Effect of gedunin on cell proliferation and apoptosis in skin melanoma cells A431 via the PI3K/JNK signaling pathway

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Abstract

Background. Melanoma is an aggressive skin malignancy with rapid metastasis and high morbidity. Gedunin (GN) is a tetranortriterpenoid belonging to the *Meliaceae* family, described for its anticancer, antiproliferative and apoptotic properties.

Objectives. In the present study, we investigated the effect of GN on A431 melanoma cell proliferation and apoptosis. The inflammatory proteins (tumor necrosis factor alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cycloxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and interleukin 6 (IL-6)) apoptosis-related proteins, such as Bax, Bcl-2 and caspase-3, and alterations in the PI3K/JNK and p38 pathways in A431 cells after GN treatment were examined.

Materials and methods. The cytotoxicity assay and cell apoptosis of GN activity on A431 cells were assessed using MTT assay, acridine orange/ethidium bromide (AO/EB), DAPI (4′,6-diamidino-2-phenylindole), propidium iodide (PI), enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR) and western blot analyses.

Results. The findings demonstrated that GN (10 and 15 μM/mL) inhibits the growth of melanoma cells, triggers apoptosis by enhancing Bax and caspase, and reduces Bcl-2, cyclin-D1, c-myc, and survivin in a concentration-reliant manner. Additionally, GN attenuated the protein expression of inflammatory proteins (TNF-α, NF-κB, COX-2, iNOS, and IL-6) and the cell proliferative PI3K/JNK/p38 signaling pathway. Due to the imbalance in the Bax/Bcl-2 ratio, apoptosis is promoted, and the caspase cascade and Cyt-c are activated. This led us to conclude that GN treatment inhibited Bcl-2, cyclin-D1, c-myc, and survivin activity through the TNF-α/NF-κB and PI3K/JNK/p38 signaling pathways, further preventing the proliferation and stimulation of apoptosis, which contributes to growth arrest in melanoma cells.

Conclusions. Gedunin has been shown to promote melanoma cell death in vitro, suggesting that it could be used as a future treatment for malignant melanoma. Our findings suggested that GN might be applied as a preventative measure in the management of skin melanoma cells.

Key words: skin cancer, gedunin, apoptosis, AROS, P13K/JNK/p38 signaling

Cite as
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Background

Skin cancer has an elevated incidence and mortality worldwide due to chemical carcinogens producing reactive oxygen species (ROS) and augmented UV radiation due to ozone diminution at the earth's surface.[1] Melanocyte-derived malignant melanoma (MM) causes the greater part of deaths ascribed to skin cancer. It is most commonly observed at the interface between the dermis and epidermis, as well as in melanocyte-rich regions of the skin, the ciliary body of the eye, the mucosa, the iris, the choroid, and the meninges. It is less frequently found in lymph nodes and internal organs.[2] Melanoma, which accounts for approx. 70% of skin cancer demises, is the most lethal form of skin cancer, featured by speedy degradation, premature metastasis and elevated mortality.[3] Melanoma has increased drastically over the past 20 years.[4] The most recent statement by the World Health Organization (WHO) indicated that there were 288,000 cases of melanoma and 61,000 deaths globally in 2018.[5] Melanoma can be treated with surgical excision, chemotherapy or immunotherapy,[6] but their efficacies are variable and the adverse effects are not negligible.[7] As a result, novel approaches to prevent melanoma formation and provide efficient protection are immediately needed.

Gedunin (GN) is one of the major tetranortriterpenoids found in the neem tree.[8] Contemporary studies have revealed that GN can reduce ovary, prostate and colon cancer cell proliferation.[9,10] Gedunin has been described to have potential anticancer activity by impeding breast cancer cell proliferation through the modulation of the assured heat shock proteins.[11] Also, the antiproliferative effects have been noticed in ovarian cancer cells in response to GN treatment via central signaling pathway regulation.[12] Furthermore, a current in silico study has shown a drug-likeness of GN for the β-catenin chain in cancer stem cells.[13] However, the apoptosis and antiproliferative effects of GN on melanoma cancer cells have not yet been investigated. The current study was conducted to evaluate anticancer and apoptotic effects of GN on A431 human melanoma cells.

Apoptosis is crucial for cellular homeostasis; hence, it became a central target for developing new anticancer remedies, influences the responsiveness to treatment, and is involved in the regulation of tumor development.[14,15] It is triggered by extrinsic and intrinsic ligands, which are regulated by various intracellular signaling pathways and coordinated by gene complexes.[16] During these processes, the instigation of the caspase cascade directs the cleavage of essential proteins that are involved in anti-apoptotic pathways, like members of the Bcl-2 protein family.[17] Bax and Bcl-2 are pro- and anti-apoptotic proteins belonging to the Bcl-2 family. These are key intermediates in mitochondrial outer membrane permeabilization supplemented by apoptosis.[18] They also play a vital role in controlling cytochrome-c discharge from the mitochondria.[19] Numerous downstream substrates of the PI3K/JNK signaling pathway, such as Bax and Bad, added to the chemotherapeutic resistance of tumor cells and reduced apoptosis.[20] Kishore et al.[21] documented that GN could suppress hamster buccal pouch carcinogenesis through the PI3K/JNK/p38 and tumor necrosis factor alpha (TNF-α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), pathways. Likewise, Tanagala et al.[22] reported that in SCC131 oral cancer cells, GN downregulated PI3K/Akt/mTOR/ERK/NF-κB signaling. While recent studies have focused on GN as an effective anti-melanogenic agent, there are still not enough papers conducted on the effects of GN in cell proliferative and its impact on apoptosis in A431 MM cells via the PI3K/JNK signaling pathway. In the current hypothesis examine that GN affected the apoptotic and proliferation of A431 melanoma cells. After GN treatment, A431 cells were treated with inflammatory proteins (tumor necrosis factor alpha (TNF-α), nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB), cycloxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and interleukin 6 (IL-6), as well as apoptosis-related proteins (Bax, Bcl-2, and caspase-3), and changes in the PI3K/JNK and p38 pathways.

