Scutellarin suppresses proliferation and increases apoptosis of nasopharyngeal carcinoma cells via MAPKs/NF-κB signaling pathway through G0/G1 cell cycle arrest – An in-vitro approach

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Abstract

Nasopharyngeal carcinoma (NPC) has a high incidence rate. It is a major public health burden in endemic areas. NPC is associated with considerable morbidity and mortality, so better treatment is needed. Scutellarin (SL) is an anticancer agent extracted from medicinal plants and it exerts anti-cancer effects through various signaling pathways. However, Scutellarin's underlying anti-proliferative and apoptotic mechanisms remain largely unknown. Thus, the current study intended to explore the molecular action of in vitro SL on CNE1 and CNE2 human NPC cells. MTT assay, DCFH-DA, Rh-123 staining, DAPI staining, flow cytometry, likewise Western blot analysis were employed to assess the proliferation and apoptosis Of NPC cells (CNE1 and CNE2) were administered SL (20 and 30 μM). Possible molecular mechanisms; intracellular ROS, MMP, cell cycle distributions, cell-cycle regulatory proteins, and MAPKs/NF-κB signaling were assessed. It was found that SL could inhibit NPC cells proliferation via enhanced intracellular ROS, MMP loss, and trigger apoptosis. SL prompted G0/G1 arrest in NPC cells by subduing cell cycle allied proteins; cyclin D1, CDK4/CDK6, pRB, and MAPKs/NF-κB signaling. Our investigation provides proof that MAPKs/NF-κB route is a possible target for treatment and may be essential in the SL-actions that are mediated against nasopharyngeal carcinoma malignancy.

1. INTRODUCTION

One kind of head and neck cancer is nasopharyngeal carcinoma (NPC) originating from the nasopharynx's epithelial cells. Genetic aberrations and virus Epstein-Barr (EBV) infections play a crucial part in the pathogenesis of NPC. A yearly incidence of 15 to 50 cases per 100,000 people is seen in several regions of South Asia, the Middle East, and North Africa, where it is far more prevalent. There are up to 21 cases per 100,000 persons in China. Additionally, it's more prevalent among Canadian and Alaskan in units. [1-3] NPC has high morbidity and mortality. Its anatomical proximity to the cervical lymph nodes increases its chance of metastasis to nearby organs. NPC is characterized by the main causes of NPC fatalities include undifferentiated carcinoma, recurrence, and distant metastases.[4] Presently, radiotherapy and chemotherapy increase the lifespan of patients with progressive NPC. Persons with the same type and stage of cancer are compared to people in the general population using the relative survival rate. The 5-year mortality rate for nasopharyngeal cancer patients usually only between 50 and 60 percent because of the prevalence of local and distant metastases, as well as the long-term side effects of radiotherapy and chemotherapy. Conventional therapies provide only short-term benefits and cause intolerable toxicity for most patients. [5]Consequently, an alternative, efficacious and safer remedial approach is urgently required. Medicinal plants may prove to be potential therapeutic drugs.

Plants-based natural agents are suitable for chemoprevention and cancer treatment owing to their anti-cancer efficacy and low toxicity.[6] Scutellarin (SL) is a glucuronide flavonoid sequestered from a range of plants such as Scutellaria altissima L, Scutellaria barbata. Don, and *Erigeron breviscapus (vant.)*Hand-Mazz.[7]

It has been proposed that SL exhibits apoptotic, anti-inflammatory, and anti-tumor biological actions.[8-9] SL stimulates ROS production triggering apoptosis in multiple myeloma cells.[10] SL has shown repressive roles in carcinomas; hepatocellular cancer, [11] breast cancer,[12] human tongue carcinoma,[13] and colorectal cancer.[14] SL causes cell cycle halt and apoptosis to impede progression of cancer cells through multiple molecular pathways.[15] Also discovered is that SL prevents cell division by arresting G2/M mobile cycle point in prostate cell cancer. [16] SL causes lung cancer cells' apoptosis by enhancing ERK1/2 activation and alleviating AKT signaling.[17] However, SL anti-cancer efficacy and its underlying regulatory mechanism in NPC remain unclear.

