



Triacontanoic ester of 5''-hydroxyjustisolin: Tumour suppressive role in cervical cancer via Bcl-2, BAX and caspase-3 mediated signalling

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ABSTRACT

Triacontanoic ester of 5''-hydroxyjustisolin, a lignan from *Justicia simplex* D. Don, possesses antitumor activity. However, the molecular mechanism underlying this is not yet clearly understood. The present study showed significant inhibition in cell viability on HeLa cell line and induced minor toxicity in normal cells. This compound induced mitotic arrest at G0/G1 phase followed by apoptosis in human cervical cancer cells and was accompanied by the upregulation of BAX, caspase-3 and downregulation of Bcl-2. Taken together, these data reveal that the title compound acts through multiple cellular/molecular pathways, which strongly suggest the role of pro and antiapoptotic Bcl-2 family proteins. Triacontanoic ester of 5''-hydroxyjustisolin may be a potential agent for the cervical cancer treatment.

1. Introduction

Cervical cancer is one of the common cancers in women. American Cancer Society reported that the estimated death from cervical cancer was 7.8% in 2014, and it is the most predominant cancer in sub-Saharan Africa and Southern Asia [1]. Surgery and chemotherapy are the two treatment options for cervical cancer. Cisplatin and 5-fluorouracil based therapies are still the main chemotherapeutic approach to these patients. In majority of patients, advances in these therapies showed an enhanced survival time. Limitations for an efficient chemotherapeutic protocol are resistance of the tumour cell and its inability to target the tumour [2]. Therefore, novel agents are needed to increase the treatment efficiency.

Small organic molecules derived from higher plants have been the backbone of cancer chemotherapy for the past half a century. An important evaluation of the anticancer drugs introduced to the market in North America, Western Europe, and Japan since 1940s has concluded that 47% of a total of the 155 anticancer drugs sanctioned up to 2006 were either natural products or directly derived from natural product lead compounds by semi-synthesis [3].

In addition to governing apoptosis the Bcl-2 family turn as a critical measure of the life–death decision process, which contains the

antiapoptotic (Bcl-2) and pro-apoptotic (BAX, BAK) proteins. There is a clear evidence that Bcl-2 family interfere with various mitochondrial components and probably affect mitochondrial bioenergetics. For understanding the governing mechanisms of apoptosis and also for treating ailments formed by the dysregulation of apoptosis, sufficient information should be obtained from studies on Bcl-2 family [4].

Justicia simplex is reported to have antibacterial [5], hepatoprotective [6] and antifertility activities [7]. A furofuran lignan and simplexoside were isolated from the petroleum ether extract of *J. simplex* [8]. Extensive chromatography of this extract sequestered asarinin, seasamin, sesamol, simplexolin [9] and triacontanoic ester of 5''-hydroxyjustisolin. Recently, the petroleum ether extract was found to have promising antitumor activity *in vivo*, by inhibiting angiogenesis via downregulation of VEGF and also by preventing the migration of cells [10].

Triacontanoic ester of 5''-hydroxyjustisolin (TE5-OHJ) is one of the biologically active components newly isolated from *J. simplex* which could be a promising anticancer compound. However, the mechanism (s) underlying its antitumor activities remain obscure. Molecular mechanism related to the effect of TE5-OHJ in cancer may be diverse and may involve different pathways. The current study investigated the mechanism behind the antitumor activity by estimating the proteins of

Abbreviations: DMEM, Dulbecco's modified eagle's medium; FBS, fetal bovine serum; MCF-7, human breast adenocarcinoma cell line; HeLa, human cervical carcinoma; HaCat, human keratinocyte immortalised; MTT, methyl tetrazolium assay; DMSO, dimethyl sulphoxide; TE5-OHJ, triacontanoic ester of 5''-hydroxyjustisolin; DLA, Daltons lymphoma ascites; RT-PCR, reverse transcriptase polymerize chain reaction

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apoptotic signalling pathway using cervical cancer cell lines (HeLa).

2. Materials and methods

2.1. Chemicals and reagents

FBS (Foetal bovine serum) and TRIzol® Reagent were acquired from Invitrogen, Bangalore, India. Sigma- Aldrich (USA) provided DMEM, Tris- EDTA Buffer and DMSO. RT-PCR kit (Verso One step, MCH15596018) was procured from Thermo-scientific, USA. Tissue culture flasks and other accessories were purchased from Tarsons, Bangalore, India.

2.2. Cell lines and maintenance

Human Keratinocyte (HaCat, Immortalised), Breast adenocarcinoma (MCF-7, ER⁺ve, tumorigenic and non-invasive) and Cervical carcinoma (HeLa, tumorigenic and invasive) cells were primarily purchased from National Centre for Cell Sciences, Pune. The cell lines were cultured and preserved as per the standard procedure.

2.3. Test materials

Triacontanoic ester of 5'-hydroxyjustisolin (TE5-OHJ) was isolated from the petroleum ether (60–80 °C) extract of *J. simplex* by silica gel column chromatography using gradient elution technique. Fractions were analysed using TLC followed by UV visualization and exposure to iodine vapour. Similar fractions were combined and recrystallized. Resultants were subjected to chemical as well as spectral analysis (Fig. 1) such as UV, IR, ¹HNMR, ¹³CNMR and LCMS [10,11].

2.4. Antiproliferative assay

MTT assay is used to determine the antiproliferative activity of the isolated compound using HeLa, MCF-7 and HaCat cells as previously described [12]. Experiments were done in triplicate and the antiproliferative potential of the compound was noted as IC₅₀ [13].

