Original article

**Swertia chirayita** suppresses the growth of non-small cell lung cancer A549 cells and concomitantly induces apoptosis via downregulation of JAK1/STAT3 pathway

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**ABSTRACT**

Lung carcinoma is the leading cause of cancer-related mortalities worldwide, and present therapeutical interventions are not successful enough to treat this disease in many cases. Recent years have witnessed a surge in exploring natural compounds for their antiproliferative efficacy to expedite the characterization of novel anticancer chemotherapeutics. **Swertia chirayita** is a valued medicinal herb and possess intrinsic pharmaceutical potential. However, elucidation of its anticancer effects at molecular levels remains unclear and needs to be investigated. We assessed the anticancer and apoptotic efficacy of **S. chirayita** ethanolic extract (**Sw**-EtOH) on non-small cell lung cancer (NSCLC) A549 cells during this exploratory study. The results elucidated that **S. chirayita** extract induced toxic effects within lung cancer cells by ~1 fold during cytotoxicity and LDH release assay at a 400 μg/ml concentration. **Sw**-EtOH extract elevates the level of ROS, resulting in the disruption of Δψm and release of cytosolic cytochrome c by 3.15 fold. Activation of caspases-3, -8 & -9 also escalated by ~1 fold, which further catalyze the augmentation of PARP cleavage (~3 folds), resulting in a four-fold increase in **Sw**-EtOH induced apoptosis. The gene expression analysis further demonstrated that **Sw**-EtOH extracts inhibited JAK1/STAT3 signaling pathway by down-regulating the levels of JAK1 and STAT3 to nearly half a fold. Treatment of **Sw**-EtOH modulates the expression level of various STAT3 associated proteins, including Bcl-XL, Bcl-2, Mcl-1, Bax, p53, Fas, Fas-L, cyclinD1, c-myc, IL-6, p21 and p27 in NSCLC cells. Thus, our study provided a strong impetus that **Sw**-EtOH holds the translational potential of being further evaluated as efficient cancer therapeutics and a preventive agent for the management of NSCLC.

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1. **Introduction**

According to the Global Cancer Observatory report, lung cancer cases constituted 18% of 18,078,957 cases during 2018 and were also responsible for 18.4% (1,761,007) fatalities, 9,555,027 casualties inclusive of different carcinomas (Taga et al., 1989). Lung cancer represents a serious cause of global health concern and is categorically delineated into small cell lung cancer and non-small cell lung cancer (SCLC/NSCLC), where the NSCLC is accountable for 80% of the cumulative lung cases (Ahmad et al., 2018; Darnell et al., 1994). Standard management of NSCLC relies on the stage at which the patient is diagnosed and commonly relies on the
surgical removal of the tumor and radiotherapy or chemotherapy (Zappa and Mousa, 2016). Like in any other type of cancer, the chemotherapeutic intervention used to treat NSCLC has longer unavoidable side effects on the patients, therefore; reinforcing the urgent need for exploring safer options, namely the natural compounds to treat this debilitating disease (Yeh et al., 2013).

Natural compounds are currently being explored for their therapeutic potential in different types of carcinomas. One such traditional herb documented in Ayurvedic literature is *Swertia chirayita* belonging to the Gentianaceae family. *S. chirayita* is commonly found in sub-tropical regions of Himayalan bed ranging from Kashmir to Bhutan within an altitude range of 1200–2100 m and is also associated with names which usually differ in languages such as in Sanskrit the plant is referred to as Anarayatika, Bhunimba, Chiratikta, Kaira whereas in Arabic and Farsi languages it is known as Qasabuzzarirah (Dutta et al., 2014; Yeh et al., 2013). Several scientific reports have earlier established that different extracts of *S. chirayita* have intrinsic antibacterial, anti-fungal, antiviral, anticancer, anti-inflammatory, anti-diabetic and antioxidant characteristics (Kumar and Van Staden, 2015). However, no exhaustive study has been reported to decipher the molecular mechanism behind *S. chirayita* ethanolic extract on NSCLC of human origin, such as A549.

The JAK/STAT (Janus kinase /signal transducer and activator of transcription) pathway plays a crucial role during proliferation, inflammation, and tumor survival. JAK/STAT pathway is involved in the downstream activation of target genes upon stimulation with cytokines (IFN-α, IFN-β, IFN-γ), IL-6, along with growth factors, namely EGF and PDGF (Jin, 2020). Earlier reports have established an aberrant expression of the JAK/STAT pathway in various solid carcinomas such as head and neck, NSCLC and SCLC (LaFave and Levine, 2012). Enhanced activation of STAT3 is frequently reported in NSCLC and is further considered to play an imperative role in inducing cancer resistance towards standard and targeted molecular therapies (Yuan et al., 2019). STAT3 increases cancer cells’ proliferation, migration, and survival and suppresses anti-cancer immune response (Yu et al., 2014).

