer remains one of the leading causes of death in the world despite of the numerous advancements. It has been discovered that some natural compounds, especially flavonoids, have anti-proliferative and apoptosis-inducing effects on cancer cells. Diosgenin, the primary steroidal sapogenin in fenugreek seeds, has been demonstrated to potently inhibit constitutively active pro-inflammatory, pro-survival signaling pathways and to induce apoptosis in a range of cancer cells [17]. Preclinical studies have shown promising effects of diosgenin on inhibiting tumor cell proliferation and growth, promoting apoptosis, inducing differentiation and autophagy, inhibiting tumor cell metastasis and invasion, blocking cell cycle, regulating immunity, and improving gut microbiome [18]. By considering the facts, we examined the anticancer activity of Diosgenin using two cancerous cell lines A549 and HCT119. The inhibitory effect of Diosgenin which was assessed in the study showed that the Diosgenin exhibited a dose-dependent inhibition of the growth of A549 and HCT116 cell lines. The dose-dependent inhibitory effect of Diosgenin on A549 and HCT116 cell proliferation (IC₅₀ of 13.96 μ M and 1.68 μ M, respectively) underscores its potent anticancer activity. Previous studies corroborate these findings, demonstrating Diosgenin's ability to suppress cell proliferation across various cancer types. For instance, Sethi et al. [17] reported significant growth inhibition in HepG2 liver cancer cells with Diosgenin, emphasizing its broad-spectrum anti-proliferative activity. The low IC₅₀ values in HCT116 cells suggest a heightened sensitivity to Diosgenin, potentially linked to the cell line's metabolic vulnerabilities [19]. These results suggest Diosgenin's promise as a therapeutic candidate, warranting further exploration of its molecular targets and mechanisms.

Diosgenin-induced apoptosis via caspase 3/7 activation aligns with its reported apoptotic potential in other cancer types. Increased caspase activity in A549 and HCT116 cells reflects classical apoptosis pathways [20]. The results mirror findings by Li et al. [21], who showed Diosgenin-induced apoptosis in MCF-7 breast cancer cells through caspase activation. The observed time- and dose-dependent increase in caspase activity emphasizes Diosgenin's role as an apoptosis inducer. Notably, higher caspase activity at 48 hours and its reduction over longer durations suggest transient apoptotic signals, a feature that could inform dosing regimens.

The modulation of NAD⁺ levels by Diosgenin highlights its impact on metabolic pathways crucial for cancer survival. The presence of NAM restored NAD⁺ levels in Diosgenin-treated cells, aligning with studies emphasizing NAD⁺ dependency in cancer metabolism [22]. The depletion of NAD⁺ in higher Diosgenin concentrations suggests potential targeting of NAD⁺-dependent enzymes such as SIRT1. These results point to Diosgenin's dual role in apoptosis induction and metabolic disruption, providing insights into its multi-faceted anticancer mechanisms.

Diosgenin-induced cleavage of PARP1 confirms its involvement in caspase-mediated apoptosis. The observed PARP cleavage aligns with Staurosporine's effects, a known apoptosis inducer, supporting the role of caspases in Diosgenin's mechanism of action. Previous research by Bürkle [23] has highlighted the critical role of PARP cleavage in apoptosis execution, linking it to reduced DNA repair and increased cell death. The absence of cleaved PARP after 24 hours of treatment suggests additional pathways beyond apoptosis, a hypothesis supported by Diosgenin's metabolic effects observed in this study.

Diosgenin's inhibition of pro-inflammatory cytokines (TNF α and IL-6) further enhances its therapeutic potential. The IC50 values observed in the human whole blood assay are consistent with previous reports of Diosgenin's anti-inflammatory activity [24]. These findings support Diosgenin's dual anticancer and anti-inflammatory roles, potentially benefiting cancer patients with inflammation-driven progression.