Apoptosis is a genetically programmed cell death. Dysregulation of apoptosis leads to tumor cells. These cells may turn malignant and are then treated with anticancer compounds. [18] A key role for reactive oxygen species (ROS) in cells homeostasis. Numerous reports have documented that elevated intracellular ROS are generally linked to apoptosis of cancer cells. [19] Kinase cascade is a well-known intracellular apoptotic signaling mechanism. It has been recognized as an apoptotic transducing pathway triggered by external stimuli, MAPKs, and their upstream kinases.[20] It has been demonstrated that ROS triggers diverse events. These comprise of Signaling routes of mitogen-activated protein kinases (MAPKs) mediated apoptosis. [21]Family relatives of MAPK; p38, controlled by extracellular signal regulated kinases (ERK) and the N-terminal c-Jun kinases (JNK) have profound action in the cellular stress. They trigger ROS,[22] and are essential for the induction of apoptotic reactions to chemotherapeutic remedies.

In this experimental study, we planned to investigate the impact of*in-vitro* SL on the NPC cells proliferation, ROS generation, apoptosis, and cell cycle regulation. We also elucidated the molecular signaling pathways involved in the anti-cancer effects of SL.

2. MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies[23].

2.1 Chemicals

Scutellarin (SL), RPMI1640, Antibiotics, Fetal Bovine Serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dichloro-dihydro-fluorescein diacetate (DCFH DA), rhodamine-123 (Rh-123), and 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), sodium dodysyl sulphate (SDS), dimethyl sulphoxide (DMSO), phosphate buffered saline (PBS), and other biochemical reagents were obtained from Germany's Merck. The primary and secondary antibodies came from Labome in the United States.

2.2 Cell lines

Human NPC CNE1 and CNE2 cell lines were procured Through ATCC, USA. Cell lines were cultured supplementing the RPMI-1640 medium with FBS (10%) and antibiotics. In order to maintain cell lines, CO_2(5%), at 37@C in a humidified atmosphere.

2.3 Cytotoxicity determination

The human NPC cells toxicity was assessed using MTT test.[24] CNE1 and CNE2 cells were sowed into 96 wells ($1x10^5$ cells/well) and cultivated at 37°C in a wet incubator having 5% CO_2. After overnight conserving, the cells were rinsed with PBS and different quantities of SL (10, 20, 30, 40, and 50 μM/ml) were added for 24 h. Subsequently, during the incubation period, MTT (10 μl) solution was included in the treated cells. Then, it was kept for 4 h to let the transformation of MTT into insoluble formazan crystals through mitochondrial dehydrogenase. It was liquefied by putting DMSO (150 μl). A 490 nm wavelength was used to test absorbance. multifunctionalreading plates (BD Biosciences, USA).

2.4 Estimation of internalized ROS

Human NPC cells (CNE1 and CNE2) were sown and left to grow for 24 hours. Then 20 and 30 μM/ml concentration of SL was added and maintained for 24 h. Therefore, the control and treated cells had stained

with DCFH-DA (10 μM) after which comes incubation at 37°C for 30 minutes. Collecting and cleaning these cells was done by PBS twice to remove the stains. The fluorescence was assessed at485±10 excitation nm and 530±12.50 emission nm. [25]

2.5 Mitochondrial membrane potential (MMP) assay

MMP was determined to identify early apoptotic stages by using Rh-123 staining. Human NPC cells (CNE1 and CNE2) were sowed in 6 wells and conserved with CO_2 (5%) at 37°C for one day in a humidified CO_2 incubator. Subsequently, CNE1 and CNE2 cells were exposed over 20 and 30 μM/ml SL for 24 h, rinsed twice with PBS cold, static using paraformaldehyde (4%), and washed. Successively, control and treated in order to stain the cells, 10 g/ml of Rh-123 in dark for half an hour at 37°C. The stained cells were washed two times with washing buffer to remove further stains. Thereafter, membrane potential was analyzed through fluorescent microscopy employed blue filter (485-530 nm).