Moreover, accumulating evidence has reported that upstream regulators of STAT3 play a vital role in lung cancer by augmenting the functional expression of IL-6 in about 40% of patients (Demaria et al., 2012). Subsequently, constitutive expression of JAK1/STAT3 is reported in cancerous cells and has been designated as a key pathway involved in the initiation and progression of cancer (Zhao et al., 2016). Consequently, JAK/STAT pathway is regarded as a promising target for exploring novel therapies. Owing to these attributes of the JAK1/STAT3 pathway, our present study was focused on elucidating the anticancer and antiproliferative effects of *S. chirayita* ethanolic extract on non-small human lung cancer A549 cells Fig. 1.

### 2. Materials

#### 2.1. Reagents and chemicals

DAPI (4, 6-diamidino-2-phenylindole), propidium iodide (P.I.), and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma (St. Louis, MO, USA). DMEM-high glucose, fetal bovine serum (FBS), 1% antibiotic–antimycotic solution, RPMI-1640, 10% FBS, 1% antibiotic–antimycotic solution, and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Thermo-Scientific, USA. All the primer sequences utilized during the study were procured from IDT, USA.

LDH release (%) = \(\frac{\text{mean absorbance treated cells}}{\text{mean absorbance untreated cells}}\) × 100

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2.2. Cell line maintenance

Human NSCLC cell line (A549) was obtained from the national repository division of the National Centre for Cell Sciences (NCCS), Pune, India. A549 cells were maintained in DMEM-high glucose enriched with 10% FBS along with 1% antibiotic-antimycotic solution. These cells were maintained under standard culture conditions at 37 °C in an atmosphere with at least 5% CO2.

2.3. Methods

2.3.1. Plant sample acquisition and preparation of *S. chirayita* ethanolic extract

*S. chirayita* whole plant was collected from the alpine area of district Pithoragarh in Uttarakhand, India, followed by its herbarium preparation. The whole plant parts of *S. chirayita* were preliminary washed with tap water, dried in the shed for nearly a week and later cut into small pieces for grinding in mortar and pestle. The obtained powdered form was stored in dry tubes. Subsequently, the powdered samples were packed in a Soxhlet apparatus and extracted with 70% ethanol at 60–65 °C for 4–5 h and subsequently sonicated (2 h). The extracted sample was filtered twice (Whatman No.1 filter), and the residual quantity of ethanol remaining within the extract was dried through evaporation under a vacuum. The resultant semi-solid ethanolic extract of *S. chirayita* (Sw-EtOH) was stored at 4 °C.

2.3.2. Evaluation of Sw-EtOH extract mediated toxicity on NSLC A549 cells

Sw-EtOH potential cytotoxic effects on A549 cells were determined using the previously-mentioned MTT assay with slight alterations (Tiwari et al., 2021). In brief, 5 × 10^3 A549 cells were seeded in each well of a 96-well plate and were treated with different Sw-EtOH concentrations (100, 200 and 400 µg/ml) for 24 h. Post incubation, media was replaced with MTT dye (5 mg/ml; 10 µl) and incubated for an additional four h. Post incubation, 100 µl of DMSO was added in each well to solubilize any formazan crystal (if formed). Eventually, the absorbance of formazan crystal was recorded at 570 nm through Bio-Rad, USA microplate reader. The viability of A549 cells treated with and without Sw-EtOH was expressed as cell viability percentage (%) by comparing the mean absorbance of cells from different groups as:

\[ \text{Cell Viability} = \frac{\text{mean absorbance treated cells}}{\text{mean absorbance untreated cells}} \times 100 \]

To further substantiate Sw-EtOH mediated toxicity, lactate dehydrogenase (LDH) assay was performed using an LDH assay kit (Sigma-Aldrich, USA) as per the protocol described earlier (Lin et al., 2017). The LDH released by treated and untreated A549 cells was monitored by recording its absorbance at 490 nm using a microplate reader (Bio-Rad, USA). The LDH release at a different dosage of Sw-EtOH was stated in percentage (%) as:

\[ \text{LDH release} = \frac{\text{mean absorbance treated cells}}{\text{mean absorbance untreated cells}} \times 100 \]
were seeded, dosed (with various Sw-EtOH concentrations) and incubated as per the protocol described in Section 2.3.2. After incubation, these treated cells were visualized to observe any changes in their appearance compared to the untreated control cells using FLoid Imaging Station, Thermo-Scientific, USA.