Figures

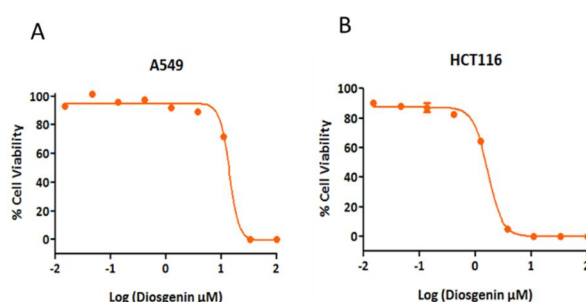


Figure 1: IC50 values for Diosgenin tested in A549 and HCT116

Diosgenin inhibited proliferation of human lung carcinoma A549 (A) and colorectal cell line HCT116 (B). 1000 cells were seeded per reaction in 96-well plates for 5 hours, post which cells were treated with Diosgenin starting from 100 μ M to 0.015 μ M and cultured for 96 hours. The untreated cell control was set at 100% viability.

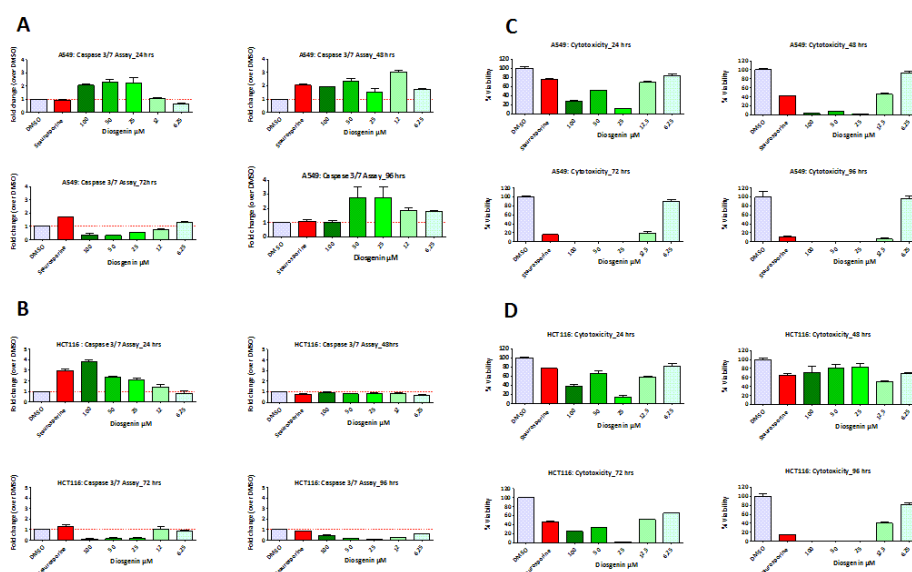


Figure 2. Detection of caspase 3/7 activity in A549 and HCT116 cells.

For assessment of caspase activity 1000 cells of A549 and HCT116 cells were exposed to Diosgenin at 100, 50, 25, 12 and 6.25 μ M for 24, 48, 72 and 96 hours. To further corroborate the caspase activity, a parallel cytotoxicity assay was performed with the same conditions as caspase assay. Staurosporine at 50nM was processed as an assay control. Diosgenin displayed dose and time dependent apoptotic activity by inhibiting proliferation of human lung carcinoma A549 (2A and 2C) and colorectal cell line HCT116 (2B and 2D). The untreated cell control was set at 100% viability. The caspase and cytotoxicity assessment was determined by two-way annova in Graph Pad prism software.

