RESEARCH ARTICLE

Editorial Process: Submission:02/18/2018 Acceptance:06/21/2018

Potential of *Aucklandia Lappa* Decne Ethanolic Extract to Trigger Apoptosis of Human T47D and Hela Cells

S S Hasson[1]*, A SH Al-Shubi[1], J Z Al-Busaidi[1], M S Al-Balushi[1], F L Hakkim[2], L Rashan[2], G M Aleemallah[3], A A Al-Jabri[1]

Abstract

Breast and cervical cancers are global health concerns and major cause of deaths among women. Current treatments such as chemotherapy are associated with several drawbacks that limit their effectiveness. Several anticancer remedies have been found with natural products in the past and the search continues for more examples. Cytotoxic natural compounds may have considerable benefits for cancer therapy either in potentiating the impact of chemotherapy or curtailment of harmful effects. Therefore, discovery and identification of new drugs for breast and cervical cancer treatment are of high priority. The present study addressed the potential role of the ALD (*Aucklandia lappa* Decne) in suppressing proliferation of T-47D, HeLa and HEp-2 cells in comparison with the non-cancer HCC1937 BL cell line. Treatment with an ALD extract of T-47D, HeLa, and HEp-2 cells resulted in reduction in cell viability in MMT assays. Furthermore, lyophilized ALD principally suppressed cancer cell line growth and proliferation through induction of either intrinsic or extrinsic apoptotic pathways as demonstrated by significantly suppressed release of LDH, and NO production in a dose-dependent manner, and activation of death receptors in T-47D and HeLa cells but not the HEp-2 cell line. Interestingly, lyophilized ALD significantly ($p<0.005$) repressed the growth of HEp-2 and T-47D cells after treatment for 48hrs while 24hrs treatment significantly suppressed T-47D and HeLa cells. We report for the first time that lyophilized ALD selectively influences apoptosis through alternative apoptotic pathways in both breast and cervical human cancer cells.

Keywords: Breast cancer- cervical cancer- T-47D cell- HeLa cell- LDH- NO- anticancer activity- Oman

Asian Pac J Cancer Prev, **19 (7)**, 1917-1925

Introduction

Cancer is a multifactorial disease resulting from multiple mutations occurred as a result from exposure to environmental carcinogens over numerous years (Levin et al., 2008) leading to mutations in a single cell, which give the cell capacity to grow uncontrollably and get away the normal controls of proliferation (Kittaneh et al., 2013). In 2008, the World Health Organisation (WHO) reported that cancer is one of the main leading causes of 7.6 million deaths around the world. In prosperous nations, around 20% or one in five individuals will die of cancer.

The increasing incidence of cancer has led to a global burden to find many different therapeutic ways including chemotherapy and surgical procedures. The stage of the disease determines the strategy of treatment including surgery, radiation or chemotherapy. A large portion of these modalities is targeting signaling pathways responsible for the proliferation of cell and survival (Domvri et al., 2013). For instance, most of the clinically used cytotoxic anticancer medications, for example, doxorubicin or cisplatin, can trigger apoptosis in cancer cells (Hassan et al., 2014).

The combination of all these drugs in treatment expands the therapeutic ampleness yet may itself instigates a potential threat of toxicity by the medication itself (Lu et al., 2014). In this way, one of the techniques for the deterrent of cancer development incorporates debilitation of anti-apoptotic genes. Consequently, new chemo-preventive agents with the limit of stifling cell proliferation and initiating apoptosis in cancer cells with less or no side effects would be pivotal. Subsequently, apoptosis remains the fundamental focus for cancer treatment by either naturally occurring or synthetic agents (Reed and Pellecchia, 2005). Presently, the focus now is shifted towards plants and animal, i.e., animal-products, sources.

Natural components and their elements have added a significant contribution to many roles in cancer chemotherapeutics. Chemoprevention for example by dietary constituents as utilitarian sustenance has a radiant role in health advancement and rose as a novel approach

*[1]Department of Microbiology and Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, [2]Research Centre, Dhofar University, Salalah, Oman, [3]Primary Health Corporation, Hamad Medical Corporation, Doha, Qatar. *For Correspondence: shyahasson@squ.edu.om*

to control cancers (Kontou et al., 2011) and other diseases (el Agamy et al., 1992; Hasson et al., 2015; Hasson et al., 2010a).