2.3.4. Assessment of Sw-EtOH extract-mediated oxidative stress within A549 cells
The efficacy of Sw-EtOH extract in instigating ROS within A549 cells was evaluated using DCFH-DA both qualitatively and quantitatively, as described earlier with slight modifications (Ahmad and Ansari, 2020). During qualitative assessment assay, 10^4 A549 cells were seeded in each well and treated with concentrations mentioned above of Sw-EtOH for six h. Subsequently, the respective wells stained with DCFH-DA (10 μM) for 30 min. The intensity of ROS-mediated fluorescence was assessed by differences within the intensity of green fluorescence (Channel excitation: emission ratio 482/18 nm: 532/59 nm) of the FLoid imaging station (Thermo-Scientific, USA).

2.3.5. Assessment of DNA damage in Sw-EtOH extract-treated A549 cells
Following the dosing schedule, as stated above in 96-well plate at varying Sw-EtOH concentration, 8-hydroxy-29-deoxyguanosine (8-OHdG) levels in A549 cells were investigated colorimetrically using OxiSelectTM Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc.) as per the manufacturer protocol.

2.3.6. Qualitative estimation of Sw-EtOH induced apoptosis
To qualitatively analyze Sw-EtOH induced apoptosis within A549 cells, double staining using DAPI and P.I. stain was performed. A549 cells were treated with stated concentrations of Sw-EtOH and incubated for 24 h. Fluorescent micrographs were then captured using FLoid Imaging station (Thermo-Scientific, USA) at blue (Excitation: Emission:390/40 nm:446/33 nm) and red filters (Excitation: Emission: 586/15 nm:646/68 nm).

2.3.7. Quantification of Sw-EtOH insinuated apoptosis through Annexin-V/FITC staining
To quantitatively assess the apoptosis induced in A549 cells post-Sw-EtOH treatment, Annexin V-FITC/PI apoptosis kit (B.D. Biosciences, USA) was used as described earlier (Wang et al., 2018). Briefly, 4X10^5 A549 cells/well were treated with different Sw-EtOH concentrations (100, 200 and 400 μg/ml) and incubated for 24 h. After incubation, the cells were washed (1X PBS) and treated with 1X binding buffer (as per manufacturers' protocol). Eventually, cells from each group were incubated with Annexin V-FITC, P.I. at R.T. in the dark for 15 min. The suspension was finally assessed through flow cytometry using FACS Calibur (B.D. Biosciences, USA).

2.3.8. Alterations in mitochondrial potential (∆Ψm) within A549 cells post-Sw-EtOH extract treatment
Alteration in ∆Ψm in Sw-EtOH treated A549 cells was assessed qualitatively using JC-1. The cells treated with or without Sw-EtOH extract were incubated for 30 min with JC-1 stain (200 μM) before

Fig. 1. Illustrative representation of Swertia chirayita mediated anti-proliferative effects on human non-small lung cancer A549 cells. Sw-EtOH was found to exert its anti-cancerous effects on A549 cells by impelling the extrinsic apoptotic pathway mediated by enhanced expression of caspase-8 enroalted to activation of Fas receptors by Fas-L. Sw-EtOH also indicated towards activation of intrinsic apoptotic pathway via augmentation of intracellular ROS levels concomitantly followed by increased levels of caspase dependent cleaved PARP protein. Furthermore, Sw-EtOH was also successful in modulating the JAK-STAT signaling pathway which eventually culminated in altered expression of signals required for cell survival and proliferation (as indicated by upwards and downwards arrow).
visualization and image acquisition using red fluorescence filter of Fluid imaging station (Thermo-Scientific, US; Excitation:586/15 nm: Emission:646/68 nm).

2.3.9. Cytochrome-c release assay

The cytochrome-c release assay was carried out as per the manufacturers' protocol described earlier (Chen et al., 2013). Briefly, 106 cells/well were co-cultured with different Sw-EtOH concentrations (100, 200 and 400 µg/ml) and incubated for 24 h. Further, the cells were centrifuged (1000 rpm; 10 min at 37 °C) and washed using ice-cold 1X PBS. Next, the total protein content of cells was obtained using T-PER Tissue Protein Extraction Reagent (ThermoFischer Scientific, U.S.). The presence of cytochrome-c within the total extracted protein of treated and untreated A549 cells was investigated using an ELISA kit (KHO1051; Thermofisher Scientific, U.S.) following manufacturers' protocol.

2.3.10. Evaluation of caspase activity within Sw-EtOH extract-treated A549 cells

Colorimetric assay kits specific for caspase-8, -9 and -3 were used to determine the caspase activities in Sw-EtOH extract-treated human NSCLC A549 cells following the manufacturer's protocol. Percent (%) increase in the caspases activity was determined by comparing the result with the level of untreated A549 cells as control.

2.3.11. Evaluation of cleaved Poly (ADP-ribose) polymerase (PARP) levels post-Sw-EtOH extract treatment

Cleaved PARP levels within Sw-EtOH extract-treated A549 cells were assessed using a Human PARP ELISA using manufacturer's instruction. Cell absorbance from Sw-EtOH was recorded at 450 nm on a microplate reader (Bio-Rad, USA).