2.6 Evaluation of apoptosis by staining with DAPI and Rh-123

To determine the apoptotic efficacy of SL on human NPC cells (CNE1 and CNE2), Rh-123 and DAPI staining were performed. CNE1 and CNE2 cells had supplemented over SL at 20 and 30 μM/ml for 1 day. NPC the rinsed cells twice using PBS, static with paraformaldehyde (4%) for 20 min, cleaned, with stains with DAPI and kept in the incubator for 20 minutes. CNE1 and CNE2 cells that have been treated were stained using Rh-123 for half an hour at 37°C. In the cells, rinsed with methanol twice to remove further stains. Finally, the cells were rinsed with PBS followed by imaging membrane potential analysis under fluorescent microscope.

2.7 PI staining for cell-cycle distribution detection

The PI staining assay was used study the spread of the cell cycle in NPC cells. [26] the two CNEs human NPC cells were given the 20 and 30 μM/ml of SL and preserved at 48 h. These treated cells had collected using trypsinization and rinsed twice using fixed and cold PBS with ice-cold 70% ethanol for at least one hour. Further, two cold phosphate-buffered saline washes were performed on the cell pellets (PBS). Then, the cells were mixed with 50 g of propidium iodide (Sigma-Aldrich, USA), 0.1 percent Triton X-100, 1 mM EDTA, and 0.5 mg RNaseA were added to 1 ml of PBS and 30 minutes at room temperature of incubation. The Flow cytometry was used to determine the cell-cycle distribution.

2.8 Western blot analysis

NPCs from humans (CNE1 and CNE2) with were added SL (20 and 30 μM/ml) and raised with 24 h. The lysates of the cells prepared by the usage of frozen lysis buffer ensuring Inhibitors of proteases and accomplished western blotting analysis. The protein content was estimated by the usage of the BCA Protein Assay Kit [27]. Briefly, then there was a protein transfer. into a the PVDF membrane. The film was prevented by the probe for 60 min and added with (CDK4, CDK6, Cyclin-D1, RB, JNK, p38 MAPK, ERK, NF-κB, GAPDH) primary antibodies in 1:1,000 dilutions and set aside overnight at 4°C. Then, secondary Antibodies were included. Protein strands were stained and protein detection visualization. The quantification of protein bands was evaluated through densitometry using Image J software.

2.9 Statistical analysis

The GraphPad Prism software, version 8.0.1, was used to statistically analyze the data. The one-way analysis of variance (ANOVA) test or the unpaired Student's t test were used to compare all groups. Any p value below 0.5 was regarded as significant.

3. RESULTS

3.1 SL effect on the cytotoxicity of CNE1 and CNE2

MTT analysis was carried out to assess the toxicology of SL on both CNE1 and CNE2 (NPC cell lines) at multiple concentrations (10, 20, 30, 40, and 50 μM/ml). SL at a concentration below 10 μM had a minimal effect on the viability of NPC cells (Figure 1A and B). However, at higher concentrations of 30, 40, and 50

μM/ml of SL effectively inhibited CNE1 and CNE2 cells proliferation. The results indicate that SL exhibits cytotoxic and anti-proliferative effects on CNE1 and CNE2 at concentration dependent manner. The IC_{50} value of SL was calculated (20, and 30 μM) concentration were selected for further study.

3.2 SL effect on the intracellular ROS accumulation in NPC cells

Various stimuli cause intracellular ROS formation by stimulating involuntary cell demise and halting the cell cycle. SL (20 and 30 μM/ml) for 24 h significantly increased intracellular ROS level in NPC cells (P <0.05). DCFH-DA-treated cells were observed under fluorescent microscope. Fluorescence intensity of ROS was found enhanced with 20, and 30 μM/ml of SL as compared to untreated control CNE1 and CNE2 cells (Figure 2A and B).