In the field of cytotoxic chemotherapy, numerous plants have provided a source of pharmaceutical products (Ivanova et al., 2005; Mothana et al., 2012; Ryu et al., 2012). A few investigations with natural compounds have demonstrated antioxidant activity in various bioassay systems (Mothana et al., 2012; Chua and Aminah 2013). Besides, chemoprevention by dietary constituents as practical nourishment has an entrenched part in health promotion and has risen as a novel approach to control cancer (Kontou, 2011) and other maladies (El Agamy et al., 1992; Hasson et al., 2010; Hasson et al., 2010b; Hasson et al., 2010c).

The World Health Organisation (WHO) has evaluated that 80% of the world population relies on depends on conventional drug for primary health care. In industrialized nations, the consumers are looking for obvious alternatives to modern medicine with its related threats of side effects and/or tranquilizes enslavement. In the last decade, the WHO health assembly has passed several of resolutions in light of investigation and utilization of traditional medicine. In 2010 the WHO revelation of health accentuated the significance of traditional medicine in achieving primary health care. As indicated by that, the utilization of plant remedies is on an expansion even in the developed nations particularly among youthful generation (Hawkins, 2007).

From what is already known about the richness of the traditional medicine including that of trials (Li et al., 2015), it is perhaps that richest resource base for the health care of mankind, but the efficacy of the most of the traditional remedies is required to be subjected to scientific verification and validation to ensure safety, efficacy, and quality. For example in previous studies the crude extract of the ALD root which is the plant of choice used in this study was found to inhibit the severity of diarrhea induced by some of the investigated bacteria strains (Irshad et al., 2012; Randhir et al., 2004) It is speculated that the extract was able to inhibit electrolyteperme ability in the intestine due to castoroiland/or through the inhibition of prostaglandins release.

Hence, the potential of herbal ALD root extract over the multidrug resistant bacteria isolates (Hasson et al., 2013) has been exploited in this study for cancer prevention and to quell the proliferation of human T47D, HeLa and HEp-2 cells and explores the underlying mechanisms to induce its apoptotic. To do that the influence of ALD crude extract on cancerous cell – Apoptosis assess using different assays.

Materials and Methods

Plant materials

Two hundred and fifty gram of ALD root was purchased from Al-Seeb, General Herbal Market, Muscat, Oman. The plant root was identified at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University. The species for the proposed study was identified and authenticated by Dr. A. Wadieh, Department of Botany, Naser College, in Lahj Governorate, University of Aden, Republic of Yemen. Voucher specimens were deposited at the Pharmacognosy Department, Faculty of Medicine, University of Science and Technology. Sana'a, Republic of Yemen.

Ethanolic extraction

The method of extraction using ethanol was followed as described Hasson et al., (2013). Briefly two hundred and fifty grams of ground samples were soaked in 400 mL of 99.9% ethanol and homogenized in an electric blender for approximately 5 min and incubated at room temperature for 4 days by vigorous stirring. The mixture was then filtered twice using a vacuum. Solvent (ethanol) in the first filtrate allowed to evaporate at room temperature and then re-extracted with another 250 mL of 99.9% ethanol with continuous shaking in the water-bath at 70°C for 6 hours. The mixture was then filtered again and the ethanol was allowed to evaporate at room temperature. The residue (extract) was collected frozen at −70°C followed by lyophilization till dryness. The dried extract was kept at 4°C. Stock solutions of the plant powdered extract were weighed and dissolved in 10% DMSO/PBS. The prepared aliquots were kept at −20°C until utilised.

Cell lines

T-47D (Human breast cancer) Sartorius et al., (1994) and HeLa (Harriet "Henrietta Lacks: An Unsung Hero," Emerge Magazine October 1994. Washington) (cervical cancer) cell lines were obtained from the National Cell Bank of Iran (NCBI). The laryngeal (HEp-2) cancer cell line was obtained from the cell bank - Department of Microbiology and Immunology. Whereas the HCC1937 BL, was given by Dr. Alawi Ubaid, College of Medicine, University of Science and Technology, Republic of Yemen, was used as a negative control (Table 1). T-47D, HeLa, and HCC1937 BL cell lines were cultured in DMEM complete medium supplemented with 10% FBS, bovine insulin (10µg/ml) 1% penicillin, and 1% amphotericin B solution (Fungizone). The HEp-2 cell line was maintained in RPMI-1640 supplemented with 10% FBS, 1% NEAA and 1% penicillin. All cell lines were maintained and grown initially in 75cm^2 tissue culture flasks in a humidified atmosphere under 5% CO_2 at 37°C. Subsequently, cells were seeded onto 24-well cell culture plates according to the need of the assay (see below). All cultures were tested for Mycoplasma contamination and were found to be negative.