2.3.12. Real-time PCR (qPCR) analysis

Total RNA content of 106 A549 cells treated with and without Sw-EtOH was extracted through HiPurATM Total RNA Miniprep Purification Kit. Subsequently, isolated RNA (2 µg) was used to prepare cDNA using a Verso cDNA synthesis kit as per the manufacturer's protocol. The primers used during the assay were as described earlier (Cui and Placzek, 2016; Karagul et al., 2020; Lee et al., 2019; Medeiros et al., 2002; Rego et al., 2011; Shamloo and Usluer, 2019; Sun et al., 2020; Tao et al., 2020; Yang et al., 2012; Zucchini et al., 2005) and mentioned in Table 1. qPCR analysis was then undertaken on ABI-7500 real-time PCR (Applied Biosystems) by applying DynAmoColorFlash SYBR Green qPCR Kit following the suppliers' protocol. The normalization of different treatment groups was done against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The comparative C.T. method was employed to analyze the data, and fold change among treatment groups were estimated using the 2−ΔΔCT method.

Table 1

| S. No. | Target gene | Forward (5'-3') | Reverse (3'-5') |
|-------|-------------|----------------|----------------|
| 1.    | GAPDH       | GAATTCCTCACTATCATCCATCTTCCAGG | GAGCCCCACGCCTTCCACATG |
| 2.    | Bcl2        | GAATTTGCGCCCTTCTTIGAG | CAAATCCAGCAGACTTCCATCT |
| 3.    | Bcl-X1      | CGAGAGTCTGGACAGAGTAG | GTCTTCGGGTTGGTGGTAGT |
| 4.    | Mcl-1       | GGACATCAAAGGGACAGACAG | GAGGTGGCTCTTGGTITAGG |
| 5.    | c-myc       | AGCGACTCTGAGGAGGAACAGG | GTCGCCACCTCTCCAGACCA |
| 6.    | Cyclin D1   | CCCCATGCTCCAACATCC | GAACAGCTCCTCCTGCCAATC |
| 7.    | p53         | CCCCCCTGGCCCTCCACCTTCCTTC | GAGCCGCGCTCAACCTGCCCTCAGT |
| 8.    | p21         | TCGAGTTCAACCCCACTGACCTT | TCAGATGACTCTGGGAAAAACCTCA |
| 9.    | p27         | CTCCTCTCCAGCAACACAGGCGG | GGGGATTTCAGCAACGGGATG |
| 10.   | Fas         | CGGCCACAAGACTAAGACTTG | CCAATGATAGTGT |
| 11.   | Fas-L       | GGGG | GTGCGCTTAT |
| 12.   | Bax         | AGTTGTTAGCTCTCCTCCTCC | TTGT CTTCACA |
| 13.   | JAK1        | GCCTTCTCGCCATGCACTTT | TCCAGTCCGACCCAGCTATG |
| 14.   | STAT3       | CTGCGCTTGCTGGTGGTAAAT | GCACGCTCCAGCCAGATC |
| 15.   | IL-6        | GAGAAAACCAGAGCTCTTGGAGA | AGGACAACCCACAGAAGAAC |

3. Results

3.1. Sw-EtOH mediated cytotoxic effects within A549 NSCLC cells

This exploratory study initially investigated the cytotoxic and antiproliferative effects instigated by Sw-EtOH on human NSCLC A549 cells. The results affirmed that Sw-EtOH extract treatment exhibited the cell growth and proliferation of A549 adenocarcinoma cells dose-dependently (p < 0.001), as shown in Fig. 2A. The dose-dependent inhibition of A549 cells after treatment with Sw-EtOH (100, 200 and 400 µg/ml) was found to be 86.07% ± 4.82, 55.88 ± 3.53% and 25.75 ± 5.58% respectively as compared to untreated control cells (Fig. 2A).

Similarly, LDH cytotoxicity assay was performed to test whether Sw-EtOH extract inhibited the proliferation of human NSCLC A549 cells. Further, A549 cells were treated with various doses (100–400 µg/ml) of Sw-EtOH extract for 24 h, LDH cytotoxicity was analyzed. As shown in Fig. 2B, Sw-EtOH extract strongly suppressed the viability of NSCLC cells in a dose-proportional manner (p < 0.001) and increased LDH release was observed to be 38.61 ± 3.54%, 57.38 ± 3.85% and 71.52 ± 4.75% at the above-stated concentrations of Sw-EtOH extract.

Moreover, alterations in the morphology of Sw-EtOH treated A549 NSCLC cells were studied by performing phase-contrast microscopy. We observed various morphological alterations such as cell shrinkage, swelling, cytoplasm and nuclear condensation, cell granulation, and withering of cell organelles indicated by pink, blue and orange arrows (Fig. 2C), suggesting dose-dependent Sw-EtOH mediated cytotoxicity in A549 NSCLC cells.