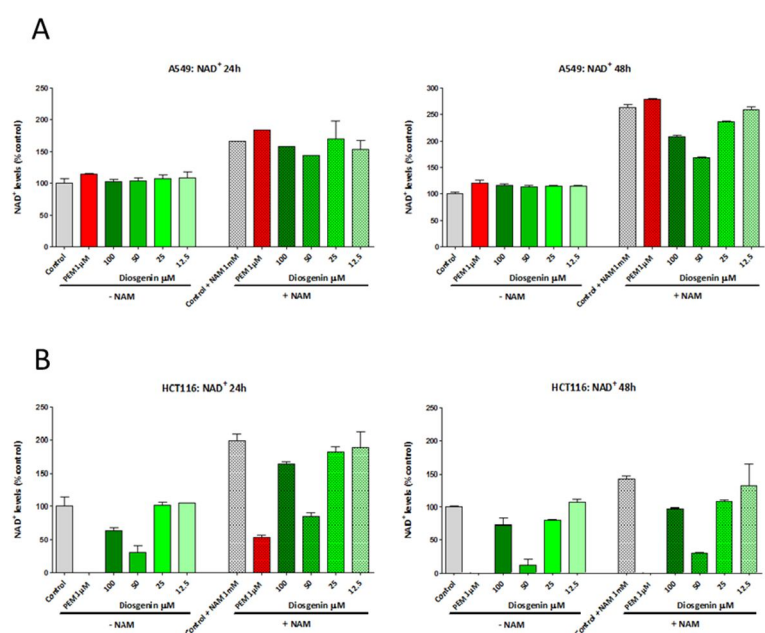
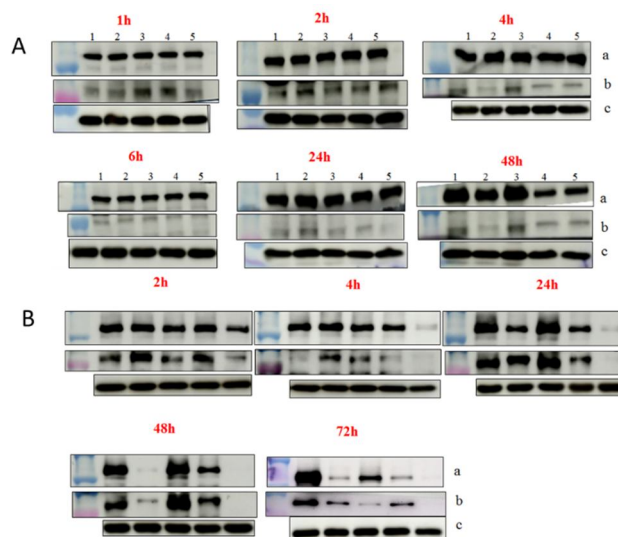


Figure 3. Detection of NAD⁺ metabolites post-supplementation of NAM in A549 and HCT116 cells.

For assessment of NAD⁺ activity, 0.1 million cells per well was seeded and incubated overnight at 37 °C in a humidifier incubator with 5% CO₂. Cells were treated with different concentrations of Diosgenin at 100, 50, 25 & 12.5μM and further incubated for 24 & 48h. Figure 3A; With NAM supplementation A549 showed modest increase in NAD⁺ levels at 24h and close to 2 fold increase at 48h restoring metabolic activity. Pemetrexed showed similar response to Diosgenin Figure 3B; In HCT116, 2 fold increase in NAD⁺ levels were seen at 24h and the response wanned down at 48h. Though Pemetrexed showed cell recovery with NAM supplementation at 24h, enhanced cytotoxicity with absolutely no cell survival was seen at 48h irrespective of NAM supplementation. The NAD⁺ assessment was determined by two-way annova in Graph Pad Prism software.



Sl. No.	Tested Conditions
1	Media control
2	Staurosporine 1μM
3	DMSO Control
4	Diosgenin 50μM
5	Diosgenin 100μM

a) PARP : 116 kDa
b) Cleaved PARP : 89 kDa
c) GAPDH: 37 kDa

Figure 4A and 4B: Detection of caspase induced PARP cleavage as a cascading effect of Diosgenin treatment.

For western blot assessments of total and cleaved PARP, A549 and HCT116 cells were seeded at 0.5 million cells per treatment condition in a 6-well plate and incubated overnight. Cells were treated with diosgenin 50 μ M and 100 μ M and staurosporine at 1 μ M, and further incubated for 1, 2, 4, 6, 24, and 48 hours for A549 and 2, 4, 24, 48 and 72 hours for HCT116 cells. Protein lysates were prepared and 10 μ g of the sample lysates were loaded on 10% SDS-PAGE for analysis. A549 and HCT116 cells indicate caspase activation induced by Diosgenin and staurosporine treatment. Detection of cleaved PARP showed enhanced caspase levels which further re-iterated the cells entering the apoptotic pathway through caspase activation in A549 (Figure 4A) and HCT116 (Figure 4B). The data shown are representative of three independent experiments.