Sterility test

To exclude any contamination, the crude plant extract sample was sterilized by filtration through 0.45µm Millipore. Each cell line, was suspended in 2ml of DMEM, plated and allowed to adhere to a culture dish (35 mm). Sterilized extract sample was then added to the culture dish and incubated at 37°C under 5% CO_2 overnight.

Cytotoxicity test

To check the drug activity of repressing/killing the cancerous cell lines and/or cell morphological changes a cytotoxicity assay were used as described by Hasson et al., (2013). Briefly, cells from the T-47D, Hela, HEp-2 and

HCC1937 BL cell cultured plates were harvested in log phase. One ml of media was removed using trypsinization (0.05% trypsin and 0.02% EDTA, in PBS) and then 1 ml of extract was added. After 24 hours incubation, the cells were observed under a microscope using a hemocytometer and the morphological changes were identified.

Influence of ALD crude extract on cancerous cell – Apoptosis

Trypan Blue Test / Dye Exclusion-Assay

Cell viability assay was carried out as described by Hasson et al., (2015), by seeding a total of 5.0 x 10^4 cells\well in 24-well plates. Cells were then incubated for 24 hours in a humidified incubator, 5% CO_2 at 37°C. Plant extract was added and cells were incubated again for 24, 48 and 72 hours. Untreated cells were use used as negative control. Following 24, 48 and 72 hours, the cells were reaped by trypsinization and 20 µL of the cell suspension was mixed with 20 µL of trypan blue. The viable cells were counted utilizing a hemocytometer as an average number of cell viability (10^4/mL). All treatments were performed in triplicate.

Effect of ALD on cellular proliferation: MTT assay Protocol

The efficacy of lyophilised extract to modify cancerous cells growth and proliferation in parallel with the non-cancerous cell line were first evaluated and determined by estimating the limit of reducing enzymes present in viable cells to convert MTT the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) salt to formazan crystals as depicted previously (Mosmann, 1983). The MTT assay was performed a few changes as portrayed by Hasson et al., (2015). Briefly, subsequent to incubating the cells for 24 hours with increasing concentrations (2.5, 10, 20, 30, 50 and 100 µg/mL) of the tested extract in a 96-well cell culture plate (5.0 x 10^4cells per well) (at 37 °C under a 5% CO_2 humidified incubator), the media was suctioned and a 100µL of serum-free medium containing 20µL MTT salt (2.5 µg/mL PBS, pH 7.4), was added to each well. The plate was then brooded assist in a 5% CO_2 incubator at 37 °C for 4hrs in the dark place. To cease the reaction and dissolve the formed crystals, the media were then disposed by inverting the plate; and a 200µL of 10% sodium dodecyl sulphate (SDS), in deionized water, was added to each well and brooded overnight at 37 °C. The absorbance was perused at 650 nm wavelength, using microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Each determination was carried out in triplicate and two independent experiments were carried out. Cell viability was calculated as a percentage and in relation to the control, wells utilizing the equation:

Inhibitory rate (%) = (1−A of experiment well/A of control well) × 100%.

A dose-survival curve was obtained for each experiment. In order to provide a positive one, a Cyclophosphamide reagent (5µg/ml) and the extract was added to the untreated cells to explore the cytotoxicity effect.

Determination of apoptosis using HOECHST 33342 DNA STAINING

Apoptosis was detected further with Hoechst 33342 (Sigma B-2262), and apoptosis percentage was calculated by observing apoptotic features under a fluorescence microscope as well as using the formula below as previously described by Vashishtha et al., (1998).

$$\%\text{Apoptotic cells} = \frac{\text{totalnumbrofcellswithapoptoticnuclei}}{\text{totalnumberofcellscounted}} \times 100$$

Briefly, the assay was carried out, by seeding a total of 2.0 x 10^5 cells\well in 24-well plates in buffered media include 2% fetal calf serum, pH 7.2. Plant extract was added and brooded for 48 hours. Homogenously suctioned and spent media was removed and 1 mL of saline was added and centrifuged at 1500 rpm for 10mins. Pellet was collected and 100µl of Hoechst stain was added to the pellet. The plate was then incubated at 37 °C for 1 hour. As the time is a critical factor due to hat the signal may begin to degrade after ~120 minutes, therefore, 30 minutes would be ideal for a minimum time. After such incubation apoptosis been analyzed under a fluorescent microscope.