3.2. Sw-EtOH induces apoptosis in A549 NSCLC cells

Various cell-based apoptosis assays were undertaken to confirm whether the cytotoxic effect of Sw-EtOH extract on lung cancer cells was due to apoptosis. DAPI/PI double fluorescent staining was undertaken to investigate apoptotic cell death in A549 NSCLC cells post-Sw-EtOH treatment. As evident in Fig. 3A, a dose-dependent increase in the number of Sw-EtOH-treated A549 cells with bright blue and red nuclei (indicating apoptosis shown by red and green arrows), whereas the untreated A549 cells exhibited...
diffuse blue and red nuclei (highly proliferating cells). Further, the merge fluorescence of DAPI/PI-stained cells was shown by white arrows demonstrating apoptosis in Sw-EtOH-treated lung cancer cells.

Furthermore, apoptosis was also assessed by Annexin-V FITC/PI assay to quantify the rates of apoptosis induced by Sw-EtOH treatment in A549 NSCLC cells. As observed in Fig. 3B, cells in the upper right quadrant (U.R.) undergo early-stage apoptosis (Annexin V-FITC +, PI-) and cells in the lower right quadrant (L.R.) witness late-stage apoptosis (Annexin V-FITC +, PI+) were referred to as apoptotic cells. The results exhibited that Sw-EtOH treatment increases dose-reliant apoptosis rates by 11.28 ± 3.74%, 16.53 ± 4.41%, 27.76 ± 3.87%, 50.99 ± 4.14% and 58.86 ± 4.20% in A549 NSCLC cells (p < 0.001) (Fig. 3C).

3.3. Sw-EtOH instigated caspase apoptotic pathways in A549 NSCLC cells

To explore the fundamental mechanism involved in Sw-EtOH-mediated apoptosis in A549 NSCLC cells carcinoma cells, the intrinsic caspase pathway and extrinsic apoptosis pathway were initially studied, as shown in Fig. 4A, caspase-3, -8 & -9 activities were significantly increased by 64.36 ± 2.85%, 153.25 ± 2.63% and 206.38 ± 3.26%; 33.21 ± 3.12%, 119.06 ± 6.40% & 155.94 ± 4.73% and 50.31 ± 5.16%, 128 ± 4.16% & 169.86 ± 4.81%, respectively as compared to the control after treatment with varying doses of Sw-EtOH (p < 0.001) (Fig. 4A).

Moreover, Sw-EtOH dose-proportionally induced the cleavage of PARP (2.74 fold; p < 0.01), which is a marker of apoptosis, coupled with an increased level of cytochrome c release (p < 0.01) in A549 NSCLC cells, thereby indicating the role of mitochondrial apoptotic pathway (Fig. 4B & 4C).

Loss of ΔΨm trigger off cytochrome c release from mitochondria to the cytosol, which is regarded as strong activators of the apoptotic caspase cascade. We, therefore, investigated the effect of Sw-EtOH on disruption in ΔΨm in JC-1 dye-stained A549 cells. The results demonstrated that Sw-EtOH treatment decreased ΔΨm in a dose reliant manner in A549 cells (Fig. 4D), substantiating the contribution of mitochondria in Sw-EtOH-induced apoptosis.

In addition, to decipher the mechanism of Sw-EtOH-induced apoptosis, we assessed the production of reactive oxygen species (ROS) (DCFH-DA staining. The fluorescent micrographs depicted that treatment of Sw-EtOH resulted in strong DCF-fluorescence intensity in A549 cells, stipulating an enhanced intracellular ROS generation (Fig. 4E). To further investigate whether DNA damage induced in Sw-EtOH extract-treated A549 lung cancer cells contributed to apoptosis induction. 8-OHdG, a product of oxidative damage to 2’-deoxyguanosine, is an established ubiquitous marker of oxidative imbalance induced by the generation of free radicals and is regarded as a critical attribute during carcinogenesis. It is physiologically formed and increased by anticancer agents (Kaushik et al., 2015). As observed in Fig. 4F, 8-OHdG was found to be substantially increased (3.58 fold; p < 0.01) in Sw-EtOH extract-treated A549 cells.