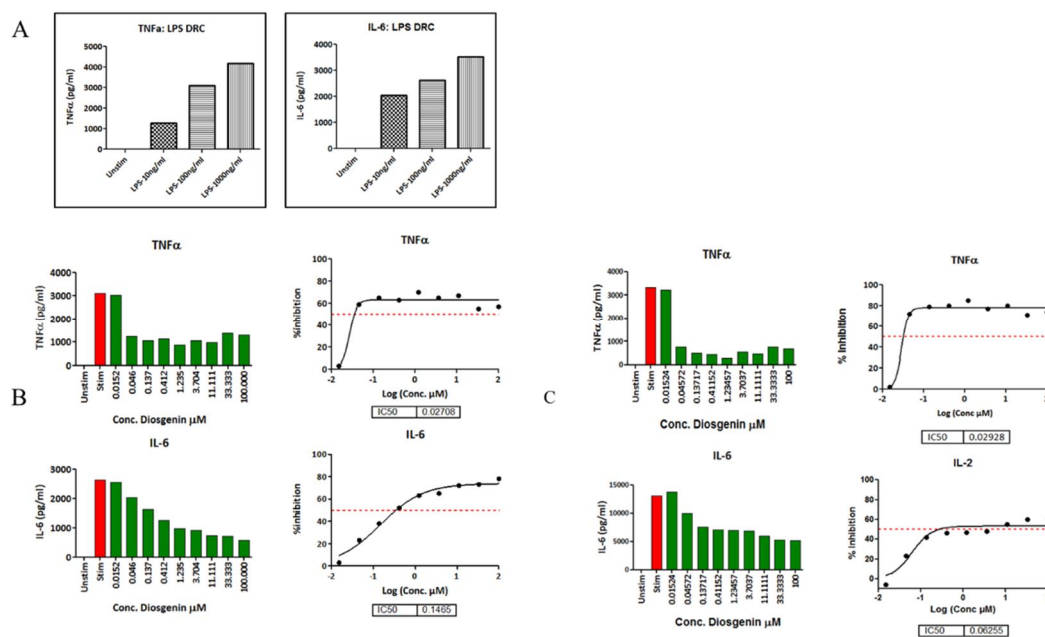


Figure 5: Impact of Diosgenin on LPS-induced release of TNF α and IL-6

Human whole blood was collected from healthy human donors in vacutainers. 180 μ l of undiluted whole blood is seeded in 96 well u-bottom plate. A pilot study in whole blood with LPS at 10, 100 and 1000ng/ml 3 different concentrations of LPS indicated a concentration dependent increase in TNF α and IL-6 release (Figure 5A). Further, 100 ng/ml of LPS stimulated whole blood in the presence of Diosgenin was treated for 5 hours (details captured in materials and method section). TNF α and IL-6 assessed by ELISA and IC50 values derived by normalizing the data to control (LPS stimulation only) for each experiment indicated similar response between donor 1 (Figure 5B) & 2 (Figure 5B). Sigmoidal dose response curves were plotted using Graph Pad Prism5 software.

V. CONCLUSION

This study underscores Diosgenin's potent anticancer properties, driven by its ability to modulate apoptosis, disrupt metabolism, and suppress inflammation. Diosgenin's dose- and time-dependent effects on caspase activation, PARP cleavage, and NAD⁺ levels highlight its multifaceted mechanism of action. The anti-inflammatory properties further position Diosgenin as a promising candidate for integrative cancer therapy. Hence current study strongly suggests that Diosgenin has a potential therapeutic application in treatment of cancer; however, further studies are required to confirm these findings.

Ethics Statement

The whole blood studies were approved by the responsible ethics committee (Ethics committee of the Medical Faculty, JSSSTU University before start. Human whole blood was drawn from healthy volunteers after obtaining written informed consent.

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Conflicts of Interest: None

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