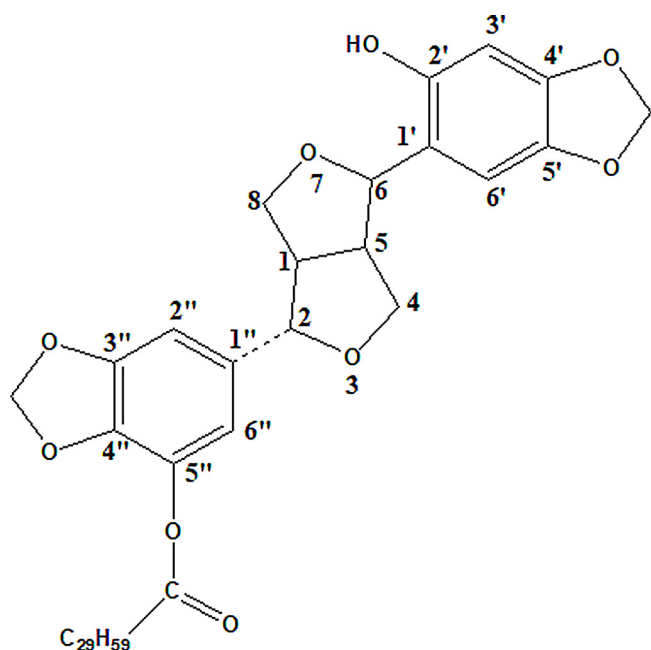


Fig. 1. Structure of triacontanoic ester of 5'-hydroxyjustisolin 2.8.

2.5. Analysis of apoptosis by acridine orange (AO) and ethidium bromide (EB) double staining

For morphological finding of apoptotic and necrotic cells, DNA-binding dyes AO and EB (Sigma, USA) were used [14]. The cells were rinsed initially with cold phosphate-buffered saline (PBS) and consequently stained with a mixture of AO (100 µg/mL) and EB (100 µg/mL), kept at room temperature for 10 min, washed and observed through a fluorescence microscope in blue filters (Olympus CKX41 with Optika Pro 5 cameras, Japan) [15].

2.6. Analysis of cell apoptosis by flow cytometry

Determination of apoptosis was done by flow cytometry (Muse™ Annexin V and dead cell reagent; MCH100105), according to the manufacturers protocol. HeLa cells (1×10^6) were seeded and incubated for 24 h. Treatments were given with and without test for 48 h. Cells were collected, washed and stained with working solution (500 µL binding buffer with 5 µL PI) for 15 min at RT in the dark and analysed using a Muse cell analyser (Muse™, Millipore, USA). The flow cytometry data obtained were gated. The percentages of cells were calculated from the mean fluorescence intensity in each of the four quadrants [16].

2.7. Cell cycle analysis by flow cytometry

Cells were treated with TE5-OHJ, washed and fixed with 66% ethanol at 4 °C overnight. On the next day, the cells were washed twice in PBS (PBS containing 25 mg/mL PI, 0.03% NP-40, 40 mg/mL RNAase A, at 37 °C in the dark for 30 min, MCH100106) and labelled with PI. The study was done according to the previously reported method [17]. The cell cycle distribution was analysed using a flow cytometer (Muse™, Millipore, USA).

2.8. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from fresh tissue samples was extracted by TRIzol Reagent® (MCH 15596018, Invitrogen co, CA) according to manufacturer's instruction [18]. 2 mg of total RNA was taken in a 20 mL reaction system using reverse transcriptase. The resulting cDNA was subjected to reverse transcriptase PCR analysis. Gene specific amplification was carried out using Verso One step RT-PCR kit (Life Technologies, USA) with a 15 mL PCR system containing 1 mL of cDNA, 200 nM of each pair of oligonucleotide primers, and 7.5 mL of Master Mix (Invitrogen, USA). Standard cycling conditions were adopted for the RT-PCR analysis. The primers (forward and backward) used were depicted below.

Bcl-2- 5'-GACGCGAAGTGCTATTGGT-3', 5'-TCAGGCTGGAAG GAG AAGAT-3'

BAX- 5'-TCCCCCGAGAGGTCTTTT-3', 5'-CGGCCCCAGTTGAAG TTG-3'

GAPDH-5'-GAAGGTGAAGGTCGGAGTC-3', 5'-GAAGATGGTGATGG GATTTC-3'

2.9. Caspase-3 measurement

Caspase estimation was done by the standard procedure [19] using the blocking buffer (200 µL, 0.2% gelatin in 0.05% tween 20 in freshly prepared PBS), 1° antibody (Caspase-3 Anti human caspase-3 in goat, Santa Cruz, USA), 2° antibody (HRP conjugated IgG 100 Santa Cruz, USA) and chromogen (O-Dianisidine – 1 mg/100 mL methanol + 21 mL citrate buffer pH 5 + 60 mL H₂O₂ fresh). Optical density was measured using an ELISA microplate reader (ERBA, Germany).

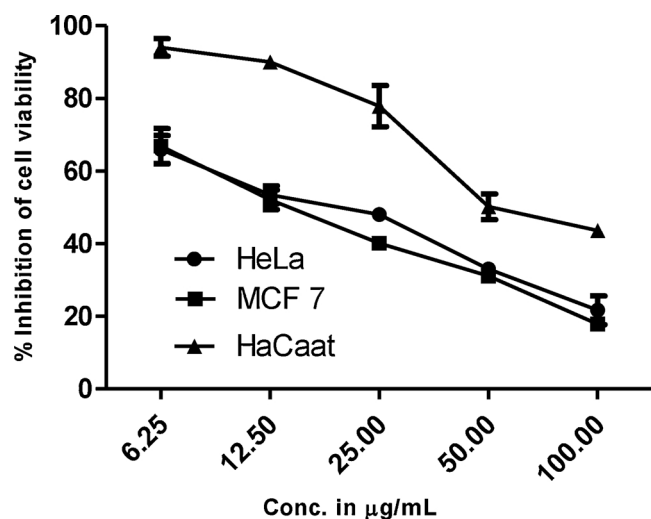


Fig. 2. Effect of TE5-OHJ on cell viability of HeLa, MCF-7 and HaCat cell line.

2.10. Statistical analysis

The results were expressed as mean \pm SD. One-way analysis of variance was used for carrying out statistical analysis, followed by Tukey's test. *t*-test was performed using Graph pad Prism software (San Diego, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. TE5-OHJ inhibits proliferation and induces apoptosis in HeLa and MCF-7 cell line

The current study investigated antiproliferative activity of TE5-OHJ in human breast cancer (MCF-7) cervical cancer cells (HeLa) and keratinocyte (HaCat) cell line. The methodical evaluation of cytotoxicity/cell proliferation in human cancer cell lines revealed that the compound had dose-dependent growth inhibitory activity (Fig. 2). Effective anticancer agents need to kill selectively the cancer cells with minimal toxicity on the normal cells. Results showed that the cytotoxicity on HaCat cells was less than its toxicity to HeLa cells, as the IC_{50} of TE5-OHJ for HeLa cells was up to 11.852 μ M. Moreover, the compound induced less cytotoxic effects on normal HaCat cells (IC_{50} is 40.41 μ M) proving its cell specificity. Hence in the further studies HeLa cells were selected. Doxorubicin had IC_{50} values of 2.523 ± 0.823 , 1.972 ± 0.547 , and 0.42 ± 0.01 μ M, respectively for HeLa, MCF-7 and HaCat cell line (Fig. 3).