3.4. Sw-EtOH treatment efficiently inhibited the JAK1/STAT3 signaling pathway

Various studies have documented that JAK/STAT signaling pathway components are regarded as plausible therapeutic targets in several cancers, including lung cancer. It documented that prolonged activation of JAK/STAT signaling contributes to the malignancy of tumors by promoting its proliferation and survival.
angiogenesis and immune evasion (Ahn et al., 2011). Thus, during our investigation, we focused on exploring the efficacy of Sw-EtOH extract on JAK1/STAT3 signaling pathway in A549 lung cancer cells. Our findings revealed that Sw-EtOH extract treatment inhibited JAK1/STAT3 pathway by deflating the expressional level of JAK1 mRNA and STAT3 mRNA in a dose-dependent manner. As observed, Sw-EtOH at concentrations of 100, 200 and 400 µg/ml reduced the gene expression level of JAK1 mRNA to 0.86 ± 0.11, 0.43 ± 0.16 and 0.32 ± 0.08 folds (p < 0.05) respectively, whereas expression of STAT3 mRNA was found to be 0.92 ± 0.03 (100 µg/ml), 0.52 ± 0.08 (200 µg/ml) and 0.42 ± 0.11 (400 µg/ml) folds (p < 0.01) in comparison with their respective control (Fig. 5A & B).

3.5. Sw-EtOH extract treatment modulated the expression of STAT3 coordinated survival proteins

3.5.1. Anti-apoptosis associated proteins

Previously, it has been established that constitutive expression of STAT3 promotes tumorigenesis by upregulating the gene expression level of certain anti-apoptotic proteins, primarily Bcl-xL, Bcl2 and Mcl1 in various solid carcinomas, including NSCLC (approximately 22% to 65%) (Yeh et al., 2013). Thus, we evaluated the efficacy of Sw-EtOH extract treatment on the gene expression level of the above-mentioned anti-apoptotic proteins. We found that anti-apoptotic markers’ mRNA expression levels, namely Bcl-xL, Bcl2 and Mcl1 were substantially downregulated post-treatment with Sw-EtOH. The mRNA expression level of Bcl-xL, Bcl2 and Mcl1 were decreased to 0.92 ± 0.07, 0.63 ± 0.17 & 0.49 ± 0.14 fold; 0.90 ± 0.03, 0.58 ± 0.05 & 0.42 ± 0.11 folds and 0.83 ± 0.16, 0.59 ± 0.25 & 0.34 ± 0.11 folds (p < 0.05, p < 0.01 and p < 0.01) respectively in a dose-dependent manner as shown in Fig. 5C, D, & E.

3.5.2. Proliferation related genes

Accumulating evidence has suggested that upstream regulators of STAT3 play an important role in lung cancer by increasing the expression of IL-6 in about 40% of patients (Demaria et al., 2012). After that, we investigated the efficacy of Sw-EtOH extract treatment on the gene expression of IL-6. We found that the mRNA expression levels of IL-6 were substantially downregulated after treatment with Sw-EtOH. The mRNA expression level of IL-6 decreased to 0.92 ± 0.20, 0.63 ± 0.15 & 0.32 ± 0.15 folds (p < 0.001) respectively in a dose-dependent manner as shown in Fig. 5F.

3.5.3. Cell cycle progression associated proteins

We performed qPCR analysis to investigate the efficacy of Sw-EtOH extract on cell cycle regulatory genes within Sw-EtOH treated A549 NSCLC cells. These results indicated that the mRNA expres-
Fig. 4. Effects of Sw-EtOH on (A) caspase-3, -8 and -9 activities (B) cleaved PARP levels and (C) cytosolic cytochrome c. (D) Fluorescent micrographs showing disrupted mitochondrial membrane potential in JC-1-stained A549 cells after treatment with Sw-EtOH (100–400 μg/ml). (E) Photomicrographs qualitatively elucidating augmented ROS levels within Sw-EtOH pre-treated A549 cells with different mentioned concentrations of Sw-EtOH for 6 h and subsequently stained with DCFH-DA (F) Quantification of 8-OHdG, a DNA damage marker, in A549 cells. Data communicated constitutes the mean ± SEM of individual experiments performed thrice in triplicates. The level of significance among different Sw-EtOH treated groups was determined using one-way Anova and Dunnett post-hoc in comparison with untreated control where *represents \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \). (Scale bar = 100 μm).

Fig. 5. Sw-EtOH mediated effect on mRNA expression of (A) JAK1 (B) STAT3 (C) Bcl-xL, (D) Bcl2 (E) Mcl1 and (F) IL-6 genes. A549 cells were treated with 0–400 μg/ml for 24 h. Fold change in expression of all target genes was normalized to GAPDH mRNA which served as internal control. Data communicated constitutes the mean ± SEM of individual experiments performed thrice in triplicates. The level of significance among different Sw-EtOH treated groups was determined using one-way Anova and Dunnett post-hoc in comparison with untreated control where *represents \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).
for its analgesic property (Leong et al., 2016). Thus, the above glycoside present within the extract of this plant, is accountable to decipher its anticancer efficacy against lung cancer cells.

