Antiproliferative and Apoptosis Inducing Effects of Non-Polar Fractions from *Lawsonia inermis* L. in Cervical (HeLa) Cancer Cells

Manish Kumar · Paramjeet Kaur · Subodh Kumar ·
Satwinderjeet Kaur

Received: 28 November 2014 / Revised: 13 February 2015 / Accepted: 2 March 2015 / Published online: 19 March 2015
© Prof. H.S. Srivastava Foundation for Science and Society 2015

Abstract Two non-polar fractions viz. hexane (Hex-LI) and chloroform fraction (CHCl_3 -LI) of *Lawsonia inermis* were studied for their antiproliferative potential in various cancer cell lines viz. HeLa, MCF-7, A549 and C6 glioma cells. Both the fractions showed more than 60 % of growth inhibition in all the tested cell lines at highest tested concentration. In clonogenic assay, different concentrations of Hex-LI and CHCl_3 -LI decreased the number and size of colonies as compared to control in HeLa cells. The apoptotic effects as nuclear condensation, fragmentation were visualized with Hoechst-33342 staining of HeLa cells using confocal microscope. Both fractions induced apoptotic cell death in human cervical carcinoma (HeLa) cells as evident from flow cytometric analysis carried out using Annexin V-FITC and propidium iodide dyes. CHCl_3 -LI treated cells significantly induced apoptosis (25.43 %) in comparison to control. Results from Neutral Comet assay demonstrated that both fractions induced double stranded breaks (DSB's) in HeLa cells. Our data indicated that Hex-LI and CHCl_3 -LI treated cells showed significant increase of 32.2 and 18.56 % reactive oxygen species (ROS) levels in DCFH-DA assay respectively. Further, experimental studies to decipher exact pathway via which these fractions induce cell death are in progress.

Keywords *Lawsonia inermis* · HeLa cells · Cytotoxicity · Apoptosis · ROS

Introduction

Cancer is characterized by several features such as uncontrolled cell division and growth, cell invasion and finally leading to metastasis. Cancer is major cause of mortality among human population globally after cardiovascular diseases (Kutluk and Kars 1998). Apoptosis is a kind of cell death characterized by various morphological alterations such as chromatin condensation, nuclear fragmentation, formation of apoptotic bodies etc. (Kerr et al. 1972). During apoptosis, caspases get activated and induce several molecular cascades which results in cell death (Allen et al. 1997; Hu and Kavanagh 2003; Wang et al. 2005; Elmore 2007; Millan and Huerta 2009). Apoptosis is the main mechanism which maintains tissue homeostasis. Alterations in the regulation of apoptosis may result in many pathological conditions and/or diseases such as cancer, autoimmune disorders, neurodegenerative diseases etc. (Kroemer and Reed 2000; Wang et al. 2012, 2013; Ramasamy et al. 2013). Once the cell loses control over apoptotic mechanism, cell evades death and divides continuously leading to neoplastic transformation (Igney and Krammer 2002; Hu and Kavanagh 2003; Reed 2003; Qi et al. 2010). Since ages, natural products are utilized in the traditional health care medicinal practices from centuries (Akerle 1988; Gurib-Fakim 2006). Plants are only sources which account for providing medicine or medicinal formulations for treating 80 % world population (Owoabi et al. 2007). Plants are considered as safe, better substitutes for various synthetic drugs and pose no side effects (Dai and Mumper 2010; Vasanthi et al. 2014). In last few years, studies on medicinal plants have shown that crude extracts as well as pure

M. Kumar · P. Kaur · S. Kaur (✉)
Department of Botanical and Environmental Sciences,
Guru Nanak Dev University, Amritsar 143005, Punjab, India
e-mail: sjkaur2011@gmail.com

S. Kaur
e-mail: sjkaur@rediffmail.com

S. Kumar
Department of Chemistry, UGC Centre for Advanced Studies,
Guru Nanak Dev University, Amritsar 143005, Punjab, India

phytochemicals possess potential to induce apoptosis (Rao et al. 2009; Wu et al. 2009; Lee et al. 2009; Xu et al. 2009). Targeting cancer cells *via* inducing apoptosis is one of the strategies for the development of anticancer drugs (Kroemer et al. 1995; Wyllie et al. 1980; Kerr et al. 1972; Kelloff et al. 2000).

Lawsonia is a monotypic genus represented by only one species *Lawsonia inermis* Linn (Lythraceae). This plant is a native of North Africa and South-West Asia. *Lawsonia inermis* Linn. is a traditional medicinal and multipurpose plant commonly known as Henna in India and commonly used for dyeing of hands and hairs. Leaves of the plant are used in traditional medicine system as expectorant, anti-inflammatory, liver tonic, haematinic, styptic, febrifuge, diuretic etc. (Reddy 1988; Ahmed et al. 2000; Bich et al. 2004; Warriar 2004; Jiny Varghese et al. 2010; Dhiman et al. 2012). Leaves of the plant were reported as modulators of antioxidant and xenobiotic metabolizing enzymes viz. phase I and II enzymes (Dasgupta et al. 2003). Extracts as well as various pure constituents from this plant have been reported with antioxidant (Hsouna et al. 2011; Guha et al. 2011; Kumar et al. 2014), antimutagenic, antigenotoxic, anticlastogenic (Raja et al. 2008; Basirian et al. 2013; Kumar et al. 2014) and antitumor activity (Dasgupta et al. 2003; Priya et al. 2011). From the last 25 years, our laboratory have been studying medicinal plants for their bioactive potential. In the present investigation, two fractions from *L. inermis* were studied for anti-proliferative and apoptosis inducing activities in cervical carcinoma HeLa cells.

Materials and methods

Chemicals

Annexin V-FITC apoptosis detection kit, 2', 7'-dichlorofluorescein diacetate and Hoechst-33342 were purchased from Sigma Chemical Co. (St Louis, MO, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and trypan blue dye were purchased from Hi-Media, Mumbai, India. All other chemicals used in the present studies were of AR grade.

Collection of plant material

The leaves of the *Lawsonia inermis* L. were obtained from local market (Majeeth Mandi) at Amritsar, Punjab, India and plant has been identified with voucher specimen (No. 6773) and has been kept in the Herbarium of the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, Punjab.