Assay for NO production

Nitric oxide (NO) is a receptive radical that plays a critical role in numerous key physiological functions for example, in immune response, neurotransmission, vascular regulation, and apoptosis. NO is quickly oxidized to nitrite and nitrate which are utilized to quantitate NO generation.

The principle of the assay in view of the response that is trailed by a colorimetric detection of nitrite as an azo color product of the Griess reaction. This assay i.e., the Griess reaction is a standout amongst the most broadly utilized assays for NO detection and represents the fundamental reaction of relatively cheap commercial kits for NO estimations. The technique was spearheaded by Johann Peter Griess (1829–1888), a German organic chemist, who was one of the founders of the azo and diazo dye industry. There is two-advance diazotization reaction in the "Griess reaction" in which acidified NO2-produces a nitrosating agent, which responds with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm (Miles, 1996). Briefly, cell lines were plated as above, following 24 hours of added the plant extract, 120 µL of the supernatant was transferred into a new tube contains120µL Griess reagent. The content mixture was mixed vigorously on a shaker at full speed for 15 minutes. Subsequently, 200µL of the sample was added in duplicate on a 96-well plate. The plate was then perused utilizing a microplate reader at absorbance wavelength from 500 to 600 nm to determine the peak wavelength (usually varies between 540 and 545 nm). Moreover, in order to provide a positive one, a Cyclophosphamide reagent (5µg/ml) and the extract was added to the untreated cells to explore the cytotoxicity effect.

Assay for Lactate dehydrogenase (LDH)

LDH is a soluble cytosolic enzyme exist in almost

all eukaryotic cells. The enzymes released into culture medium upon cell demise due to damage to the plasma membrane. The increase of the LDH activity in culture supernatant is correlated to the number of lysed cells. LDH Cytotoxicity Assay gives a colorimetric strategy to quantify LDH activity utilizing a mixed reaction containing lactate, NAD+, diaphorase, and INT. LDH catalyzes the diminishment of NAD+ to NADH within the presence of L-lactate, while the formation of NADH can be estimated in a coupled reaction in which the tetrazolium salt INT is decreased to a red formazan product.

The quantity of the very hued and soluble formazan can be estimated at 490 nm spectrophotometrically. Upon completion of the incubation, 50 µL of the upper phase was harvested from each well. The cells were then lysed with a cell lysis solution for 40 minutes at room temperature and the lysate was collected. LDH activity was estimated utilizing LDH release quantification (Fischer scientific, India) Cytotoxicity Assay Kit, as per producer's guideline. At that point the level of LDH released surrounding environment was determined. In order to provide a positive one, a Cyclophosphamide reagent (5µg/mL) and the extract was added to the untreated cells to explore the cytotoxicity effect.

DNA ladder assay

Cell lines (2×10^6 cells/mL in 6-well plate) were collected after 24h treatment with the plant crude extract at 80 µg/mL. The cells were centrifuged at 1,000 rpm for 15 min and washed with PBS containing 20 mmol/L EDTA. The pellet was lysed in 250µL of lysis buffer (100 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0, and 5% Triton X-100) containing 400 µg/mL DNase-free RNase and incubated at 37 °C for 90 min, followed by incubation with proteinase K (200 µg/mL) at 50 °C for 2h. The DNA was extricated utilizing phenol-chloroform-isoamyl alcohol with a proportion 25: 24: 1 for 1 min and centrifuged at 15,000 rpm for 2 min. The aqueous phase was additionally extricated utilizing chloroform-isoamyl alcohol with a proportion of 24: 1 and centrifuged at 15,000 rpm for 5 min. The DNA was permitted to settled and after that precipitated overnight with chilled alcohol (3v:1v) and 0.3 mol/L sodium acetate at -20 °C. The precipitate was centrifuged at 15,000 rpm for 10 min. The DNA pellet was washed in with 80% Alcohol, air-dried for 30 mins and dissolved in 50 µL of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.4). Evaluation of the quantification of the extracted DNA was finished by a Thermo ScientificTM Nanodrop 2,000c spectrophotometer machine, (NanoDrop, USA). The DNA samples were subjected to electrophoresis "BioRad, Germany" in 1.8 % agarose gel at 50V for 1.5h as depicted by Saxena et al., (2012).