Further, swertiamarin, a secoiridoid is soluble in an aqueous solution and well-known for its anti-inflammatory, anaesthetic, antihistamine, anticonvulsant properties (Lim et al., 2006). It is reported that STAT3 activation mediates down regulation of important proteins such as P53, Fas, Fas-L and Bax involved in inducing apoptosis (Al Zaid Siddiquee and Turkson, 2008). To examine whether Sw-EtOH treatment could up regulate the expressional levels of these proteins, qPCR analysis was undertaken. Our findings found that the mRNA expression of P53, Fas, Fas-L and Bax increased by 1.10 ± 0.14, 1.99 ± 0.12 & 2.30 ± 0.18 (p < 0.001) folds; 2.24 ± 0.18, 3.46 ± 0.21 & 4.80 ± 0.42 (p < 0.001) folds; 1.65 ± 0.25, 2.79 ± 0.16 & 3.67 ± 0.18 (p < 0.01) folds and 2.65 ± 0.20, 3.93 ± 0.63 & 4.77 ± 0.61 (p < 0.01) folds respectively post Sw-EtOH extract treatment as shown in Fig. 6C & D.

3.5.5. Apoptosis induction related proteins

It is reported that STAT3 activation mediates down regulation of important proteins such as P53, Fas, Fas-L and Bax involved in inducing apoptosis (Al Zaid Siddiquee and Turkson, 2008). To find whether Sw-EtOH treatment could up regulate the expressional levels of these proteins, qPCR analysis was undertaken. Our findings found that the mRNA expression of P53, Fas, Fas-L and Bax increased by 1.10 ± 0.14, 1.99 ± 0.12 & 2.30 ± 0.18 (p < 0.001) folds; 2.24 ± 0.18, 3.46 ± 0.21 & 4.80 ± 0.42 (p < 0.001) folds; 1.65 ± 0.25, 2.79 ± 0.16 & 3.67 ± 0.18 (p < 0.01) folds and 2.65 ± 0.20, 3.93 ± 0.63 & 4.77 ± 0.61 (p < 0.01) folds respectively post Sw-EtOH extract treatment as shown in Fig. 6C & D.

4. Discussion

Many chemical constituents with propitious pharmacological activities have been identified from the extract of S. chirayita plant; however, such compounds have not been well characterized, specifically against the human application. The plant consists of a number of pharmacologically important phytochemicals such as xanthones, ursolic acid, flavonoids, terpenoids, iridoids, amarogentin, glycosides, secoiridoids and swertiamarin (Khanal et al., 2015). It is soluble in an aqueous solution and well-known for its anti-inflammatory, anaesthetic, antihistamine, anticonvulsant properties (Lim et al., 2006). Further, swertiamarin, a secoiridoid glycoside present within the extract of this plant, is accountable for its analgesic property (Leong et al., 2016). Thus, the abovementioned nutraceutical potential of this plant compels us to decipher its anticancer efficacy against lung cancer cells.

In our present research, we provide evidence that Sw-EtOH exerts antiproliferative and anticancer effects by growth inhibition, induction of apoptosis and altered expression of JAK1 and STAT3 associated proteins in A549 NSCLC cells to establish the possible implication of extract mediated inhibition of JAK1/STAT3 signaling pathway (He et al., 2017). We primarily determined the cytotoxic effect of Sw-EtOH on A549 NSCLC cells as it is a key step in evaluating the apoptotic potential of the targeted anticancer agent. Our findings based on MTT and LDH assays suggested that treatment of A549 NSCLC cells with Sw-EtOH extract blocked their proliferation, which was directly proportional to the different concentrations of Sw-EtOH. The morphological assessment further reaffirmed morphological alterations, including cellular shrinkage, membrane blebbing and cell detachment, which altogether indicates apoptosis in Sw-EtOH-treated A549 cells.

Induction of apoptosis by drugs has emerged as an efficient therapeutic intervention against cancer cells (Wu et al., 2019). DNA fragmentation and chromatin condensation are the prime characteristics of apoptosis, and these were observed in NSCLC 8 serves to be the initiator of this pathway and is activated by the TNF family, or via mitochondrial apoptotic pathway resulting in the release of cytochrome c (Nica et al., 2008). Both pathways eventually result in the subsequent cleavage and activation of major downstream effector protease such as caspase-3. In line with these observations, our study also elucidated that Sw-EtOH extract-induced apoptosis is accompanied by caspase activation, PARP cleavage, loss of Δψm, cytochrome c release, ROS generation,
increased 8-OHdG amount, upregulated expression of apoptotic proteins (Bax), reduced expression of anti-apoptotic proteins (Bcl-2, Bcl-XL & Mcl1) and increased expression of Fas and Fas-l in NSCLC A549 cells. Thus, our data revealed that Sw-EtOH induces apoptosis via both intrinsic and extrinsic apoptosis pathways in NSCLC cells.

p53 also serves an important role in regulating tumor metastasis and invasion in NSCLC (Ahn et al., 2011). Consequently, activation or gain of function by p53 may serve to be an effective therapeutic target in several carcinomas. Our present findings demonstrated that the treatment of Sw-EtOH extracts increased the functional expression of p53 and its downstream components such as p21 and p27 in A549 cells. Therefore, implicating that Sw-EtOH may also exert its anticancer activity by inducing the activation of the p53 cascade.