Preparation of extracts

Leaves were crushed to fine powder and dipped into 80 % methanol for 2 days at room temperature with constant shaking so that phytoconstituents get dissolved into solvent. The miscella (solvent containing phytoconstituents) so obtained was concentrated using rotary vacuum evaporator and finally lyophilized. The mother extract (MeOH-LI) i.e., 120 g was dissolved in doubled distilled water and fractionated with hexane and chloroform in series. The respective fractions so obtained were named as Hex-LI and CHCl₃-LI fractions.

Antiproliferative studies

Procurement and maintenance of cancer cell lines

HeLa (Human cervical cancer), MCF-7 (human breast adenocarcinoma), A549 (Human alveolar adenocarcinoma) and C6 glioma (Rat glioblastoma) cell lines were purchased from the National Centre for Cell Science (NCCS, Pune, India). Different cell lines were cultured in DMEM medium containing 10 % FBS and antibiotic-antimycotic solution. Cells were grown at 37 °C and 5 % CO₂ using CO₂ incubator.

Measurement of cell viability

Before carrying out all the experiments, cell viability was checked using method of Militao et al. (2006) with slight modifications. Cells were washed with PBS (pH 7.4), trypsinised and then finally centrifuge at 2000 rpm. Cell pellet was suspended in media and stained with trypan blue dye (0.4 % in PBS) to determine number of viable cells (not stained). After determining viability, cells were used to carry out different experiments.

Cytotoxicity

MTT assay was carried out to assess cytotoxic potential of both the fractions using method of Mickisch et al. (1990) with slight modifications. HeLa cells were seeded at density of 8000 cells per well of the 96 well plate. After time period of 24 h, cells were treated with extract concentrations for next 24 h. On the completion of treatment time, MTT was added and incubated for 2 h. Finally media was removed from the wells and DMSO (100 µl) was added per well to dissolve the purple coloured formazan crystals (MTT metabolic product) and finally reading was taken at 570 nm.

Colonogenic assay

Colonogenic potential of both the fractions was assessed using protocol of Gundala et al. (2013) with slight modifications.

The HeLa cells at a density of 4000 cells/well were seeded into 6 well plates containing 3 ml complete growth medium. Cells were treated with different concentrations of test samples for 24 h and incubated in CO₂ incubator for 8 days. Finally, the media was removed and colonies were stained with crystal violet stain. The numbers of colonies were counted for control as well as for each concentration.

Nuclear morphology assessment using confocal microscopy

The morphology of nucleus was examined using Hoechst 33342 dye. Nuclear condensation, fragmentation etc. are the distinct attributes of apoptosis (Lizard et al. 1995; Zhang and Xu 2002). HeLa cells at a density of 2×10^5 cells/well were cultured into 6-well plates. After the treatment of different extract/fractions to HeLa cells (24 h), cells were observed under inverted microscope. Finally stained with Hoechst 33342 for 10 min at 37 °C and scanned under a Nikon A1R Laser Scanning Confocal Microscope system (Nikon Corporation, Japan) using NIS Elements AR analysis software version 4.11.00 for observing nuclear morphology.

Determination of apoptosis using flow cytometry

Apoptosis inducing potential of Hex-LI and CHCl₃-LI fractions was evaluated by using Annexin V-FITC Apoptosis Detection Kit (Sigma) as described in Haneef et al. (2012). About 1×10^6 HeLa cells were seeded and allowed to attach for 24 h. After 24 h, cells were treated with Hex-LI (250 µg/ml) and CHCl₃-LI (450 µg/ml). After treating the cells for 24 h, all adhering as well as floating cells were collected in tubes and centrifuged to obtain cell pellet. Cell were washed twice with PBS and centrifuged again to obtain cell pellet. After that cell pellet was suspended in binding buffer followed by addition of 5 µl of Annexin V-FITC conjugate and 10 µl of Propidium iodide to 500 µl of cell suspension. After incubation of 10 min, cells were analyzed by flow cytometry (BD Accuri C6 Flow Cytometer, BD Biosciences) using BD Accuri software. [Viable cells (annexin V and PI negative), early apoptotic cells (annexin V positive and PI negative), necrotic cells (annexin V negative, PI-positive) and late apoptotic cells (annexin V positive and PI positive)].

Single cell gel electrophoresis assay (neutral comet assay)

Neutral comet assay was carried out to determine double strand breaks in DNA using the method of Wojewodzka et al. (2002) and Matkar et al. (2006) with slight modifications. HeLa cells were seeded at the density of 4×10^5 cells in 6 well plates. After the complete adherence of cells, cells were

treated with Hex-LI (250 µg/ml) and CHCl₃-LI (450 µg/ml) for 24 h. After the treatment, cells were washed with PBS, trypsinised, centrifuged to get pellet and finally suspended in 500 µl of PBS. Frosted slides were coated with normal melting point agarose (NMPA). After solidification of agarose, next layer of low melting point agarose (LMPA) containing treated and untreated HeLa cells was laid and allowed for solidifying followed by the third layer of LMPA and covered with coverslip. After agarose layering, coverslip was removed and slides were lowered down in lysing jars containing lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, pH 8.3, 10 % DMSO, 1 % Triton X-100) for 45 min. After that slides were removed from lysing jar and placed in electrophoretic buffer (300 mM sodium acetate, 100 mM Tris-HCl, pH 8.3) followed by electrophoresis for 45 min (20 V). The slides were stained with 20 µg/ml ethidium bromide and placed in moist chamber before scoring. To measure the DNA damage induced in HeLa cells on treatment with fractions, 50 comets from each slide were examined under an Epifluorescent Nikon microscope. Analysis of results was done with the visual scoring method; a total of 50 comets per slide were categorized into one to five categories according to level of DNA damage in the comets. Each comet was given grade between 0 (undamaged) and 4 (completely damaged) (Collins et al. 1997).

Reactive oxygen species measurement

ROS measurement was carried out by method of Deeb et al. (2010) with slight modifications. HeLa cells were seeded in 6-well plates and allowed to adhere completely. The cells were treated with test fractions Hex-LI (250 µg/ml) and CHCl₃-LI (450 µg/ml) for 24 h. At the end of treatment period, cells were incubated in 2', 7'-dichlorofluorescein diacetate (DCFH-DA) dye for 30 min at 37 °C. Cells were then washed twice with PBS and finally analyzed by BD Accuri C6 Flow Cytometer (BD Biosciences) (ExCitation 488 nm; Emission 530 nm) for 2,7-dichlorofluorescein (DCF) positive cells.