Objectives

We aimed to assess the anticancer impact of GN on A431 melanoma cells and clarify the molecular mechanisms underlying the PI3K/AKT/p38 apoptotic signaling pathway.

Materials and methods

Chemicals

Gedunin, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics, phosphate-buffered saline (PBS), MTT, acridine orange/ethidium bromide (AO/EB), 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), sodium dodecyl sulphate (SDS), and dimethyl sulfoxide (DMSO) were provided by Merck (Darmstadt, Germany). JNK and p38 antibodies, the phosphorylated form and total PI3K, and horseradish peroxidase (HRP)-conjugated β-actin were acquired from Labome (Cambridge, USA).

Cell culture

Shanghai Aiyan Biotechnology Co., Ltd. (Shanghai, China) provided the A431 melanoma cell lines, which were obtained in Shanghai, China. The cells were cultivated in DMEM medium supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) at 37°C in an environment of 5% CO_2 and less than 95% humidity.

MTT cell viability assay

The vitality of human melanoma cells was determined using the MTT assay.[23] After seeding A431 cells into 96 wells

(1×10^5 cells/well), they were grown at 37°C in a moist incubator with 5% CO_2. After overnight incubation, cells were dipped in PBS and exposed to GN at various doses (5, 10, 15, and 20 μM/mL) for a day. The cells were treated with a 10 μL MTT solution and left for 4 h to allow mitochondrial dehydrogenase to convert into insoluble formazan crystals. The formazan crystals were then dissolved using 150 μL of DMSO. The optical density (OD) at 490 nm was determined using an enzyme-linked immunosorbent assay (ELISA) plate Reader (Bio-Tek Instruments, USA). The IC_{50} values for 4-parameter logistic function dose-response curves were calculated using Sigma PlotTM software (Systat, San Jose, USA; GN concentrations that caused a 50% reduction in MTT assay). The concentrations selected for further investigation were used.

Apoptosis evaluation by AO/EB staining

The study used AO/EB staining and labeling to observe apoptotic morphological differentiation in melanoma cells exposed to GN at 10 and 15 μM doses.[24] For a full day, A431 cells were treated to 10 and 15 μM/mL of GN. The treated and control cells received the same amount of AO/EB dye mixture (100 g/mL). Following the incubation, the cells were examined using an Olympus BX51 fluorescence microscope (Olympus Corp., Tokyo, Japan) and left at room temperature in the dark for 20 min to ensure that all unbound dye had been removed by PBS washing.

Apoptosis assessed by DAPI staining

Six well plates with 1×10^5 human melanoma cells each were seeded with A431, and GN (10 and 15 μM/mL) was added as a supplement. These treated cells were labeled with DAPI in accordance with the previously described methodology to study the nuclear changes associated with apoptosis.[25] After that, the materials were placed on a glass slide and examined using an Olympus BX51 fluorescence microscope (Olympus Corp.).

Evaluation of apoptosis by PI staining

The PI staining test was used to identify apoptotic nuclei in A431 melanoma cells. The cells were treated to various GN concentrations (10 and 15 μM/mL) and stored for 48 h. The samples were subsequently extracted and stained with PI according to the described procedure.[26] A microscope (Olympus BX51) was used to look at the red fluorescence emitted by the nuclei.

Measurement of caspase-9 and -8

Caspase-9 and caspase-8 activity in GN-treated A431 cells was measured using the caspase-9 and -8 colorimetric assay kit (Biovision, Exton, USA) according to the manufacturer's instructions. The cells were homogenized in a lytic buffer (ethanesulfonic acid (HEPES) Ph 7.4; 5 mm 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) and 5 mm dithiothreitol (DTT)) and kept in ice for 10 min. The homogenate was centrifuged in a microcentrifuge (10,000 × g) for 1 min. The supernatant (cytosolic extract) was transferred to a fresh tube and kept on ice for immediate assay. Fifty microliters of each sample containing 50–200 μg of protein were placed into microtiter plate wells. Fifty microliters of 2X Reaction Buffer (Biovision) (containing 10 mm DTT) were added to each well. Five microliters of 4 mm LEHD-pNA (caspase-9 substrate) substrate were added to each well and incubated at 37˚C for 1–2 h. After incubation, the samples on the microtiter plate were read at 400 or 405 nm in a microtiter plate reader. The concentration of pNA released from the substrate was calculated from the absorbance value. Fold increases were determined by comparing the results with the levels of the control samples. Caspase-9 and -8 activity was expressed as μmol of pNA formed/min/mL of cell lysate.

mRNA expressions detected by qRT-PCR

Total RNA was extracted from A431 human melanoma cells using the TRIzol® reagent (Invitrogen, Thermo Fisher Scientific, Waltham, USA) following the manufacturer's instructions. To reverse transcribe the isolated RNA into cDNA, a High-capacity cDNA Reverse Transcription kit (Bio-Rad, Hercules, USA) was used, following the manufacturer's instructions. Lastly, the cDNAs were evaluated in accordance with the company's instructions using a FastStart SYBR Green master mix (Invitrogen, Thermo Fisher Scientific). The band intensity was measured on 1.5% agarose gels using electrophoresis with ImageJ v. 1.48 software (National Institutes of Health (NIH), Bethesda, USA).