3.3 SL effect on MMP loss in NPC cells

Integrity Rh-123, a green fluorescent dye contained in active mitochondria, can be used to measure of the mitochondrial membrane potential. The control CNE1 and CNE2 cells had higher Rh-123 intensity. Treatment with SL (20 and 30 μM/ml), caused significant ($p < 0.05$) reduction in mitochondrial membrane potential in theCNE1 and CNE2 cells and shown that SL harm to the mitochondria. This indicates amplified mitochondrial depolarization. Thus, results confirm that SL modified the MMP in NCP (CNE1 and CNE2) cells (Figure 2C and D).

3.4 SL effect on the mitochondrial mediated apoptosis during NPC cells

The use of DAPI staining was analyze the effect of SL (20 and 30 μM/ml) on NPC cells morphology (Figure 3A and B). Staining with DAPI is known to create adducts over dual-DNA strands. The SL-triggered cell death was established through various signs condensed nuclei morphology, nuclear body fragmentation, and loss of membrane integrity as compared to untreated NPC cells. These findings suggest that SL exhibits anti-proliferative and apoptotic activity towards CNE1 and CNE2 cells.

The molecules and permeability cause a potential electric anomaly in MMP. The staining with Rh-123 generates mitochondrial membrane depolarization. This causes apoptosis in a timely manner. A lipophilic cationic fluorescent stain Rh-123 involves neighboring active mitochondria highlighting the crucial role of mitochondria in the apoptotic model. More there was fluorescence (488/525 nm) detected when untreated CNE1 and CNE2 regulating cells along with enhanced MMP and yellow and green Rh-123 fluorescence. No fluorescence was detected in the SL (20 and 30 μM/ml) treated NPC cells. These results indicate that SL -preserved NPC cells attenuate MMP, thereby triggering the mitochondrial apoptosis (Figure 3A-B).

3.5 SL effect on cell cycle arrest of NPC cells

Using flow cytometry, the NPCs cell cycle was discovered. After 24 hours of intervention, we discovered that the proportion of NPC cells in G0/G1 phase gradually increased with increasing dose of SL, and the proportion of NPC cells in G2 and S phase gradually decreased. However, the difference between the two groups was not statistically significant (P <.05) when compared to the control group. The proportion of CNE1 and CNE2 cells in the G2 and S phases of the SL (20 and 30 μM), however, reduced when compared to the control group (P 0.05). G0/G1 phase cells in CNE1 and CNE2 cells increased from 50.2 to 60.1%, cell cycle G2/M phase declined from 18.7% to 15.30% and S period cells varied from 23.15% to 27.2% (Figure 4A-B).

3.6 SL effect on protein expression in NPC cells

It was observed that SL (20 and 30 μM/ml) treated CNE1 and CNE2 cells down-regulated the levels of cell-cycle regulatory key proteins; cyclin-D1, CDK4, CDK6, and pRB compared to the control cells. These findings indicate that SL has a strategic governing action on NPC cell cycle (Figure 5).

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JNK, Jun, p38, and ERK, and NF-χB protein expressions were down-regulated on SL-treated NPC cells (CNE1 and CNE2) compared to untreated control cells. Exposure of SL (20 and 30 μM/ml) reduced protein

expression of cells CNE1 and CNE2. This indicates inhibition of MAPKs and NF-ϰB signaling abridged cell metastasis, invasion, and inflammation. It further indicates that SL triggered NPC cells' apoptosis uses MAPKs pathways (Figure 6).

4. DISCUSSION

An endemic NPC with high morbidity has an ethnic and geographical preponderance. [1-2] The early metastasis of NPC and the toxic effects of existing treatment strategies limit treatment effectiveness. [4] Plants provide an assorted repository of bioactive compounds that may be crucial for the safer chemotherapy for NPC. [28] This study established the anti-cancer effect of SL in *in-vitro* NPC cells (CNE1 and CNE2). Cytotoxicity analysis revealed that CNE1 and CNE2 cells viability reduced in a manner dependent on dose. These outcomes agree with prior results that have shown that SL impedes the development of human HepG2 cells [11] and MCF-7 cells.[12]