Statistical analysis

The results were expressed as the average and standard error. GI₅₀ values were calculated using regression equation. The data were also analyzed for statistical significance using Student's *t* test and analysis of variance (One-way ANOVA) and the difference among means was compared by high-range statistical domain (HSD) using Tukey's test. The significance of results was checked at $*p \leq 0.05$.

Results

Measurement of cell viability

The cells showing viability above 90–95 % were used to carry out experiments. Viable cells are colourless while dead cells are stained blue in color after treatment with trypan blue dye.

Cytotoxicity among different cancer cell lines

The antiproliferative effect of both fractions viz. Hex-LI and CHCl_3 -LI was studied against HeLa, MCF-7, A549 and C6 glioma cell lines. The antiproliferative potency of fractions was seen in dose dependent manner (Figs. 1, 2, 3 and 4). Hex-LI showed GI_{50} (concentration at which 50 % inhibition of growth has been observed) of 382.44, 186.47, 378.73, 204.27 $\mu\text{g/ml}$ in HeLa, MCF-7, A549, C6 cancer cell lines respectively whereas CHCl_3 -LI showed GI_{50} of 741.44, 524.46, 469.80 and 387.14 $\mu\text{g/ml}$ in HeLa, MCF-7, A549, C6 cancer cell lines respectively.

Colonogenic assay

Colonogenic assay is an in vitro experiment used to check ability of cell to grow indefinitely and form colony. Such type of cell is known as colonogenic as it retains the capacity of proliferation after treatment with chemotherapeutic agents (Munshi et al. 2005; Franken et al. 2006). In the colony formation assay, both the fractions viz. Hex-LI and CHCl_3 -LI effectively inhibited the growth of colonies in dose dependent manner. It was observed that there was complete inhibition of colony formation at highest concentration of 400 $\mu\text{g/ml}$. Along with decrease in the number of colonies, it was also observed that there was decrease in the size of colonies as compared to size of control colonies (Figs. 5 and 6). Both fractions were taken forward to study apoptotic cell death in HeLa cells.

Fig. 1 Growth inhibitory effects on HeLa cells treated with various concentrations of Hex-LI and CHCl_3 -LI. [Hex-LI (Regression equation: $y=23.03\ln(x)-86.95$; $*r=0.9664$) and CHCl_3 -LI (Regression equation: $y=0.059x+6.255$; $*r=0.9767$)] $*p\leq 0.05$

Fig. 2 Growth inhibitory effects on MCF-7 cells treated with various concentrations of Hex-LI and CHCl_3 -LI. [Hex-LI (Regression equation: $y=25.23\ln(x)-81.91$; $*r=0.9859$) and CHCl_3 -LI (Regression equation: $y=0.095x+0.176$; $*r=0.9576$)] $*p\leq 0.05$

Nuclear morphology assessment using confocal microscopy

Hoechst 33342 is a DNA specific fluorescent dye which enters easily into the cell and intercalates in A-T regions of DNA. After treatment of 24 h, HeLa cells were observed for morphology changes under inverted microscope (Figs. 7 and 8). For nuclear morphological assessment, cells were washed with PBS followed by fixation with paraformaldehyde. After fixation, cells were stained with Hoechst 33342 dye to observe nuclear morphological alterations such as DNA condensation, fragmentation etc. It was observed that on treatment with Hex-LI and CHCl_3 -LI, nuclei showed condensation, fragmentation (Figs. 9 and 10). However, the control cells showed prominent nucleus without any type of condensation and fragmentation.

Determination of apoptosis using flow cytometry

To evaluate, whether the fractions induce cell death through apoptotic or necrotic mode, flow cytometric analysis was carried out by using Annexin V-FITC and PI dye. During apoptosis, there is translocation of phosphatidylserine to outer surface of plasma membrane. Annexin V is Ca^{2+} dependent

Fig. 3 Growth inhibitory effects on A549 cells treated with various concentrations of Hex-LI and CHCl_3 -LI. [Hex-LI (Regression equation: $y=0.071x+23.11$; $*r=0.9648$) and CHCl_3 -LI (Regression equation: $y=0.086x+9.597$; $*r=0.9481$)] $*p\leq 0.05$

Fig. 4 Growth inhibitory effects on C6 glioma cells treated with various concentrations of Hex-LI and CHCl₃-LI. [Hex-LI (Regression equation: $y=26.92\ln(x)-93.20$; $*r=0.9555$) and CHCl₃-LI (Regression equation: $y=22.82\ln(x)-85.98$; $*r=0.9643$)] $*p\leq 0.05$

phospholipid-binding protein possesses high affinity for phosphatidylserine (Vermes et al. 1995). Translocation of phosphatidylserine to outer surface of plasma membrane also takes place during necrosis. So the use of both dyes viz. Annexin V-FITC and PI helps in estimation of early apoptotic, late apoptotic and necrotic cell population. From the Fig. 11, it is clear that Hex-LI and CHCl₃-LI treatment caused 7.63 and 15.53 % of early apoptosis as compared to control value of 4.66 %. However, percentage of late apoptotic cells were 7.46 and 9.9 % in Hex-LI and CHCl₃-LI treated cells. The total number of apoptotic cells was 15.1 and 25.43 % as compared to control value of 11.93 %.

Single cell gel electrophoresis assay (neutral comet assay)

After evaluating apoptosis in Hex-LI and CHCl₃-LI treated HeLa cancer cells, same concentrations of the both fractions were used to determine double stranded breaks in HeLa cells. From the results it was seen that both fractions showed DSB's breaks with DNA damage (arbitrary units) of 137.3 and 75 in Hex-LI and CHCl₃-LI treated HeLa cancer cells respectively (Fig. 12). Positive control i.e., camptothecin showed more DNA damage (153.3) as compared to both fractions.