Western blot analysis

Human melanoma cells A431 were treated with 10 and 15 μM/mL of GN and grown for 1 day. Protease inhibitors were added, and the cell lysates were generated using an ice-cold lysis solution. Subsequently, a western blot analysis was conducted. The protein content was determined with the BCA Protein Assay Kit (Thermo FIsher Scientific). To summarize, the proteins were electrophoretically dispersed and then transferred to a polyvinyl difluoride (PVDF) film. After blocking the film for 1 h at room temperature with the probe, primary antibodies (TNF-α, NF-κB, COX-2, iNOS, and interleukin 6 (IL-6)) PI3K, JNK, and p38) were added at a dilution of 1:1,000 and the film was left overnight at 4°C. After that, secondary antibodies were added. To detect proteins, protein bands were colored and shown. Densitometry was used to quantify the protein bands using ImageJ software.

Statistical analyses

The statistical analysis of the data from each group was conducted using GraphPad Prism v. 8.0.2 (GraphPad Software, San Diego, USA) and IBM SPSS v. 25 (IBM Corp., Armonk, USA). Measurement data were presented as the median (min and max). As the sample size was too small to verify normal data distribution, the differences between the groups were analyzed with the non-parametric Kruskal–Wallis test and Dunn's post hoc test. Subsequently, significant differences among multiple groups were examined using the Kruskal–Wallis test and Dunn's post hoc test was employed for multiple comparisons. A statistically notable data divergence was considered at a p-value < 0.05. All tests in this study were bilateral.

Results

Cytotoxicity effect of GN on A431 melanoma cells

The cytotoxicity of GN (5, 10, 15, and 20 µM/mL) on human melanoma cells A431 was assessed using the MTT test. A431 proliferation could not be significantly affected by GN administration at concentrations less than 10 µM. However, GN doses of 5, 10, 15, and 20 µM/mL inhibited A431 cell survival. The findings revealed that GN had dose-dependent cytotoxic and antiproliferative effects on A431 melanoma cells. The IC_{50} value of GN was 15 µM, hence, 10 and 15 µM were chosen for further research (Fig. 1, Table 1).

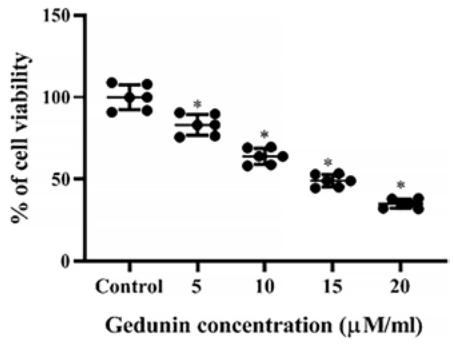

Fig. 1. Gedunin inhibits A431 human melanoma cell proliferation. Human A431 melanoma cells for a full day were exposed to varying concentrations of gedunin (GN) (5–20 µM/mL). Cell viability was assessed using the MTT test. A * p-value < 0.05 indicates significance compared to melanoma control cells that have not received treatment. The figure presents data (black dots) and the medians (horizontal lines). A * p-value < 0.05 compared to the control group (Table 1)

Assessment of apoptosis using AO/EB staining

Dual acridine orange/ethidium bromide staining revealed apoptotic changes in A431 melanoma cells (Fig. 2A). The untreated A431 melanoma cells were consistently labeled green and alive. In a concentration-dependent manner, GN (10 and 15 µM/mL) treated A431 cells revealed more apoptotic alterations than controls. Compressed chromatin, membrane blebbing, and early apoptotic cells were observed in A431 melanoma cells treated with 10 µM GN. The treatment of 15 µM GN caused late apoptotic alterations in A431 cells, including chromatin condensation, fragmented nuclei and orange-red membrane blebbing.

Influence of GN on A431 melanoma cell apoptosis using DAPI staining

Normal, live cells can be seen in the DAPI-stained human melanoma A431 cells (Fig. 2B). Gedunin-induced apoptosis-treated A431 melanoma cells were shown to have improved nuclei shape and nuclear body fragmentation compared to untreated control cells. Chromatin condensation, membrane blebbing, nuclear envelope damage, and cellular collapse were observed in A431 cells treated with GN (10 and 15 µM/mL). These results point out GN-induced involuntary cell demise in a concentration-dependent manner.

Influence of GN-induced apoptosis in A431 melanoma cells evinced using PI staining

In A431 cells, PI labeling was used to detect apoptotic nuclei (Fig. 2C). Propidium iodide (PI) stain enters the cells when membrane integrity is lost, which is associated with membrane polarity damage, guiding apoptosis. The findings demonstrate that GN caused dose-dependent apoptosis in A431 cells. Apoptotic activity was elevated by GN treatments (10 and 15 M/mL) compared to untreated A431 control cells. Hence, GN-prompted apoptosis could be one mechanism for averting melanoma cell proliferation.

Measurement of caspase-9 and -8 activity using ELISA

Treatment with GN of A431 melanoma cells exhibited augmented activity of caspase-9 and -8 compared to untreated control cells (Fig. 3, Table 2,3). The amount

Table 1. Compare groups with each other

Variables	Control	5 µM	10 µM	15 µM	20 µM	Test value (H)**	p-value*
MTT	100.03 (90.98–108.98)	83.15 (75.63–90.59)	63.93 (58.15–69.65)	49.07 (44.64–53.46)	35.12 (31.94–38.26)	27.87	<0.001

Data were presented as median (min and max); * p-value was generated from Kruskal–Wallis test with Dunn's post hoc test; ** degrees of freedom value is equal to 4.