Cyclin D1 is a member of the G1 cyclin family, which regulates G1-S transition during the cell cycle (Chiang et al., 2019) and is regarded as the most important checkpoint within the mammalian cell cycle (Motokura et al., 1991). Moreover, cyclin D1 mediates the phosphorylation and functional inactivation of retinoblastoma (R. B.) protein associated with cyclin-dependent kinases (CDK) such as CDK4 and CDK6. Earlier reports have shown that the overexpression of cyclinD1 is involved in the development and progression of NSCLCs. Our data demonstrated that Sw-EtOH extract effectively decreased the expression levels of cyclinD1 mRNA in A549 cells, indicating G1 phase arrest. c-Myc is yet another multifunctional oncogene involving cell growth, proliferation, tumorigenesis, and therefore is found overexpressed in different carcinomas. One of the key biological functions of c-Myc is to promote cell-cycle progression in several cancers. Interestingly, Sw-EtOH extract substantially downregulated the expression of c-Myc mRNA in A549 cells, demonstrating that inhibition of c-Myc post-Sw-EtOH treatment could be related to apoptosis induction and G1 phase arrest in NSCLCs.

JAK/STAT is also an important signaling pathway involved in tumorigenesis and found frequently dysregulated in diverse types of carcinomas (Sansone and Bromberg, 2012). Activated JAK/STAT leads to downstream activation of genes, namely cyclin D1, survivin and Bcl-2, which regulates cell proliferation and invasion and cell survival (Sakamoto et al., 2007). Our study established that Sw-EtOH instigated the suppression of JAK1 mRNA within NSCLC cells.

Previous studies have established STAT3 as an important downstream effector molecule of JAK/STAT signaling pathway. STAT3, a transcription factor, regulates the expression of various genes and consequently exerts its effects on several cellular processes such as growth, migration, apoptosis, and autophagy (Baxter et al., 2005). Constitutive phosphorylation of STAT3 correlates with cell proliferation, resistance to chemotherapy and poor prognosis during lung carcinoma (Kralovics et al., 2005). We extended our interest in studying the potency of Sw-EtOH extract on the gene expression of STAT3 in treated A549 lung cancer cells. Our qPCR analysis substantiated that Sw-EtOH extract treatment significantly downregulated the expression level of STAT3 mRNA in NSCLCs. Earlier, activation of STAT3 is known to enhance the expression level of key anti-apoptotic proteins, namely Mcl-1, Bcl-2 and Bcl-XL. These proteins are well-known for their role in enhancing the proliferation of cancerous cells (Gao et al., 2005).

Furthermore, cyclin D1 and c-Myc have been reported to enhance the proliferation required for the tumor growth (Yokogami et al., 2013). Contrasting, studies have also documented that STAT3 down-regulate the expression of several key such as p53, Fas, Fas-L and Bax involved in apoptosis induction (Niu et al., 2005). Therefore, we investigated the effect of Sw-EtOH extract on the expression level of STAT3 associated genes in NSCLCs.

Intriguingly, higher levels of IL-6 are found in the serum of advanced and metastatic cancer patients, including lung cancer patients (Scott et al., 1996). As a result, we inspected the effect of Sw-EtOH extract on the expression level of IL-6 in lung cancer cells. Our qPCR findings exhibited that Sw-EtOH extract downregulated IL-6 mRNA expression in a dose-dependent manner. In sum, it can be stated that Sw-EtOH extract suppresses JAK1/STAT3 signaling pathway and thereby induced apoptosis to obstruct the growth and viability of A549 NSCLCs. Taken together, Sw-EtOH extract holds the potential of being a plausible novel therapeutic for lung cancer; however, supporting in vivo and clinical exploration are still warranted.

5. Conclusions

In conclusion, our present findings indicated for the first time that Sw-EtOH extracts inhibited the JAK1/STAT3 signaling pathway and induce apoptosis via regulating the expression of apoptotic proteins such as Bcl-2, Bcl-XL, Mcl-1, Fas, Fas-L, p53 & Bax along with modulating the expression of cyclin D1, c-Myc, p21 & p27 which would eventually halt the progression of the cell cycle. Thus, Sw-EtOH extract could be a potential agent for targeting NSCLC cells with constitutively activated JAK1/STAT3 signaling pathway owing to its ability to modulate STAT3 associated proteins, thereby presses the need for preclinical and clinical explorations of Sw-EtOH as an anticancer agent.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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