Fig. 5 Colony formation inhibition in HeLa cells on treatment with Hex-LI. Cell survival was determined by considering the control cells survival as 100 %

Fig. 6 Colony formation inhibition in HeLa cells on treatment with CHCl₃-LI. Cell survival was determined by considering the control cells survival as 100 %

Reactive oxygen species measurement

2', 7'-dichlorofluorescein diacetate (DCFH-DA), was used to measure ROS in treated cells. DCFH-DA, a non polar compound when it enters into the cells, it gets cleaved by cellular estrases into DCFH. Later on DCFH get oxidized by hydrogen peroxide/low molecular weight peroxides into 2,7-dichlorofluorescein (DCF) (highly fluorescent) which is analyzed by flow cytometry. Both the fraction viz. Hex-LI and CHCl₃-LI significantly induced generation of reactive oxygen species. It has been observed that ROS levels were found to be 32.2 % as compared to its control (10.4 %) in Hex-LI and 18.56 % as compared to its control (11.03 %) in CHCl₃-LI treated cells (Fig. 13).

Discussion

Apoptosis is a well regulated process, characterized by cell shrinkage, chromatin condensation, fragmentation, formation of apoptotic bodies. It plays a vital role in cellular homeostasis by controlling cell proliferation (Millan and Huerta 2009; Ramasamy et al. 2013). Numerous plants and their constituents are known to possess apoptosis inducing potential (Hsieh et al. 2006; Hsu et al. 2011; Kim et al. 2008). Any compound could possibly be developed into an anti-cancer drug, if it shows less inhibitory effects on normal cells than the cancer cells (Taraphdar et al. 2001). In recent years, it has been seen that scientific community is more engaged in isolating and screening natural molecules for drug discovery (Moure et al. 2001; Krishnaiah et al. 2011). Apoptosis can be exploited as an important target to develop anticancer drugs (Hu and Kavanagh 2003; Camero 2002). In the present work, we studied the growth inhibitory and apoptosis inducing effects of popular medicinal plant *L. inermis* on cancer cell lines viz. HeLa, MCF-7, A549 and C6. We found that two fractions viz. Hex-LI, CHCl₃-LI were found to exhibit considerable dose dependent growth inhibitory activity i.e., more than

Fig. 7 Morphological features visualized under phase contrast inverted microscope. The cells were incubated with various Hex-LI concentrations for 24 h. **a** Control; **b** 100 $\mu\text{g/ml}$; **c** 250 $\mu\text{g/ml}$; **d** 350 $\mu\text{g/ml}$

Fig. 8 Morphological features visualized under phase contrast inverted microscope. The cells were incubated with various CHCl_3 -LI concentrations for 24 h. **a** Control; **b** 350 $\mu\text{g/ml}$; **c** 400 $\mu\text{g/ml}$; **d** 450 $\mu\text{g/ml}$

Fig. 9 Nuclear alterations observed in HeLa cells after treatment with Hex-LI (24). Cells were stained with Hoechst 33342. *Arrows* designates cells exhibiting nuclear condensation, fragmentation and formation apoptotic bodies. The cells were incubated with various concentrations for 24 h. **a** control; **b** 100 $\mu\text{g}/\text{ml}$ of Hex-LI; **c** 250 $\mu\text{g}/\text{ml}$ of Hex-LI; **d** 350 $\mu\text{g}/\text{ml}$ of Hex-LI

Fig. 10 Nuclear alterations observed in HeLa cells after treatment with CHCl_3 -LI (24 h). Cells were stained with Hoechst 33342. *Arrows* designates cells exhibiting nuclear condensation, fragmentation and formation apoptotic bodies. The cells were incubated with various concentrations for 24 h. **a** control; **b** 350 $\mu\text{g}/\text{ml}$ of CHCl_3 -LI; **c** 400 $\mu\text{g}/\text{ml}$ of CHCl_3 -LI; **d** 450 $\mu\text{g}/\text{ml}$ of CHCl_3 -LI

Fig. 11 Induction of apoptosis in HeLa cells after 24 h of treatment with Hex-LI and CHCl₃ analyzed by flow cytometry. **a** Control; **b** Hex-LI (250 µg/ml); **c** CHCl₃-LI (450 µg/ml) and **d** Graph showing number of

live cells (LL quadrant), early apoptotic (LR quadrant), late apoptotic (UR quadrant), necrotic (UL quadrant) and total apoptotic cells. * $p \leq 0.05$

60 % in all the tested cell lines (Figs. 1–4). Antiproliferative activity of the extract depends upon the extraction procedure and the choice of solvent used for extraction (Chirinos et al. 2007; Lafka et al. 2007, 2013). Sadeghi-aliabadi et al. (2008) reported that chloroform extract of *Convolvulus arvensis* is quite effective in inducing anticancer effects in HeLa cells. Machana et al. (2012) reported that combination of hexane and water fraction of *Polyalthia evecta* showed high anticancer activity in HepG2 cells. Pacifico et al. (2013) reported CHCl₃ extract from *Laurus nobilis* leaves induced strong antiproliferative activity in nervous system cell lines. Kim et al. (2012) studied hexane extract of garlic cloves and reported that the extract has the ability to induce apoptosis in Hep3B cells. Colonogenic assay was used to check the potential of cell to divide and form colony after the removal of test compound (Kama et al. 2011). Our results depicted that both the fractions inhibited the colony formation potential of cervical carcinoma cells (Figs. 5 and 6). According to Drees et al.

(1997) test sample was regarded as effective if it decreased the colony formation potential to ≤ 30 % in comparison to control. Our data showed that both the fractions completely inhibited colony formation potential of HeLa cells as compare to control indicating high effectivity of test fractions. Pratumvinit et al. (2009) investigated *Ficus hispida* L. extract for colony formation inhibition in T47D breast cancer cell line and found that extract treatment showed reduction in number of colonies. Leaves of *Moringa oleifera* significantly inhibited colonogenicity in A549 cells in dose dependent manner (Jung 2014).