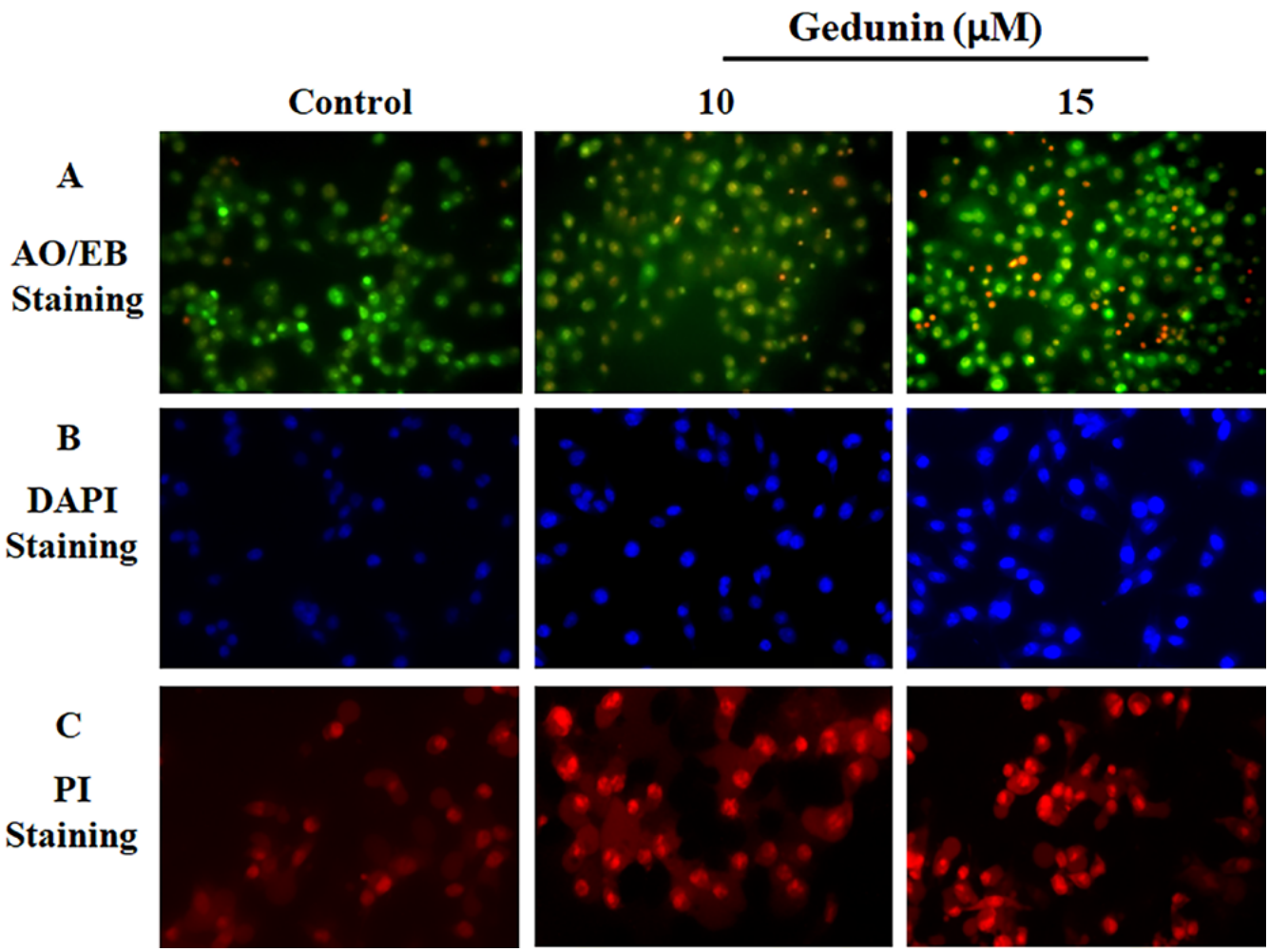

Fig. 2. The effect of gedunin (GN) on the apoptosis of A431 human melanoma cells. A. acridine orange/ethidium bromide (AO/EB); B. DAPI (4',6-diamidino-2-phenylindole), and C. Propidium iodide (PI) staining. Gedunin (10 and 15 μM/mL) treated with A431 melanoma cells for 24 h. Using AO/EB, DAPI and PI staining, apoptosis in melanoma cells was investigated and observed under a fluorescence microscope

Table 2. Comparisons of measured parameters between groups

Variables	Control (n = 6)	15 μM (n = 6)	20 μM (n = 6)	Test value (H)**	p-value*
Caspase-9	100.05 (91–109)	158.18 (143.87–172.33)	183.29 (166.71–199.69)	13.66	0.001
Caspase-8	100.05 (91–109)	116.14 (105.63–126.53)	130.20 (118.43–141.85)	11.97	0.003
Cyclin-D1	1.00 (0.91–1.09)	0.75 (0.68–0.82)	0.52 (0.47–0.57)	15.20	<0.001
Bcl-2	1.00 (0.91–1.09)	0.80 (0.73–0.87)	0.48 (0.44–0.52)	15.23	<0.001
Bax	1.00 (0.91–1.09)	2.15 (1.96–2.34)	3.50 (3.19–3.82)	15.20	<0.001
Caspase-3	1.00 (0.91–1.09)	2.02 (1.00–2.30)	3.08 (2.80–3.36)	14.07	<0.001
C-Myc	1.00 (0.91–1.09)	0.58 (0.53–0.63)	0.45 (0.41–0.49)	15.26	<0.001
Survivin	1.00 (0.91–1.09)	0.53 (0.48–0.58)	0.40 (0.36–0.44)	15.20	<0.001
TNF-α	1.00 (0.91–1.09)	0.71 (0.65–0.77)	0.48 (0.44–0.52)	15.26	<0.001
NF-κB	1.00 (0.91–1.09)	0.68 (0.62–0.74)	0.46 (0.42–0.50)	15.23	<0.001
COX-2	1.00 (0.91–1.09)	0.79 (0.72–0.86)	0.53 (0.48–0.58)	15.20	<0.001
iNOS	1.00 (0.91–1.09)	0.61 (0.56–0.66)	0.38 (0.35–0.41)	15.26	<0.001
IL-6	1.00 (0.91–1.09)	0.70 (0.64–0.76)	0.42 (0.38–0.46)	15.23	<0.001
p-PI3K/PI3K	1.00 (0.91–1.09)	0.80 (0.73–0.87)	0.53 (0.48–0.58)	15.20	<0.001
p-JNK/JNK	1.00 (0.91–1.09)	1.43 (1.30–1.56)	2.51 (2.28–2.74)	15.20	<0.001
p-P38/P38	1.00 (0.91–1.09)	1.52 (1.38–1.66)	2.68 (2.44–2.92)	15.20	<0.001

Data were presented as median (min and max); * p-value was generated from Kruskal–Wallis test with Dunn's post hoc test; ** degrees of freedom is equal to 2. TNF-α – tumor necrosis factor alpha; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; iNOS – inducible nitric oxide synthase; IL-6 – interleukin 6.