Intracellular ROS has been known for its association with physiological and pathological central intracellular signals. Raised ROS is linked to genomic uncertainty. It is a cause for tumorigenesis and its progression. [29-30] The current study finds that SL increased intracellular ROS level, decreased MMP, and triggered mitochondrial-mediated apoptosis in CNE1 and CNE2 cells. It had earlier been documented that SL treatment increases ROS generation in colon cancer cells, thereby disrupting MMP, inducing apoptosis, and attenuating HCT116 cells proliferation. SL could harm the mitochondrial functions by excess formation of ROS, the main cause of HCT116 cells death. [31] Both ROS and mitochondria exhibited a strategic role in apoptosis. [32] Still, emerging evidence specifies that anti-cancer natural compounds could prompt apoptosis in malignant cells by accumulative ROS formation. ROS further causes MMP distraction to cause cell death in tumors.[33] Thus, natural anti-cancer agents such as SL can enhance intracellular ROS generation and death of tumor cells.

In our study, we also assessed the molecular anti-cancer actions of SL using in vitro NPC cells. Our results established that SL subdued the two CNEs cells by augmenting cell apoptosis and blocking cells in the G0/G1 stage. It is well known that tumor cell apoptotic generation is a fundamental therapeutic approach for malignant treatment.[34] Modifications in the cell cycle may increase metabolism and affect viability. [35] Currently, we noticed that SL reduced cyclin-D1, CDK 4, CDK 6, and pRB expression levels. The cell cycle regulatory mechanism is a switch of cyclins, CDKs, and CDK4/6 is linked and it has cyclin-D phosphorylates protein RB. They trigger evolution during the G1 phase restraint socket.[36-37] CDK4/6/Cyclin-D acts it is in the late-middle G1 phase requisite in the phosphorylation an RB gene product (pRb), which restricts a constraint on progression at the late G1 restriction point chief controller of the G1/S switch.[38] Modifications of any constituent of this route, including hyper expression that cyclin-D associated CDKs or CDK mutations that alter binding at p16direct to phosphorylation of Rb and ensure Phase G1 through S progression. [37] These variations have been established in many human cancers, proposing that cell cycle pathway inactivation plays a significant role in cancer pathogenesis. ROS plays key role in the regulation of proliferation, cellular transformation, and apoptosis. However, excessive amount of ROS can cause cell death cancerous cells. [39]

In our research, we established that ROS is closely linked with cell cycle arrest induced by SL. Similar results have been documented in renal cancer cells (RCC) arrested at G0/G1 phase with a high dose of SL. Also, SL has been found to suppress Cyclin-D1 and CDK2 expression.[40] Both of these caused G1 to S shift and enhanced p21 resulting Phase G0/G1 halt RCC cells.[41]

Conversely, Lin *et al.* [40] have demonstrated that SL triggered Cell cycle G2/M detention by subduing cyclin B and CDK1 expression Hela cell culture. Gao and co.[22] have also stated that SL enhanced mobile cycle block at the by G2/M phase declining cyclin-B1 and CDK2 in prostate cancer cells. Thus, we assumed that SL might trigger different molecular mechanisms between assorted cancer types to exert its anti-cancer effects.

MAPK signaling is facilitated by p38, JNK, and ERK1/2, which are significant in the mechanism of cell proliferation, diversity, transformation and assembly of pro-inflammatory mediators and NF-ϰB inflection.[42] MAPK signaling cascade initiates the transcription of cellular inflammatory retort accompanying genes

arbitrated by NF-ϰB and pro-inflammatory molecules synthesis.[43] NF-ϰB pathway has been found to halt apoptosis through death receptors and stimulate malignant cells proliferation. Constitutive instigation of NF-ϰB has been noticed in diverse cancer cells. [44] These findings reveal that cell cycle arrest hedge the MAPK signaling through the suppression of cell cycle proteins. Presumably, the underlying mechanism of action of SL caused growth arrest through repression of MAP kinases and NF-ϰB. These in turn direct a decline in G1-correlated CDKs (CDK4/CDK6), cyclin-D1, and p-Rb.[43] Previous studies recommended that SL might constrain MAPKs phosphorylation by hindering the upstream kinase.[45] It has been stated that more ROS endogenous levels are associated with the down-regulation of the MAPK signaling and cause ROS-mediated mitochondrial dysfunction-related apoptosis in human cancer cells.[46] Our research confirm that SL brought down the expressiveness of numerous constituents of the MAPKs and NF-ϰB signaling and also restrained NPC cells development and cell cycle movement the G1 to the S phase. Further, we recommend *in vivo*analysis with mice model to study the bioavailability of SL and rate of tumor inhibition in mice tumor xenograft model.