Plant extracts are centre of attraction for the search of anti-cancer agents because of minimal side effects and their potential to inhibit growth and proliferation of cancerous cells (Rao et al. 2009). Herbs or combinations of extracts of these herbs can act synergistically resulting in increased effectivity and can also have potential to reduce toxic effects. (Vickers 2002; Li et al. 2000). In the present study, after evaluating

Fig. 12 **a** Various kinds of comets in different grades (1–4) by visual scoring. **b** DNA damage induced in Hex-LI (250 $\mu\text{g}/\text{ml}$) and CHCl_3 -LI (450 $\mu\text{g}/\text{ml}$) treated HeLa cells evaluated by visual scoring. * $p \leq 0.05$

cytotoxicity, Hex-LI and CHCl_3 -LI were analyzed for cell morphological changes, nuclear changes, DNA fragmentation and formation of apoptotic bodies which are the characteristic features of apoptosis. It was observed that treated cells showed morphological alterations such as cell shrinkage, rounding off along with decline in cell number (Figs. 7 and 8). Further, to observe nuclear changes, cells were stained with Hoechst 33342 dye and observed under confocal microscope (Figs. 9 and 10). With increase in the concentration of both the

Fig. 13 ROS level analyzed by flow cytometry in HeLa cells treated with Hex-LI (250 $\mu\text{g}/\text{ml}$) and CHCl_3 (450 $\mu\text{g}/\text{ml}$) in comparison to untreated cells (Control). * $p \leq 0.05$

extracts, nuclei of the cells showed shrinkage, condensation, nuclear fragmentation and formation of apoptotic bodies. Further, flow cytometric studies confirmed that both fractions induced apoptosis in HeLa cells. Hex-LI induced 15.1 % and 25.43 % of apoptosis in HeLa cells (Fig. 11). Earlier we reported that both the fractions were rich in polyphenolic constituents. HPLC revealed that Hex-LI contains ellagic acid in good proportion while CHCl_3 -LI contains ellagic acid along with kaempferol in high amounts (Kumar et al. 2014). Apoptosis inducing effects of these fractions might be a contribution of these phytochemicals as these have been reported as potential anticancer agents in many studies. Edderkaoui et al. (2008) reported that ellagic acid induced apoptosis in pancreatic cancer cell by suppressing NF-kappa B activity. Han et al. (2006) showed that ellagic acid induced apoptosis in human osteogenic sarcoma (HOS) through modulating expression of caspase 3 and bax proteins. Kaempferol stimulated apoptosis in human osteosarcoma U-2 OS cells (Huang et al. 2010). Detailed study revealed the involvement mitochondrial dependent and endoplasmic reticulum stress signaling pathway in kaempferol induced apoptosis. In another study, kaempferol induced apoptosis in human bladder cancer cells by upregulating tumor suppressor PTEN i.e., Phosphatase and tensin homolog deleted on chromosome 10 and downregulating Akt phosphorylation (Xie et al. 2013). HeLa cells were evaluated using neutral comet assay for double stranded breaks (DSB's) on treatment with Hex-LI and CHCl_3 -LI for 24 h. It was observed that cells treated with both the fractions showed DSB's in DNA which may result from endonucleolytic activity of enzymes involved in apoptosis. These DSB's were resulted from the apoptotic process occurring in the treated cells. Lim et al. (2011) reported that *Acalypha wilkesiana* extracts caused apoptotic cell death via DNA single and double stranded breaks in U87MG and A549 cells. Lam et al. (2012) studied that *Fagonia cretica* extract treatment induces DNA damage in human breast cancer (MCF-7) cells. As compared to normal cells, cancer cells show high metabolic activities which results in elevated levels of reactive oxygen species (Gundala et al. 2014; Schumacker 2006; Szatrowski and Nathan 1991; Trachootham et al. 2009). Various polyphenolic compounds are known to exert anticancer effects via generating oxidative stress in cancer cells (Azam et al. 2004). ROS in excess can cause damage to DNA, proteins, lipids and cell membranes. In response to ROS, mitochondrial membrane become permeable leading to the release of cytochrome *c* which induces caspases cascades ultimately resulting in apoptosis (Conklin 2004; Circu and Aw 2010; Simon et al. 2000; Thayyullathil et al. 2008). Sharma et al. (2007) reported that Kaempferol showed apoptogenic activity in human glioblastoma cells by elevating the levels of ROS. Ellagic acid caused apoptosis of tsgh8301 human bladder cancer cells (Ho et al. 2014). Increased ROS and Ca^{2+} production along with other factors played important role in inducing

apoptosis in tsgh8301 human bladder cancer cells. Several natural products targets cancer cells by elevating ROS levels (Ding et al. 2009; Jeong et al. 2010; Widodo et al. 2010). Hex-LI and CHCl_3 -LI significantly increased production of ROS in HeLa cells as compared to their respective controls. Increased ROS production can be a one of the mechanism along with other signaling cascades responsible for the apoptosis inducing effects of Hex-LI and CHCl_3 -LI fractions of *L. inermis*.

Conclusion

In conclusion, fractions isolated from *L. inermis* possess good anticancer activity which can be exploited for the development of chemotherapeutic agents for the treatment of cancer after further toxicity studies. Studies are also in progress to explore the mechanism responsible for these activities.

Acknowledgments This work was supported by the Council of Scientific and Industrial Research (CSIR) [38(1265)/10/EMR-II], New Delhi (India) and the UPE programme of University Grants Commission (UGC), New Delhi.

Conflict of interest None.