The purpose of AO and EB assay was to determine whether apoptosis or necrosis was responsible for the antiproliferative activity of the compound. Doxorubicin hydrochloride and DMSO were used in the study design as positive control and vehicle control. AO can permeate cells and stain the nuclei green, whereas EB stains the nuclei orange-red, which can be taken up only by cells that have lost cytoplasmic membrane integrity, and hence points to dead cells. Rupture of cell membrane which releases the cell content is an evidence of necrosis. On analysing the dead cells population in the different treated groups, it was clear that TE5-OHJ caused cell death through apoptosis and not by necrosis.

3.2. TE5-OHJ induces apoptosis and mitotic arrest on HeLa cells

Apoptosis was observed by flow cytometry using Annexin V FITC/PI staining method, after 12 h of treatment with TE5-OHJ. It resulted in an increase in the apoptotic cell populations (Fig. 4). The percentage of live, dead, early apoptotic and late apoptotic cells were calculated. As shown in the Figure FACS suggested that treatment increased the lower-

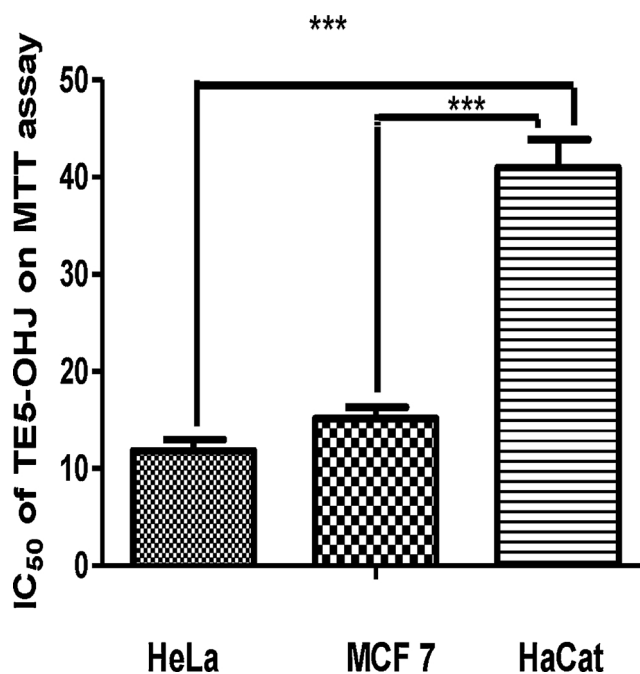


Fig. 3. IC_{50} of TE5-OHJ on HeLa, MCF-7 and HaCat cells by MTT assay.

right quadratic cell population in FITC channel compared with the control group, i.e., 27.2% and 10.2% for test and control respectively, which was visible from the percentage increase in red fluorescence intensity in the early and late apoptotic stages of the treated cells. Apoptotic effect was not significantly altered in MCF-7 cell line in presence of test groups (Fig. 5).

HeLa cells were treated with TE5-OHJ to assess the cell cycle distribution by flow cytometry. The proportion of cells at the G₀/G₁ increased slightly from 60% to 62.9%, with a relative decrease in S-phase and G₂/M phases, indicating cell cycle arrest at G₀/G₁ (Fig. 6). Comparative decline in S-phase and G₂/M-phase indicated the inhibition of DNA replication.

3.3. TE5-OHJ inhibits apoptotic marker Bcl-2 and induces BAX

Bcl-2 expression studies were carried out in MCF-7 and HeLa cells. The results on HeLa cells revealed 40% downregulation of Bcl-2 on treatment with TE5-OHJ when compared with the untreated group (Fig. 6 A). The inhibition of Bcl-2, the major antiapoptotic proteins, could be the chief mechanism behind apoptosis. MCF-7 cells did not exhibit statistically significant downregulation.

The results of RT-PCR also revealed the TE5-OHJ induced upregulation of BAX (Fig. 6B), which favours apoptosis. Cells were normalised with the housekeeping gene, GAPDH (Fig. 6C).

3.4. TE5-OHJ induces caspase-3 dependent apoptosis on cell lines

The IHC analysis exhibited an increase in caspase levels on treatment with TE5-OHJ in both HeLa and MCF-7 cell lines when compared with their respective controls (Fig. 7). The overexpression of caspase revealed the role of TE5-OHJ in apoptotic death. The outcome of the experiment exhibited a sharp increase of caspase-3 levels in HeLa cells than MCF-7 cell line. This is responsible for the cancer cell demise by generating DNA damage and fragmentation (Fig. 8).

4. Discussion

Cervical cancer is one of the common cancers worldwide. 5 FU and Cisplatin based treatments continued to be the main treatment choices