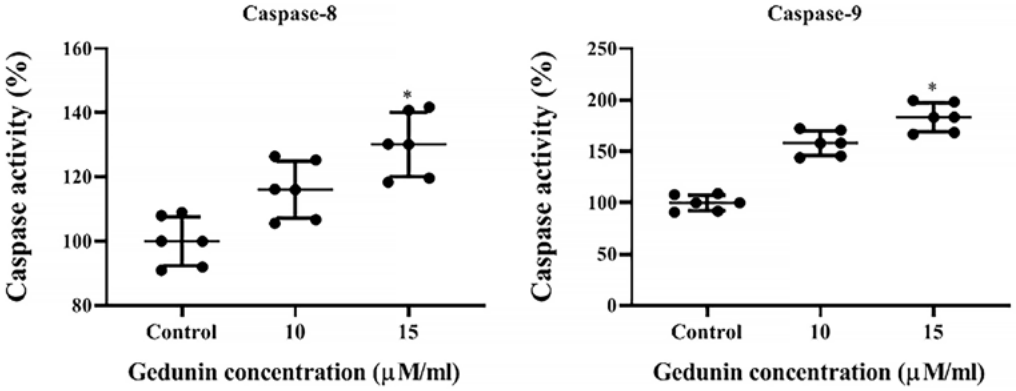

Fig. 3. Measurement of caspase-9 and -8 in A431 human melanoma cells treated with gedunin (GN). For a whole day, control, 10 and 15 μM/mL GN doses were administered to human melanoma A431 cells. Using enzyme-linked immunosorbent assay (ELISA), the activity of caspase-9 and -8 was quantified. The figure presents data (black dots) and the medians (horizontal lines); * $p < 0.05$ compared to the control group (Table 2,3)

Table 3. The results of the Dunn's post hoc test

Figure	Variable	C vs 15 μM	C vs 20 μM	15 μM vs 20 μM
Fig. 3	caspase-9	0.092	p < 0.001	0.390
Fig. 3	caspase-8	0.251	p < 0.002	0.251
Fig. 4	cyclin-d1	0.154	p < 0.001	0.154
Fig. 4	Bcl-2	0.153	p < 0.001	0.153
Fig. 4	Bax	0.154	p < 0.001	0.154
Fig. 4	caspase-3	0.312	p < 0.001	0.103
Fig. 4	C-Myc	0.152	p < 0.001	0.152
Fig. 4	survivin	0.154	p < 0.001	0.154
Fig. 5	TNF-α	0.152	p < 0.001	0.152
Fig. 5	NF-κB	0.153	p < 0.001	0.153
Fig. 5	COX-2	0.154	p < 0.001	0.154
Fig. 5	iNOS	0.152	p < 0.001	0.152
Fig. 5	IL-6	0.153	p<0.001	0.153
Fig. 6	p-PI3K/PI3K	0.154	p < 0.001	0.154
Fig. 6	p-JNK/JNK	0.154	p < 0.001	0.154
Fig. 6	p-P38/P38	0.154	p < 0.001	0.154

TNF-α – tumor necrosis factor alpha; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; AO/EB –acridine orange/ethidium bromide; iNOS – inducible nitric oxide synthase; IL-6 – interleukin 6.

of caspase-9 and -8 was significantly ($p < 0.05$) increased by GN at a dose of 15 μM compared to 10 μM GN treatment. Gedunin improved caspase activities in a concentration-dependent way.

Impact of GN on mRNA expression of cyclin-D1, Bcl-2, Bax, caspase-3, c-myc, and survivin in A431 melanoma cells

A431 cells were treated with GN (10 and 15 μM/mL) to measure the mRNA expression of proteins involved in apoptosis (Fig. 4, Table 2,3). While Bax and caspase-3 showed decreased mRNA expression, the untreated A431 control cells showed increased mRNA expression of cyclin-D1, Bcl-2, c-Myc, and survivin. When compared to untreated control melanoma cells, GN significantly decreased the expression of mRNAs for cyclin-D1, Bcl-2, c-Myc, and survivin, while increasing the expression of Bax and caspase-3 in a concentration-dependent manner.

Gedunin suppressed the NF-κB signaling pathway analyzed using western blot

The NF-κB/TNF-α signaling has a substantial role in cancer cell growth and development. Figure 5 depicts increased protein expression of TNF-α, NF-κB, COX-2, iNOS, and IL-6 in human A431 melanoma cells (Table 2,3). Treatment with GN (10 and 15 μM/mL) in the A431 cells decreased TNF-α, NF-κB, COX-2, iNOS, and IL-6 levels. In human melanoma cells, GN reduced these protein expressions in a concentration-dependent manner.

Gedunin suppressed the P13K/JNK/p38 signaling pathway analyzed using western blot

p-PI3K, PI3K, p-JNK, JNK, p38, and p38 signaling have a substantial role in cancer cell growth and development. Human A431 melanoma cells indicated intensified protein expression of P13K, JNK, and p38 (Fig. 6, Table 2,3). Treatment with GN (10 and 15 μM/mL) in the A431 cells decreased P13K/JNK/p38 levels. Gedunin reduced the expression of these proteins in the human melanoma cells in a concentration-dependent manner.