References

- Ahmed S, Rahman A, Alam A, Saleem M, Athar M, Sultana S (2000) Evaluation of the efficacy of *Lawsonia alba* in the alleviation of carbon tetrachloride induced oxidative stress. *J Ethnopharmacol* 69:157–164
- Akerle O (1988) Medicinal plants and primary health care: an agenda for action. *Fitoterapia* 59:355–363
- Allen RT, Hunter WJ 3rd, Agarwal DK (1997) Morphological and biochemical characterization and analysis of apoptosis. *J Pharmacol Toxicol Methods* 37:215–228
- Azam S, Hadi N, Khan NU, Hadi SM (2004) Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties. *Toxicol in Vitro* 18:555–561
- Basirian M, Manjula SN, Mruthunjaya K (2013) In vitro anti-clastogenic activity of different fractions of roots of *Lawsonia inermis* on chromosomal aberration of human lymphocytes against doxorubicin and cyclophosphamide as clastogens. *Int J Med Med Sci* 46:1239–1244
- Bich DH, Chung DQ, Chuong BX et al (2004) The medicinal plants and animals in Vietnam. Science and Technology Publishing House, Hanoi
- Camero A (2002) Targeting the cell cycle for cancer therapy. *Br J Cancer* 87:129–133
- Chirinos R, Rogez H, Campos D, Pedreschi R, Larondelle Y (2007) Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruiz & Pavón) tubers. *Sep Purif Technol* 55:217–225
- Circu ML, Aw TY (2010) Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 48:749–762
- Collins A, Dusinska M, Franklin M, Somorovská M, Petrovská H, Duthie S, Fillion L, Panayiotidis M, Raslová K, Vaughan N (1997) Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ Mol Mutagen* 30:139–146
- Conklin KA (2004) Free radicals: the pros and cons of antioxidants. Cancer chemotherapy and antioxidants. *J Nutr* 134:3201–3204
- Dai J, Mumper RJ (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15:7313–7352
- Dasgupta T, Rao AR, Yadava PK (2003) Modulatory effect of Henna leaf (*Lawsonia inermis*) on drug metabolising phase I and phase II enzymes, antioxidant enzymes, lipid peroxidation and chemically induced skin and forestomach papillomagenesis in mice. *Mol Cell Biochem* 245:11–22
- Deeb D, Gao X, Jiang H, Janic B, Arbab AS, Rojanasakul Y, Dulchavsky SA, Gautam SC (2010) Oleanane triterpenoid CDDO-Me inhibits growth and induces apoptosis in prostate cancer cells through a ROS-dependent mechanism. *Biochem Pharmacol* 79:350–360
- Dhiman A, Sharma K, Goyal J, Garg M, Sharma A (2012) Determination of Lawsone content in leaves of *Lawsonia inermis* Linn. *Int J Pharm Pharm Sci* 1:17–20
- Ding H, Han C, Guo D, Chin YW, Ding Y, Kinghorn AD, D'Ambrosio SM (2009) Selective induction of apoptosis of human oral cancer cell lines by avocado extracts via a ROS-mediated mechanism. *Nutr Cancer* 61:348–356
- Drees M, Dengler WA, Roth T, Labonte H, Mayo J, Malspeis L, Grever M, Sausville EA, Fiebig HH (1997) Flavopiridol (L86-8275): selective antitumor activity in vitro and activity in vivo for prostate carcinoma cells. *Clin Cancer Res* 3:273–279
- Edderkaoui M, Odinkova I, Ohno I, Gukovsky I, Go VL, Pandol SJ, Gukovskaya AS (2008) Ellagic acid induces apoptosis through inhibition of nuclear factor kappa B in pancreatic cancer cells. *World J Gastroenterol* 14(23):3672–3680
- Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35:495–516
- Franken NAP, Rodermond HM, Stap J, Haveman J, Bree CV (2006) Clonogenic assay of cells in vitro. *Nat Protoc* 5:2315–2319
- Guha G, Rajkumar V, Ashok Kumar R, Mathew L (2011) Antioxidant activity of *Lawsonia inermis* extracts inhibits chromium(vi)-induced cellular and DNA toxicity. *Evid Based Complement Alternat Med*. doi:10.1093/ecam/nep205
- Gundala SR, Yang C, Lakshminarayana N, Asif G, Gupta MV, Shamsi S, Aneja R (2013) Polar biophenolics in sweet potato greens extract synergize to inhibit prostate cancer cell proliferation and in vivo tumor growth. *Carcinogenesis* 34(9):2039–2049
- Gundala SR, Yang C, Mukkavilli R, Paranjpe R, Brahmabhatt M, Pannu V, Cheng A, Reid MD, Aneja R (2014) Hydroxychavicol, a betel leaf component, inhibits prostate cancer through ROS-driven DNA damage and apoptosis. *Toxicol Appl Pharmacol* 280:86–96
- Gurib-Fakim A (2006) Medicinal plants: traditions of yesterday and drugs of tomorrow. *Mol Aspects Med* 27:1–93
- Han DH, Lee MJ, Kim JH (2006) Antioxidant and apoptosis-inducing activities of ellagic acid. *Anticancer Res* 26(5A):3601–3606
- Haneef J, Parvathy M, Thankayyan RSK, Sithul H, Sreeharshan S (2012) Bax translocation mediated mitochondrial apoptosis and caspase dependent photosensitizing effect of *Ficus religiosa* on cancer cells. *PLoS ONE* 7(7):e40055. doi:10.1371/journal.pone.0040055
- Ho CC, Huang AC, Yu CS, Lien JC, Wu SH, Huang YP, Huang HY, Kuo JH, Liao WY, Yang JS, Chen PY, Chung JG (2014) Ellagic acid induces apoptosis in tsgh8301 human bladder cancer cells through the endoplasmic reticulum stress- and mitochondria-dependent signaling pathways. *Environ Toxicol* 29:1262–1274
- Hsieh TJ, Liu TZ, Lu FJ, Hsieh PY, Chen CH (2006) Actinodaphnine induces apoptosis through increased nitric oxide, reactive oxygen species and down regulation of NF-kappa B signaling in human hepatoma Mahlavu cells. *Food Chem Toxicol* 44:344–354
- Hsouna AB, Trigui M, Culioli G, Blache Y, Jaoua S (2011) Antioxidant constituents from *Lawsonia inermis* leaves: isolation, structure elucidation and antioxidative capacity. *Food Chem* 125:193–200