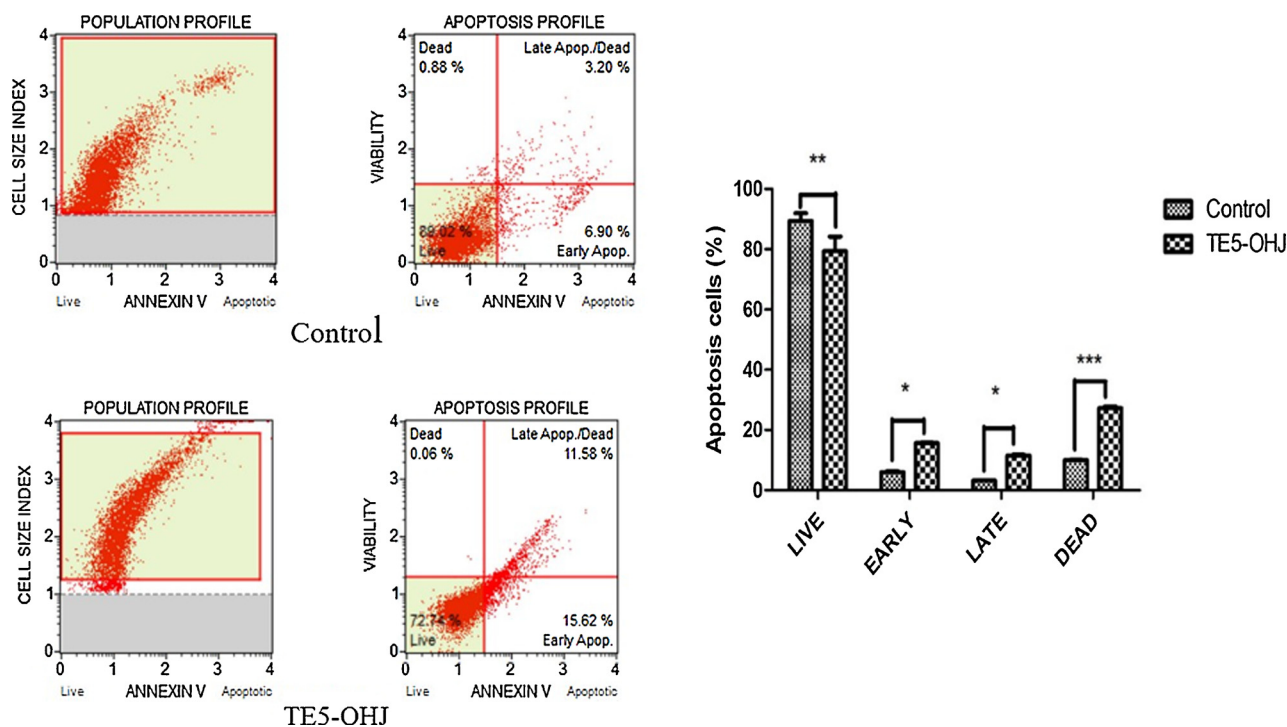


Fig. 4. FACS analysis of Annexin V/Propidium iodide apoptosis induced by control and TE5-OHJ.

for patients. Molecular targeted drugs like bevacizumab and cetuximab have been used for treatment nowadays [20]. However, the results suggest that those drugs are not very satisfactory and novel agents are desirable for effective treatment. Conventional medicines such as gambroic acid have shown to have exceptionally good anticancer activity with limited side effects [21].

The present study investigated the antiproliferative effect of TE5-OHJ on both HeLa and MCF-7 cell lines. The desirable trait for a potential anticancer agent is that the compound mediated suppression of cell viability and/or growth should be cancer cell selective. Results revealed that TE5-OHJ possessed cytotoxic and cell growth inhibitory effects on human normal epithelial cells (HaCat), an immortalized keratinocyte cell type. However, cell viability was not significantly

affected by treatment at concentrations that were cytotoxic to MCF-7 and HeLa cells. Moreover, an increased IC₅₀ value for test compound against HaCat cells as compared to MCF-7 and HeLa cells indicate less cytotoxic effects. These data suggest that the compound imparted cancer cell-specific cytotoxic effects on human breast and cervical cancer cells.

Removing the unwanted cells by apoptosis maintains homeostasis of healthy tissues through a normal physiological process of cell death [22]. In the elimination of cancer cells, induction of apoptosis plays a very active role. However, cell proliferation increases when cancer cells escape the apoptosis process, which eventually leads to tumour formation. Hence controlling the cancer without causing damage to the normal cells is the main barrier of the current therapy [23]. As a result,

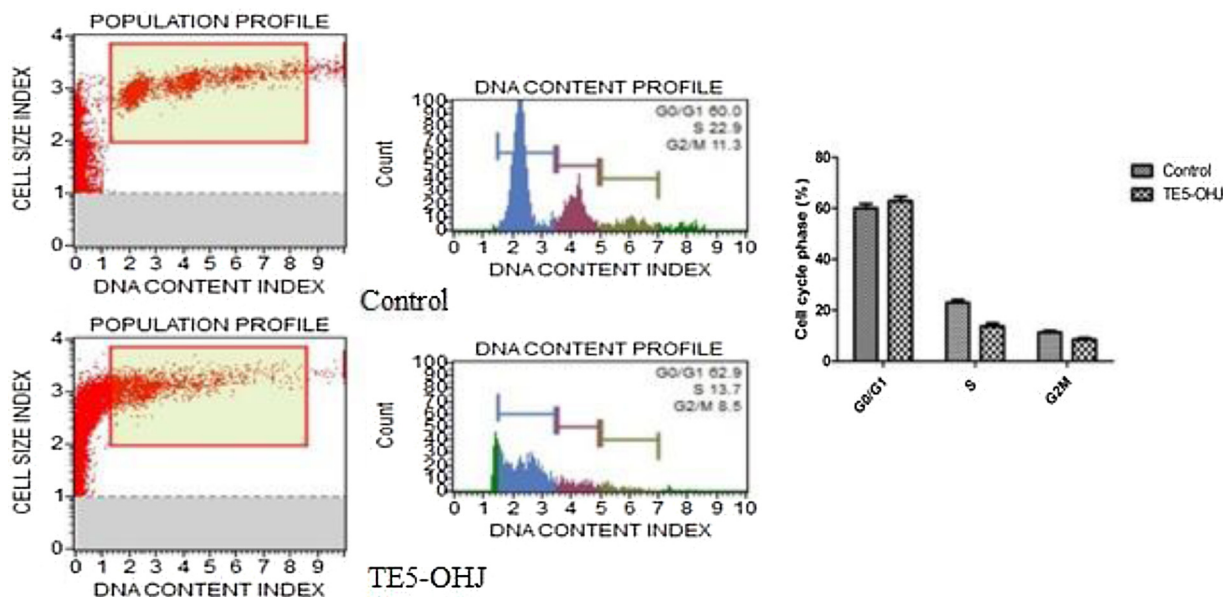


Fig. 5. Cell cycle progression pattern of control and TE5-OHJ on HeLa cells using Flow cytometry.