Discussion

Herbal plant-based natural products are still one of the greatest reservoirs for unique compounds with pharmacological activities.[27] Research is enduring to recognize a natural agent with the selective ability to block or impede cancer initiation and converse promotional stages by promoting apoptosis and arresting cancer cell growth deprived of typical cell cytotoxic effects.[28] The contemporary data indicate that several tumours are caused by anti-apoptotic protein genes

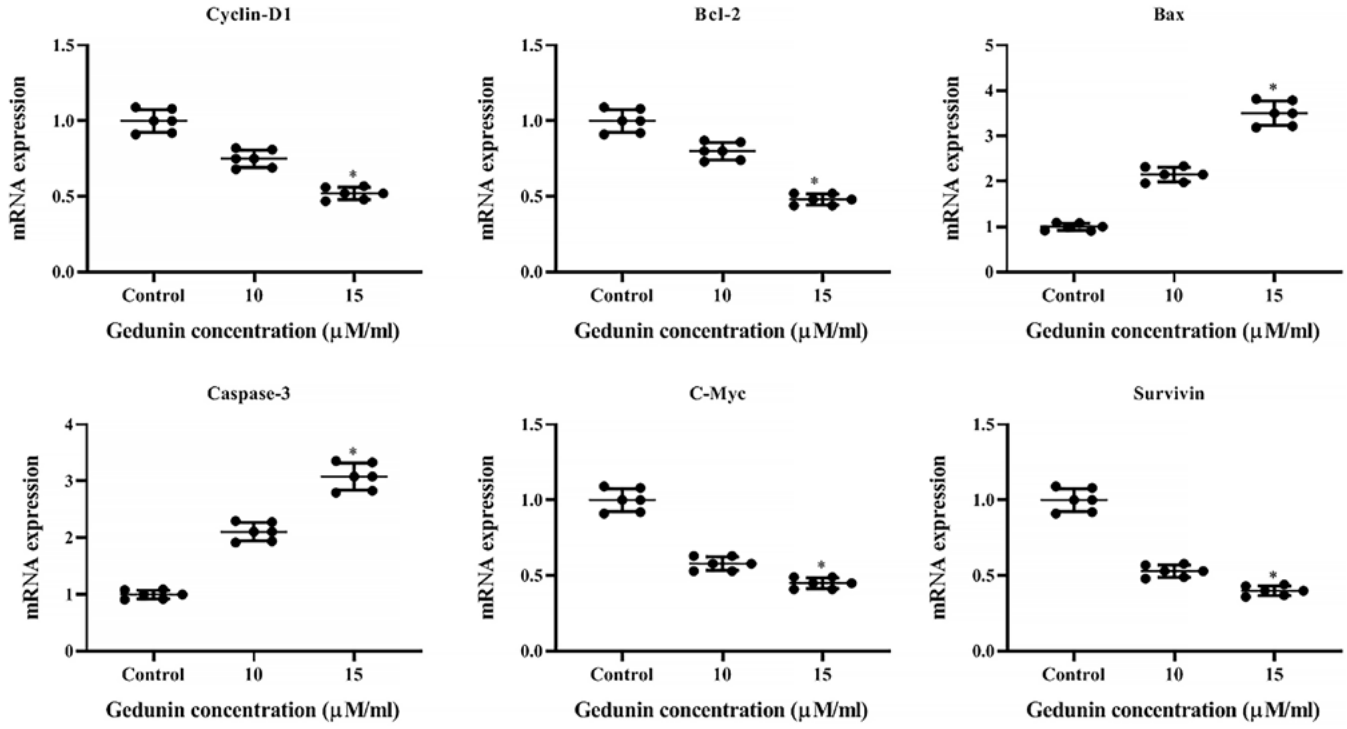

Fig. 4. Influence of gedunin (GN) on the mRNA expression of A431 human melanoma cells, including cyclin-D1, Bcl-2, Bax, caspase-3, c-Myc, and survivin. For a full day, A431 melanoma cells were exposed to control and 10 and 15 μM/mL doses of GN. The mRNA expression of cyclin-D1, Bcl-2, Bax, caspase-3, c-Myc, and survivin in A431 cells was determined using reverse transcription polymerase chain reaction (RT-PCR). The figure presents data (black dots) and the medians (horizontal lines); * $p < 0.05$ compared to the control group (Table 2,3)