- Hsu H-F, Huang K-H, Lu K-J, Chiou S-J, Yen J-H, Chang C-C, Hwang J-Y (2011) *Typhonium blumei* extract inhibits proliferation of human lung adenocarcinoma A549 cells via induction of cell cycle arrest and apoptosis. *J Ethnopharmacol* 135(2):492–500
- Hu W, Kavanagh JJ (2003) Anticancer therapy targeting the apoptotic pathway. *Lancet Oncol* 4:721–729
- Huang WW, Chiu YJ, Fan MJ, Lu HF, Yeh HF, Li KH, Chen PY, Chung JG, Yang JS (2010) Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria-dependent pathway in human osteosarcoma U-2 OS cells. *Mol Nutr Food Res* 54(11):1585–1595
- Igney F, Krammer P (2002) Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2:277–288
- Jeong JC, Jang SW, Kim TH, Kwon CH, Kim YK (2010) Mulberry fruit (Morus fructus) extracts induce human glioma cell death in vitro through ROS-dependent mitochondrial pathway and inhibits glioma tumor growth in vivo. *Nutr Cancer* 62:402–412
- Jiny Varghese K, Silvipriya KS, Resmi S, Jolly CI (2010) *Lawsonia inermis* (Henna): a natural dye of various therapeutic uses - a review. *Inventi Rapid: Cosmeceuticals* 1:1–5
- Jung IL (2014) Soluble extract from *Moringa oleifera* leaves with a new anticancer activity. *PLoS ONE* 9(4):e95492. doi:10.1371/journal.pone.0095492
- Karna P, Gundala SR, Gupta MV, Shamsi SA, Pace RD, Yates C, Narayan S, Aneja R (2011) Polyphenol-rich sweet potato greens extract inhibits proliferation and induces apoptosis in prostate cancer cells in vitro and in vivo. *Carcinogenesis* 32(12):1872–1880
- Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Malone WA, Boone CW, Kopelovich L, Hawk ET, Lieberman R, Lawrence JA, Ali I, Viner JL, Sigman CC (2000) Progress in cancer chemoprevention: development of diet-derived chemopreventive agents. *J Nutr* 130(2S):467S–471S
- Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26:239–257
- Kim EJ, Lim SS, Park SY, Shin HK, Kim JS, Park JHY (2008) Apoptosis of DU145 human prostate cancer cells induced by dehydrocostus lactone isolated from the root of *Saussurea lappa*. *Food Chem Toxicol* 46(12):3651–3658
- Kim HJ, Han MH, Kim GY, Choi YW, Choi YH (2012) Hexane extracts of garlic cloves induce apoptosis through the generation of reactive oxygen species in Hep3B human hepatocarcinoma cells. *Oncol Rep* 28:1757–1763
- Krishnaiah D, Sarbathy R, Nithyanandam R (2011) A review of the antioxidant potential of medicinal plant species. *Food Bioprod Process* 89:217–233
- Kroemer G, Reed JC (2000) Mitochondrial control of cell death. *Nat Med* 6(5):513–519
- Kroemer G, Petit PX, Zamzami N, Vayssiere JL, Mignotte B (1995) The biochemistry of programmed cell death. *FASEB J* 9:1277–1287
- Kumar M, Kumar S, Kaur SJ (2014) Identification of polyphenols in leaf extracts of *Lawsonia inermis* L. with antioxidant, antigenotoxic and antiproliferative potential. *Int J Green Pharm* 8:23–36
- Kutluk T, Kars A (1998) General knowledge about cancer. Ankara; Turkey cancer investigation and fight society publications, pp: 7–15
- Lafka TI, Sinanoglou VJ, Lazos ES (2007) On the extraction and antioxidant activity of phenolic compounds from winery wastes. *Food Chem* 104:1206–1214
- Lafka TI, Lazou AE, Sinanoglou VJ, Lazos ES (2013) Phenolic extracts from wild olive leaves and their potential as edible oils antioxidants. *Foods* 2:18–31
- Lam M, Carmichael AR, Griffiths HR (2012) An aqueous extract of *Fagonia cretica* induces DNA damage, cell cycle arrest and apoptosis in breast cancer cells via FOXO3a and p53 expression. *PLoS ONE* 7(6):e40152. doi:10.1371/journal.pone.0040152
- Lee HZ, Liu WZ, Hsieh WT, Tang FY, Chung JG, Leung HWC (2009) Oxidative stress involvement in *Physalis angulata*-induced apoptosis in human oral cancer cells. *Food Chem Toxicol* 47:561–570
- Li XK, Motwani M, Tong W, Bornmann W, Schwartz GK, Huanglian A (2000) Chinese herbal extract, inhibits cell growth by suppressing the expression of cyclin B1 and inhibiting CDC2 kinase activity in human cancer cells. *Mol Pharm* 58:1287–1293
- Lim SW, Ting KN, Bradshaw TD, Zeenathul NA, Wiart C, Khoo TJ, Lim KH, Loh HS (2011) *Acalypha wilkesiana* extracts induce apoptosis by causing single strand and double strand DNA breaks. *J Ethnopharmacol* 138(2):616–623
- Lizard G, Fomel S, Genestier L (1995) Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. *Cytometry* 21:275–283
- Machana S, Weerapreeyakul N, Barusux S, Thumanu K, Tanthanuch W (2012) Synergistic anticancer effect of the extracts from *Polyalthia evecta* caused apoptosis in human hepatoma (HepG2) cells. *Asian Pac J Trop Biomed* 2:589–596
- Matkar SS, Wrischnik LA, Jones PR, Hellmann-Blumberg U (2006) Two closely related nickel complexes have different effects on DNA damage and cell viability. *Biochem Biophys Res Commun* 343:754–761
- Mickisch G, Fajta S, Keilhauer G, Schlike E, Tschada P, Alken P (1990) Chemosensitivity testing of primary human renal cell carcinoma by tetrazolium based microculture assay (MTT). *Urol Res* 18:131–136
- Militao GCG, Dantas INF, Pessoa C, Falcão MJC, Silveira ER, Lima MAS, Curi R, Lima T, Moraes MO, Costa-Lotufo LV (2006) Induction of apoptosis by pterocarpanes from *Platymiscium floribundum* in HL-60 human leukemia cells. *Life Sci* 78:2409–2417
- Millan A, Huerta MD (2009) Apoptosis-inducing factor and colon cancer. *J Surg Res* 151:163–170
- Moure A, Cruz JM, Franco D, Dominguez JM, Sineiro J, Dominguez H, Núñez MJ, Parajó JC (2001) Natural antioxidants from residual sources. *Food Chem* 2:145–171
- Munshi A, Hobbs M, Meyn RE (2005) Clonogenic cell survival assay. From: *Methods in Molecular Medicine*, vol 110: Chemosensitivity vol. 1. In: Blumenthal RD (ed) *In vitro Assays*. Humana Press Inc, Totowa, NJ
- Owoabi J, Omogbai EKI, Obasuyi O (2007) Antifungal and antibacterial activities of the ethanolic and aqueous extract of *Kigella africana* (Bignoniaceae) stem bark. *Afr J Biotechnol* 6:882–885
- Pacifico S, Gallicchio M, Lorenz P, Potenza N, Galasso S, Marciano S, Fiorentino A, Stintzing FC, Monaco P (2013) Apolar *Laurus nobilis* leaf extracts induce cytotoxicity and apoptosis towards three nervous system cell lines. *Food Chem Toxicol* 62:628–637
- Pratumvinit B, Srisapoomi T, Worawattananon P, Opartkiattikul N, Jiratchariyakul W, Kummalue T (2009) In vitro antineoplastic effect of *Ficus hispida* L. plant against breast cancer cell lines. *J Med Plant Res* 3(4):255–261
- Priya R, Ilavenil S, Kaleeswaran B, Srigopalram S, Ravikumar S (2011) Effect of *Lawsonia inermis* on tumor expression induced by Dalton's lymphoma ascites in Swiss albino mice. *Saudi J Biol Sci* 18:353–359
- Qi F, Li A, Zhao L, Xu H, Inagaki Y, Wang D, Cui X, Gao B, Kokudo N, Nakata M, Tang W (2010) Cinobufacini, an aqueous extract from Bufo bufo gargarizans Cantor, induces apoptosis through a mitochondria mediated pathway in human hepatocellular carcinoma cells. *J Ethnopharmacol* 128:654–661
- Raja W, Agrawal RC, Ovais M (2008) Evaluation of antimutagenicity effect of *Lawsonia inermis* (henna) leaf extract in Swiss albino mice. *Res J Pharm Technol* 1(3):278–279
- Ramasamy S, Wahabb NA, Abidina NZ, Manickam S (2013) Effect of extracts from *Phyllanthus watsonii* Aizy Shaw on cell apoptosis in cultured human breast cancer MCF-7 cells. *Exp Toxicol Pathol* 65:341–349