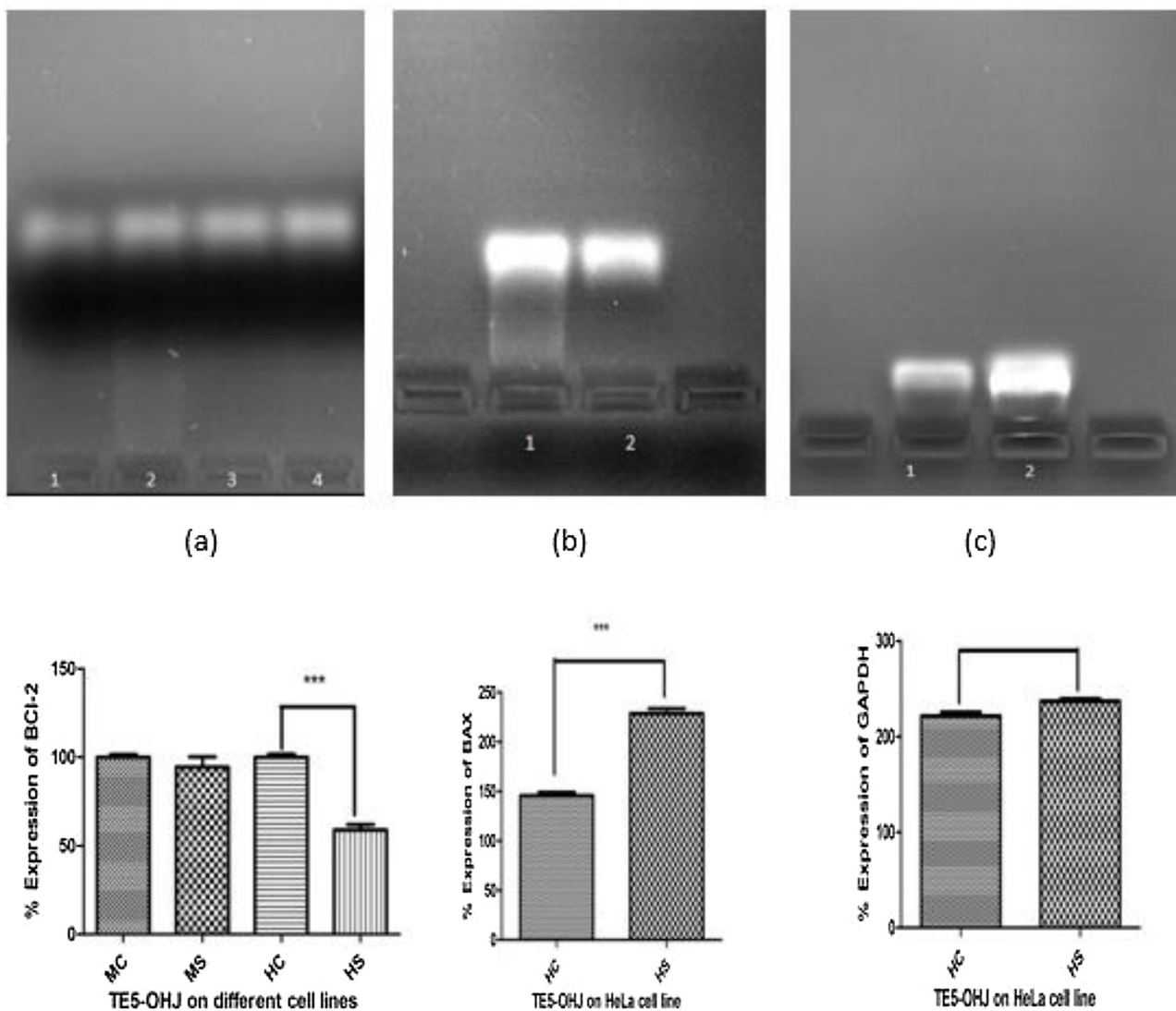


Fig. 6. Electrophoretic separation of RT-PCR samples and its relative expression. a. Bcl-2 expression Lane1: TE5-OHJ treated HeLa cells; Lane 2: Control HeLa cells; Lane 3:TE5-OHJ treated MCF-7 cells; Lane 4: control MCF-7 cells. b. BAX expression Line 1: Control HeLa cells; Line 2: TE5-OHJ treated HeLa cells. c. GAPDH expression, Line 1: TE5-OHJ treated HeLa cells; Line 2: Control HeLa cells.

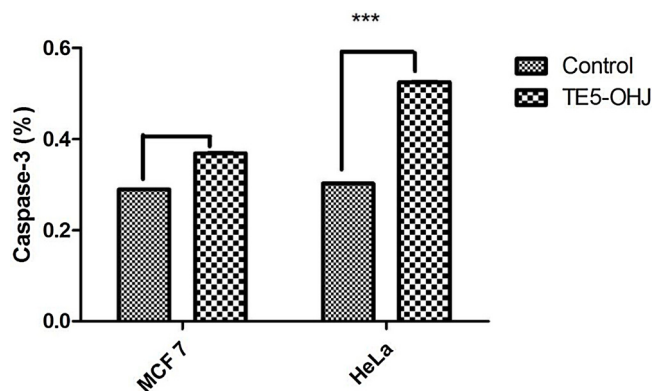


Fig. 7. IHC analysis of caspase-3 levels in HeLa and MCF-7 cell line.

selective induction of apoptosis is considered as one of the most important cancer prevention strategy.

A calcium-dependent phospholipid-binding protein show great affinity for phosphatidyl serine (PS). PS is a membrane element usually located to the internal face of cell membrane, initially in the apoptotic

pathway [24]. Annexin V can freely bind to the PS molecule, which are transported to the exterior face of the cell membrane. The Muse™ Annexin V & dead cell assay uses Annexin V to identify PS on the exterior membrane of apoptotic cells. Annexin V can also act as a dead cell marker; 7-AAD, which excludes the dead cells.

Many of the anticancer compounds exert their effect on cell cycle [25]. Effect of TE5-OHJ on cell cycle was studied by flow cytometry using HeLa cells. Treatment of cervical cancer cells with the TE5-OHJ significantly inhibited the cell proliferation and induced mitotic arrest in G0/G1 phase in HeLa cells. Elevated Sub-G0 phase indicate resting phase and diminished S phase indicate inhibition of DNA replication [26]. These results revealed the cell cycle-specific activities of TE5-OHJ in cervical cancer cells.