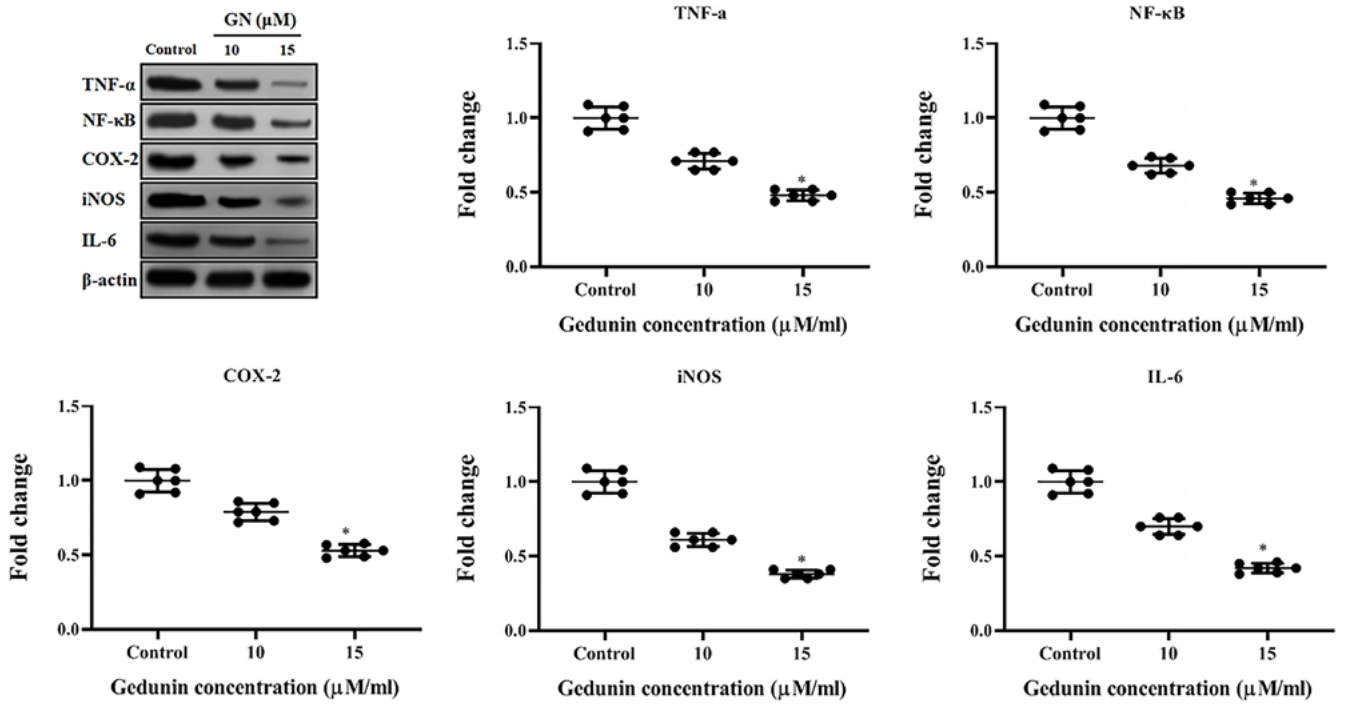

Fig. 5. Impact of gedunin (GN)-treated A431 human melanoma cells on tumor necrosis factor alpha (TNF-α), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cyclooxygenase-2 (COX2), inducible nitric oxide synthase (iNOS), and interleukin 6 (IL-6) inflammatory markers. For 1 day, melanoma A431 cells were exposed to 10 and 15 μM/mL of GN. Using western blot analysis, the protein expression of TNF-α, NF-κB, COX-2, iNOS, and β-actin was determined. The figure presents data (black dots) and the medians (horizontal lines); * $p < 0.05$ compared to the control group (Table 2,3)

that code for dysfunction, growth receptors, growth factors, tumour suppressors, and transcription factors. These genes constitute cancer treatment targets.[29,30]

In the current work, we assessed the capability of GN to constrain A431 cell proliferation as well as its apoptosis effects.

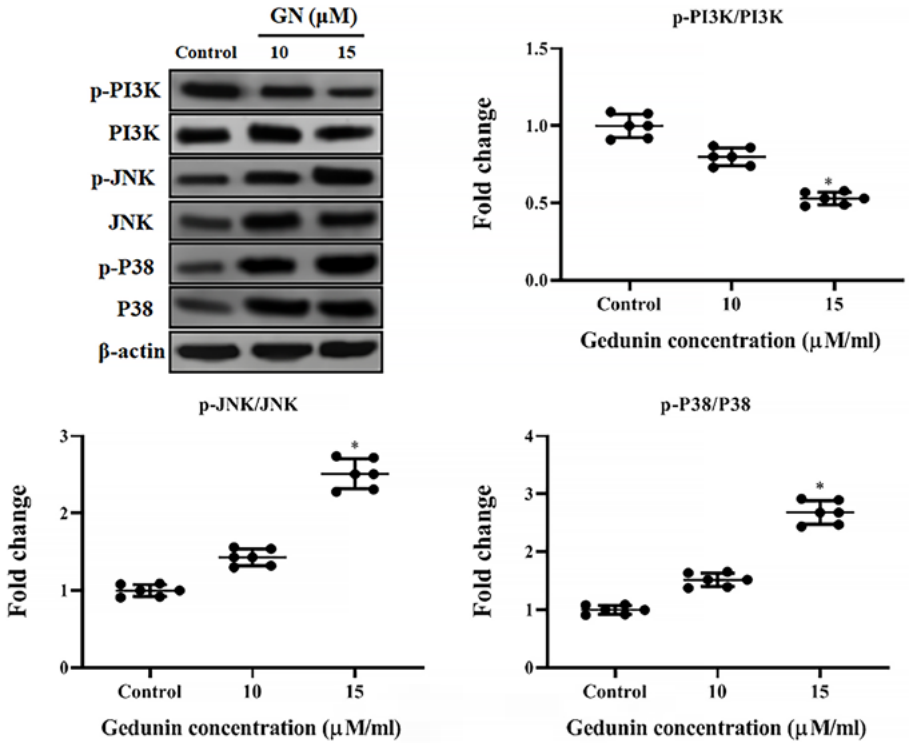

Fig. 6. Impact of gedunin (GN)-treated A431 cells on the P13K/AKT/p38 signaling pathway. For a whole day, melanoma A431 cells were exposed to 10 and 15 μM/mL of GN. Using western blot analysis, the protein expression of p-P13K, P13K, p-AKT, AKT, p-p38, p38, and β-actin was determined. The figure presents data (black dots) and the medians (horizontal lines); * $p < 0.05$ compared to the control group (Table 2,3)

Natural compounds are reported to have the competencies for reticence proliferation and triggering apoptosis in numerous malignant cells.[31] Gedunin is a bioactive limonoid, a derivative of the neem tree that seems to be an active natural chemotherapeutic agent due to its powerful anticancer efficacy on various kinds of malignancies, including oral, prostate, ovarian, and colon cancers.[9,10] It has been documented that GN is an effective anticancer drug.[32] Brandt et al.[33] claimed that GN has an antiproliferative impact on the breast cancer cell lines MCF-7 and SkBr3. Lamb et al.[34] demonstrated that GN modulated Hsp90 to demonstrate antiproliferative action. Our current study found that GN is an effective anticancer and antiproliferative agent against A431 human melanoma cells, evidenced by the MTT cytotoxicity assay. These results imply that GN may be a viable chemotherapeutic medication for the treatment of melanoma skin cancer.