- Rao PS, Ramanadham M, Prasad MNV (2009) Anti-proliferative and cytotoxic effects of *Strychnos nux-vomica* root extract on human multiple myeloma cell line – RPMI 8226. *Food Chem Toxicol* 47: 283–288
- Reddy KR (1988) Folk medicine from Chittoor district Andhra Pradesh, India used in the treatment of jaundice. *Int J Crude Drug Res* 26: 137–140
- Reed JC (2003) Apoptosis-targeted therapies for cancer. *Cancer Cell* 3:17
- Sadeghi-aliabadi H, Ghasemi N, Kohi M (2008) Cytotoxic effect of *Convolvulus arvensis* extracts on human cancerous cell line. *Res Pharm Sci* 3:31–34
- Schumacker PT (2006) Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell* 10:175–176
- Sharma V, Joseph C, Ghosh S, Agarwal A, Mishra MK, Sen E (2007) Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. *Mol Cancer Ther* 6(9):2544–2553
- Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species in apoptosis induction. *Apoptosis* 5:415–418
- Szatrowski TP, Nathan CF (1991) Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 51:794–798
- Taraphdar AK, Roy M, Bhattacharya RK (2001) Natural products as inducers of apoptosis: implication for cancer therapy and prevention. *Curr Sci* 80:1387–1396
- Thayyullathil F, Chathoth S, Hago A, Patel M, Galadari S (2008) Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. *Free Radic Biol Med* 45:1403–1412
- Trachootham D, Alexandre J, Huang P (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov* 8:579–591
- Vasanthi P, Ganapathy M, Evanjelene VK, Ayyavuv N, Angamuthu J (2014) Phytochemical screening and antioxidant activity of extracts of the leaf and bark of *Albizzia lebbek* (Benth). *Acad J Med Plant* 2: 026–031
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis, flow cytometric detection of phosphatidylserin expression on early apoptotic cells using fluorescein labeled annexin-V. *J Immunol Methods* 184:39–51
- Vickers A (2002) Botanical medicines for the treatment of cancer: rationale, overview of current data, and methodological considerations for phase I and II trials. *Cancer Investig* 20:1069–1079
- Wang ZB, Liu YQ, Cui YF (2005) Pathways to caspase activation. *Cell Biol Int* 29:489–496
- Wang CZ, Calway T, Yuan CS (2012) Herbal medicines as adjuvants for cancer therapeutics. *Am J Chin Med* 40:657–669
- Wang CZ, Zhang Z, Huang WH, Du GJ, Wen XD, Calway T, Yu C, Nass R, Zhao J, Du W, Li SP, Yuan CS (2013) Identification of potential anticancer compounds from *Oplopanax horridus*. *Phytomedicine* 20(11):999–1006
- Warrier PK (2004) Indian medicinal plants-a compendium of 500 species. Orient longman private limited, Chennai, pp 303–304
- Widodo N, Priyandoko D, Shah N, Wadhwa R, Kaul SC (2010) Selective killing of cancer cells by Ashwagandha leaf extract and its component Withanone involves ROS signaling. *PLoS ONE* 5:e13536
- Wojewodzka M, Buraczewska I, Kruszewski M (2002) A modified neutral comet assay: elimination of lysis at high temperature and validation of the assay with anti-single-stranded DNA antibody. *Mutat Res* 518:9–20
- Wu SJ, Chang SP, Lin DL, Wang SS, Hou FF, Ng LT (2009) Supercritical carbon dioxide extract of *Physalis peruviana* induced cell cycle arrest and apoptosis in human lung cancer H661 cells. *Food Chem Toxicol* 47:1132–1138
- Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* 68:251–306
- Xie F, Su M, Qiu W, Zhang M, Guo Z, Su B, Liu J, Li X, Zhou L (2013) Kaempferol promotes apoptosis in human bladder cancer cells by inducing the tumor suppressor, PTEN. *Int J Mol Sci* 14(11):21215–21226
- Xu Y, Ge R, Du J, Xin H, Yi T, Sheng J, Wang Y, Ling C (2009) Corosolic acid induces apoptosis through mitochondrial pathway and caspases activation in human cervix adenocarcinoma HeLa cells. *Cancer Lett* 284:229–237
- Zhang J, Xu M (2002) Apoptotic DNA fragmentation and tissue homeostasis. *Trends Cell Biol* 12(2):84–89