Bcl-2 was recognized as an oncogene which is originated from human follicular lymphoma of B cell. The antiapoptotic activity of Bcl-2 was invented by Vaux *et al.* in 1988. The intrinsic regulation of apoptotic cell decease were explored by Bcl-2 family, which is the best characterized protein family consists of antiapoptotic and proapoptotic members [27]. The antiapoptotic (Bcl-2, Bcl-XL) and proapoptotic proteins (BAX, Bid and Caspases). The BAX/Bcl-2 ratio is considered a measure of cell susceptibility to apoptosis [28] as well as proapoptotic index [29]. This ratio was found to be significantly decreased in the

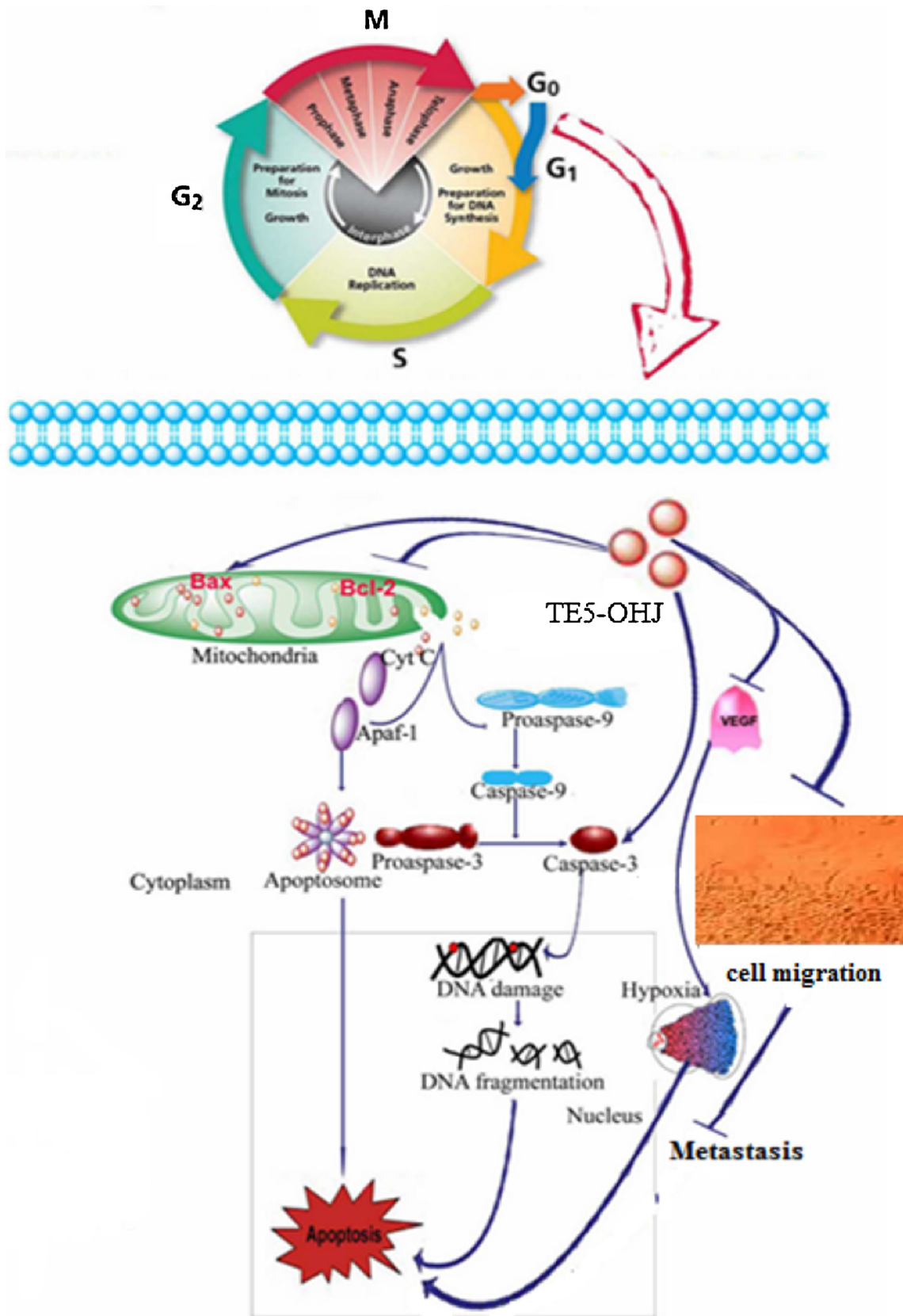


Fig. 8. Proposed mechanism of triacontanoic ester of 5-hydroxy justisolin.

current study as reported earlier and may be due to the increased apoptosis via increasing proapoptotic Bax and decreasing antiapoptotic Bcl-2 expression in an animal model [30,31].

The expression of these genes are firmly controlled by complex mechanism [4]. Bcl-2, BAX and caspase-3 have a main role in the intrinsic pathway. The resistance to chemotherapy and radiotherapy are due to the overexpression of Bcl-2 [32] by inhibiting the liberation of cytochrome c, caspase and mitochondria derived activators [24]. TE5-OHJ was capable of causing the downregulation of Bcl-2, thereby preventing the resistance to chemotherapy and radiotherapy.

The release of cytochrome c was increased by the proapoptotic protein BAX following a death signal, which can interact with Apaf-1, leading to the activation of caspase-9. Caspase-3 shows a vital role in apoptosis [31], and is a member of Aspartate specific cysteinyl proteases [4]. The caspase-3 is triggered by caspase-9 and produces DNA damage or prevents DNA repair. Subsequently, activating the caspase cascade produces apoptosis [33,34].

Treatment of HeLa cells with TE5-OHJ at its IC₅₀ concentration brought about induction of apoptosis, mainly due to overexpression of caspase-3 and BAX. Simultaneously, it blocked the expression of Bcl-2. Concurring to previous reports the release of mitochondrial cytochrome c, and the consequential triggering of caspase-9 and caspase-3 were important in the signal transduction pathway induced apoptotic cell death (Fig. 8). The mitochondrial membrane damage and reactive oxygen species production are involved in the caspase-3 dependent cell death. These converge to common events, involving, blebbing, cell shrinkage, chromatin condensation and DNA fragmentation which lead to apoptosis [35].