5 CONCLUSIONS

To summary, the present *in-vitro* a study showed that SL (20 and 30 μM) successfully subdued viability and triggered apoptosis in CNE1 and CNE2 human NPC cells. SL unveiled its cytotoxicity by the accumulation of ROS. SL caused MMP cessation leading to DNA impairment and down-regulation of MAPKs/NF-ϰB. Thus, SL signaled reduced cell cycle proteins and thereby arrested cell cycle at the G0/G1. This research aimed to research the mechanism of anti-cancer of SL and the outcomes show that SL inhibits the growth of nasopharyngeal cancer MAPKs/NF-ϰB. Hence, we conclude that SL may serve as a promising therapeutic drug for nasopharyngeal carcinoma.

DATA ACCESSIBILITY DECLARATION

The corresponding author will provide the data sets utilized and examined during the current investigation upon reasonable request.

CONFLICT OF INTEREST

The authors say they have no competing interests.

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None

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AUTHORS' CONTRIBUTIONS

The study's inception, design, data analysis, and paper writing were all done in collaboration with each of the mentioned authors. The submitted work was read and approved by all authors.

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FIGURE LEGENDS

FIGURE 1 SL inhibits Cells NPC proliferation. A-B) Cells CNE1 and CNE2 were given SL in several dosages (10-50 μM/ml) for 24 hours. MTT test was used to analyze the cell proliferation. The outcomes were indicated as mean SD for three copies experiments. *p< 0.05 is used to indicate significance when compared to the untreated control.

FIGURE 2 A-B) SL enhances the accumulation of ROS in NPC cells. Human CNE1 and CNE2 cells were given the SL (20 and 30 μM/ml) for 24h. The DCFH-DA fluorescence accumulation was identified below an inverted fluorescence microscope. *p <0.05 is used to indicate significance when compared to the untreated control.

FIGURE 2 C-D) Assessment of SL artificial apoptosis on the two CNEs NPC cells using the Rh-123 stain under a fluorocytmic microscope. Human CNE1 and CNE2 cells were given the SL (20, and 30 μM/ml) for 24 h. An apoptotic effect of SL has Fluorescence microscopy was used to examine the MMP variation employed blue filter (485-530 nm). *p<0.05 is used to indicate significance when compared to the untreated control.

FIGURE 3 SL induces apoptosis on NPC human cells. A-B) Human NPC cells had treated with SL (20 and 30 μM/ml) for 24h. Apoptosis occurred in scrutinized by Rh-123 and DAPI staining uptake. photomicrograph of the combined DAPI and Rh-123 staining on the CNE1 and CNE2 cells preserved for 24 hours while taking pictures with a fluorescent microscope.

FIGURE 4 A-B) Influence of SL on cell cycle dissemination of NPC cells. The cell cycle delivery was assessed using flow cytometry. The significance is assessed at *p<0.05 in comparison to the control.

FIGURE 5 Influence of SL treated NPC cells on cell cycle regulatory proteins. Human CNE1 and CNE2 cells were given the SL (20 and 30 μM/ml) for 24h. The expression of the proteinCDK4, CDK6, cyclin D1, p-Rb, and GAPDH were evaluated by western blot assay.

FIGURE 6 SL attenuates MAPKs/NF-ϰB signaling pathway on NPC human cells. Human the two CNEs cells were given the SL (20 and 30 μM/ml) for 24h. The manifestation of JNK, p38MAPK, ERK, and NF-ϰB proteins was assessed from Western blotting.

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