Apoptosis is a gene-delimited spectacle triggered by many chemotherapeutic mediators.[14,15] It has been well established that both the intrinsic and extrinsic pathways are involved in apoptosis.[16] We observed augmented mRNA expressions of Bax and caspase-3 and levels of caspase-8, and -9, while cyclin-D1, c-Myc, survivin, and Bcl-2 expressions were reduced in GN treated melanoma cancer cells. The Bcl-2 family is generally elaborate in controlling the intrinsic apoptosis pathway. This depends on Bcl-2 and Bax expression as the main apoptotic molecules. Alternatively, an elevated expression points out the stimulation of the apoptotic extrinsic pathway.[35] Apoptosis can trigger cell cycle arrest. Proteins that regulate the cell cycle and cell cycle checkpoint transitions are regulated by cyclin-dependent kinases, which are regulated by cyclins. A cyclin involved in the G1 to S phase cell cycle transition is cyclin-D1. Numerous tumors have been linked to cyclin-D1 hyperactivity, and it has been shown that cyclin-D1 expression inhibition may help treat cancer.[36–38] Previously, Johnson et al.[39] have shown that, in comparison to ovarian cancer cells that were not treated, GN-treated cells had reduced the inhibitory phosphorylation (Y15) of CDK1 and increased levels of cyclin-B1. They also created double-strand breaks, increased the ratio of Bcl-2 to Bax proteins, and ultimately caused the release of cytochrome-c from the mitochondria. Research has shown a connection between apoptosis and cyclin-D1 attenuation.[40] The melanoma skin cancer cells in our study had high expression levels of cyclin-D1, C-Myc and survivin. Furthermore, GN administration significantly inhibited survivin, C-Myc and cyclin-D1 mRNA expression as well as cell proliferation. The expression of cyclin-D1, C-Myc and survivin were attenuated, which may have facilitated GN-mediated antiproliferative and apoptotic actions.

The regulation of essential elements accountable for an inflammatory response in skin cancer necessitates the activation of NF-κB.[41] The purpose of the current investigation was to ascertain how GN affects NF-κB transcription. As expected from the western blot analysis, the results demonstrated that NF-κB/p65 was activated and translocated into the nucleus upon TNF-α stimulation. However, this impact could be countered by GN pretreatment. These results unequivocally demonstrated that GN specifically blocked TNF-α-induced NF-κB translocation, which is believed to be the main mechanism triggering the inflammatory response.

Strong evidence suggests that MAPK has a role in GN treatments. JNK, p38 and the other 3 members of the MAPK family are frequently associated with GN processes in humans and mice, and their signaling pathways play a major role in the initiation of inflammation and death of a variety of cell types.[42] The findings were in line with the western blot analysis and demonstrated that NF-κB/p65 was activated and translocated into the nucleus following TNF-α stimulation. Gedunin pretreatment, however, had the ability to reverse this impact. These results unequivocally demonstrate that GN specifically blocked TNF-α-induced NF-κB translocation, which is believed to be the main mechanism causing the inflammatory response. Despite sharing 60–70% of their structural similarities, PI3K, JNK and p38 have distinct biological effects because of variations in their sizes and activation loop sequences.[43] Our results showed that TNF-α-induced endothelial cell injuries were accompanied by an increase in the phosphorylation levels of all MAPK family components, which is in line with previous findings. However, the only activities that were significantly lowered by GN pre-incubation were JNK and p38. Notably, anisomycin (AM), an activator of JNK and p38, totally undid the protective effects of GN treatment. These results unequivocally demonstrate that GN inhibits the p38, MAPK, and JNK signaling pathways, which mediates its protective properties.

Limitations

Within the current hypothesis, we examined the anticancer potential of GN on A431 melanoma cells and clarified the PI3K/AKT/p38 signaling pathway only, but more studies are required apoptotic and cell adhesive pathway molecular marker levels.

Conclusions

In vitro studies have demonstrated that GN has the capacity to avert the inflammatory markers TNF-α, NF-κB, COX-2, iNOS, and IL-6, to inhibit cell proliferation, and to trigger apoptosis of A431 human melanoma cells. This is achieved via the upregulation of the expression of Bcl-2, cyclin-D1, c-Myc, and survivin via the PI3K/JNK/p38 signaling pathway, and the subsequent decrease in the expression of Bax and caspases. Our findings point out that GN can be reflected as a promising anticancer remedy for treating human melanoma skin cancer. The results data from this section succinctly summarized the key findings of the study. This study effectively communicates that GN inhibited melanoma cell growth and induced apoptosis in a concentration-dependent manner. Additionally, it highlights the modulation of various proteins and signaling pathways involved in inflammation, cell proliferation and apoptosis by GN treatment. Based on our findings, we recommended GN as a therapeutic drug for carrying out an in vivo study in the future.

Supplementary data

The supplementary materials are available at https://doi.org/10.5281/zenodo.11392666. The package includes the following files:

Supplementary Fig. 1. Results of Kruskal–Wallis test as presented in Fig. 3.

Supplementary Fig. 2. Results of Kruskal–Wallis test as presented in Fig. 4.

Supplementary Fig. 3. Results of Kruskal–Wallis test as presented in Fig. 5.

Supplementary Fig. 4. Results of Kruskal–Wallis test as presented in Fig. 6.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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