5. Conclusion

The DNA content and cell cycle distribution analysis by flow cytometry revealed that the HeLa cells treated with the compound produced cell cycle arrest at G0/G1 phase. Bcl-2 family are the key regulators of apoptosis and are overexpressed in many types of cancer. They are located in the endoplasmic reticulum and mitochondrial membrane. Overexpression of Bcl-2 causes antiapoptotic effect by suppressing the release of cytochrome c, Apaf-1 and caspase-3, which are necessary for the cell demise. This is one of the major reasons for the resistance to chemotherapy and radiation therapy. TE5-OHJ caused the downregulation of Bcl-2 expression and upregulation of proapoptotic protein BAX, which promotes this pathway results in apoptosis. ELISA test also revealed the direct stimulation of caspase-3. In addition to this, the title compound revealed as an antiangiogenic agent and thereby it could prevent cancer metastasis. Hence Triacontanoic ester of 5'-hydroxy justisolin may be a potential agent for the cervical cancer treatment.

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Author's contribution

The study was designed, Cell culture maintenance, MTT assay, AO and EB assay, Annexin V FITC/ Propidium iodide (PI) staining assay, RT-PCR and Caspase-3 were performed by Litty Joseph (littyjimmyalappatt@gmail.com). Data analysis, and interpretation of results were done by Litty Joseph and KK Srinivasan (pansrini@yahoo.co.in). Both authors were involved in preparation of the manuscript.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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References

- [1] S. Alam, R.S. Yadav, A. Pal, S.K. Purshottam, B.P. Chaudhari, M. Das, K.M. Ansari, Dietary administration of Nexrutine inhibits rat liver tumorigenesis and induces apoptotic cell death in human hepatocellular carcinoma cells, *Toxicol. Rep.* 2 (2015) 1–11.
- [2] X.-J. Liang, C. Chen, Y. Zhao, P.C. Wang, Circumventing tumor resistance to chemotherapy by nanotechnology, *Methods Mol. Biol.* 596 (2010) 467–488, https://doi.org/10.1007/978-1-60761-416-6_21.
- [3] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs over the last 25 years, *J. Nat. Prod.* 70 (2007) 461–477 doi:Chronic ischaemic mitral regurgitation. Current treatment results and new mechanism-based surgical approaches*.
- [4] Y. Tsujimoto, Role of Bcl-2 family proteins in apoptosis: Apoptosomes or mitochondria? *Genes to Cells.* 3 (1998) 697–707, <https://doi.org/10.1046/j.1365-2443.1998.00223.x>.
- [5] M.G. Eswari, R.L. Rathi, J. Harini, R. Aruna, V. Balakrishnan, Phytochemical screening of *Justicia simplex* D. Don a valuable medicinal plant extract against dental pathogens, *Int. Lett. Nat. Sci.* 21 (2014) 10–21, <https://doi.org/10.18052/www.scipress.com/ILNS.21.10>.
- [6] S. Jasemine, R.S. Srivastava, S.K. Singh, Hepatoprotective effect of crude extract and isolated lignans of *Justicia simplex* against CCl₄-Induced hepatotoxicity, *Pharm. Biol.* 45 (2007) 274–277, <https://doi.org/10.1080/13880200701214698>.
- [7] Y. Govindaraj, V. Melanaphuru, S. Gupta, V. Agrahari, R.K. Nema, Antifertility activity of the ethanolic extract of *Cassia occidentalis*, *Derris brevipes* variety braves and *Justicia simplex*, *World J. Chem. Educ.* 4 (2009) 118–122.
- [8] S. Banerjee, D.K. Jaiswal, Shibnath Ghosal, New furofuran lignans from *justicia simplex*, *Phytochemistry* 19 (1980) 332–334.
- [9] S. Ghosal, A.K. Srivastava, R.S. Srivastava, S. Chattopadhyay, M. Maitra, Justicisaponin-I, a new triterpenoid saponin from *Justicia simplex*, *Planta Med.* 42 (1981) 279–283, <https://doi.org/10.1055/s-2007-971641>.
- [10] L. Joseph, J.M. Aranjani, S.R. Pai, K.K. Srinivasan, Promising anticancer activities of *J. simplex* in cellular and animal models, *J. Ethnopharmacol.* 199 (2017) 231–239, <https://doi.org/10.1016/j.jep.2017.01.046>.
- [11] J.B. Harborne, *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, Third, Springer, 1998 (Accessed November 20, 2015), <http://trove.nla.gov.au/work/9080354?q&versionId=45450216>.
- [12] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J. Immunol. Methods* 65 (1983) 55–63 (Accessed July 17, 2014), <http://www.ncbi.nlm.nih.gov/pubmed/6606682>.
- [13] K. Shien, S. Toyooka, H. Yamamoto, J. Soh, M. Jida, K.L. Thu, S. Hashida, Y. Maki, E. Ichihara, H. Asano, K. Tsukuda, N. Takigawa, K. Kiura, A.F. Gazdar, W.L. Lam, S. Miyoshi, Acquired resistance to EGFR inhibitors is associated with a manifestation of stem cell-like properties in cancer cells, *Cancer Res.* 73 (2013) 3051–3061, <https://doi.org/10.1158/0008-5472.CAN-12-4136>.
- [14] J.H. Zhang, J. Yu, W.X. Li, C.P. Cheng, Evaluation of Mn²⁺ stimulated and Zn²⁺ inhibited apoptosis in rat corpus luteal cells by flow cytometry and fluorochromes staining, *Chin. J. Physiol.* 41 (1998) 121–126 (Accessed March 12, 2016), <http://www.ncbi.nlm.nih.gov/pubmed/9801843>.
- [15] D. Ribble, N.B. Goldstein, D.A. Norris, Y.G. Shellman, A simple technique for quantifying apoptosis in 96-well plates, *BMC Biotechnol.* 5 (2005) 12, <https://doi.org/10.1186/1472-6750-5-12>.
- [16] I. Vermes, C. Haanen, H. Steffens-Nakken, C. Reutelingsperger, A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V, *J. Immunol. Methods* 184 (1995) 39–51, [https://doi.org/10.1016/0022-1759\(95\)00072-1](https://doi.org/10.1016/0022-1759(95)00072-1).
- [17] X.F. Zhang, Q.Z. Pan, K. Pan, D.S. Weng, Q.J. Wang, J.J. Zhao, J. He, Q. Liu, D.D. Wang, S.S. Jiang, H.X. Zheng, L. Lv, C.L. Chen, H.X. Zhang, J.C. Xia, Expression and prognostic role of ubiquitination factor E4B in primary hepatocellular carcinoma, *Mol. Carcinog.* 55 (2016) 64–76, <https://doi.org/10.1002/mc.22259>.
- [18] Y. Cheng, A.K.L. Cheung, J.M.Y. Ko, Y.P. Phoon, P.M. Chiu, P.H.Y. Lo, M.L. Waterman, M. Lung, Physiological β -catenin signaling controls self-renewal networks and generation of stem-like cells from nasopharyngeal carcinoma, *BMC Cell Biol.* 14 (2013) 44, <https://doi.org/10.1186/1471-2121-14-44>.
- [19] K.P. Laladhas, V.T. Cheriyan, V.T. Puliappadamba, S.V. Bava, R.G. Unnithan, P.L. Vijayammal, R.J. Anto, A novel protein fraction from *Sesbania grandiflora* shows potential anticancer and chemopreventive efficacy, in vitro and in vivo, *J. Cell. Mol. Med.* 14 (2010) 636–646, <https://doi.org/10.1111/j.1582-4934.2008.00648.x>.
- [20] *Cervical Cancer Treatment Protocols: Treatment Protocols*, (2014) (Accessed March 25, 2017), <http://emedicine.medscape.com/article/2005259-overview>.
- [21] X. Li, S. Liu, H. Huang, N. Liu, C. Zhao, S. Liao, C. Yang, Y. Liu, C. Zhao, S. Li, X. Lu, C. Liu, L. Guan, K. Zhao, X. Shi, W. Song, P. Zhou, X. Dong, H. Guo, G. Wen, C. Zhang, L. Jiang, N. Ma, B. Li, S. Wang, H. Tan, X. Wang, Q.P. Dou, J. Liu, Gambogic acid is a tissue-specific proteasome inhibitor in vitro and in vivo, *Cell Rep.* 3 (2013) 211–222, <https://doi.org/10.1016/j.celrep.2012.11.023>.
- [22] C.A. Schmitt, Senescence, apoptosis and therapy—cutting the lifelines of cancer, *Nat. Rev. Cancer* 3 (2003) 286–295, <https://doi.org/10.1038/nrc1044>.

- [23] M.T.G. Liam Portt, Grant Norman, Caitlin Clapp, Matthew Greenwood, Anti-apoptosis and cell survival: a review, *Mol. Cell Res.* (2011) 238 (Accessed March 12, 2016), http://ac.els-cdn.com/S0167488910002764/1-s2.0-S0167488910002764-main.pdf?_tid=873ace72-e83c-11e5-9e53-00000aacb35e&acdnat=1457778417_8f590278779ff6828ddfb19d06f2c256.
- [24] W.G. Telford, L.E. King, P.J. Fraker, Comparative evaluation of several DNA binding dyes in the detection of apoptosis-associated chromatin degradation by flow cytometry, *Cytometry* 13 (1992) 137–143, <https://doi.org/10.1002/cyto.990130205>.
- [25] H. Gali-Muhtasib, N. Bakkar, Modulating cell cycle: current applications and prospects for future drug development, *Curr. Cancer Drug Targets* 2 (2002) 309–336 (Accessed November 20, 2015), <http://www.ncbi.nlm.nih.gov/pubmed/12470209>.
- [26] M. Archana, T.L. Yogesh, K.L. Kumaraswamy, Various methods available for detection of apoptotic cells—a review, *Indian J. Cancer* 50 (2013) 274–283, <https://doi.org/10.4103/0019-509X.118720>.
- [27] F. Llambi, D.R. Green, Apoptosis and oncogenesis: give and take in the BCL-2 family, *Curr. Opin. Genet. Dev.* 21 (2011) 12–20, <https://doi.org/10.1016/j.gde.2010.12.001>.
- [28] L. Ouyang, Z. Shi, S. Zhao, F.T. Wang, T.T. Zhou, B. Liu, J.K. Bao, Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis, *Cell Prolif.* 45 (2012) 487–498, <https://doi.org/10.1111/j.1365-2184.2012.00845.x>.
- [29] T. Song, J.Y. Liu, J.Y. Yang, NKAP plays an oncogenic function partly through signalling pathway in hepatocellular carcinoma, *Neoplasia* 66 (5) (2019) 792–800.
- [30] G. Bagheri, R. Rezaee, K. Tsarouhas, A. Docea, Magnesium sulfate ameliorates carbon monoxide-induced cerebral injury in male rats, *Mol. Med. Report.* 19 (2) (2019) 1032–1039.
- [31] M.A. Rezaee, A.H. Mohammadpour, M. Imenshahidi, M. Mahmoudi, M. Sankian, A. Tsakalof, A.M. Tsatsakis, S.A. Moallem, Protective effect of erythropoietin on myocardial apoptosis in rats exposed to carbon monoxide, *Life Sci.* 1 (148) (2016) 118–124.
- [32] A.G. Porter, R.U. Janoke, Emerging role of caspase 3 in Apoptosis, *Cell Death Differ.* (1999) 99–104 (Accessed March 13, 2016), https://www.researchgate.net/profile/Reiner_Jaenicke/publication/13096781_Emerging_role_of_caspase_3_in_apoptosis/links/0deec531904ffdc35a000000.pdf.
- [33] M. Fenech, W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, *Mutat. Res.* 534 (2003) 65–75 (Accessed December 6, 2015), <http://www.ncbi.nlm.nih.gov/pubmed/12504755>.
- [34] S. Elmore, Apoptosis: a review of programmed cell death, *Toxicol. Pathol.* 35 (2007) 495–516, <https://doi.org/10.1080/01926230701320337>.
- [35] S.A. Lowndes, A.L. Harris, The role of copper in tumour angiogenesis, *J. Mammary Gland Biol. Neoplasia* 10 (2005) 299–310, <https://doi.org/10.1007/s10911-006-9003-7>.