Statistical analysis

Data are presented as mean +/- ± SEM and each experiment was carried out in triplicate and two independent experiments were carried out. All the data were analyzed by using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) for comparison between different treatment groups. Differences were considered statistically significant when P values are<0.05. All computations were made by employing the statistical package for Social Sciences software (SPSS Inc., v.20, Chicago, IL, USA).

Results

The anticancer activity of the extract was affirmed by Trypan blue, Hochest staining, and MTT assays. The negative control HCC1937BL cells indicated high proliferation that has been taken as 100%. In order to provide a positive one, a Cyclophosphamide reagent (5µg/ml) and the extract was added to the untreated cells to explore the cytotoxicity effect.

Viability exclusion assay showed that all cells were treated with different concentration (10, 25, 50, 75 and 100 µg/ml) of the ALD ethanolic extract for 24 h. The viability of T-47D, HeLa, and HEp-2 cells were declined in a measurements subordinate way (Figure 1) and demonstrated cytotoxic activity with the IC_{50} concentration of the extract observed to be between 50µg/mL to 75µg/mL.

ALD extract induced DNA fragmentation: The ALD ethanolic extract was found to be killing the cells by apoptosis with significant results found for both T-47D and HeLa cells after 24 hours (Figure 2). Only a few number of apoptotic cells were seen as shown after 48 hours of treatment. Apoptotic cells are characterized by DNA fragmentation and cytoplasmic shrinkage. Moreover, the assay showed that T-47D and HeLa cells were found to be more sensitive to ALD ethanolic extract than HEp-2 and with no potential effects on HCC1937 BL cells and this finding clearly evidenced antiproliferative potential

Table 1. Cell Lines Used in the Study

	Origin	Cell type	Tissue	Disease	Estrogen Receptor
T47D	46 years/ breast tumor from human	Adherent Epithelial	Luminal A	Human breast tumor	ER+, PR+/-, HER2-
HeLa	31 years Henrietta Lacks cervical cancer cells	epithelial	cervix	Adenocarcinoma	
HEp-2	56 years/ Caucasian male	Epithelial	Larynx	Carcinoma	Not expressed
HCC1937 BL	24 years / Homosapines, Human female caucasian	B lymphoblast; Epsein-Barr virus (EBV) transformed	Peripheral blood	Normal	Not expressed

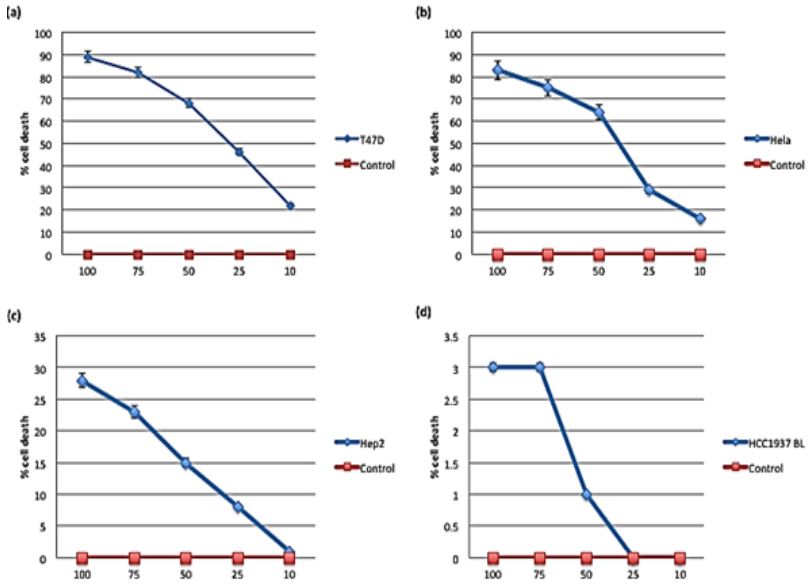

Figure 1. Effect of ALD Extract on Cell Viability of (a) T-47D, (b) HeLa, (c) HEp-2 and (c) HCC1937 BL using Dye Exclusion Test "Typan Blue". Cell viability was tested 24 h after treatment with different concentrations of ALD extract (25, 50, 75, 100µg/mL), using the Dye Exclusion assay. Data are expressed as percent of untreated control, which is set at 100%, ± S.E.M. (n = 3). Arrow represents the significance decline in cell death. (*) =p<0.05 compared to treated cell lines.

selectivity of the ALD ethanolic extract against cancerous cell lines only.

Assessing cell metabolic activity: Results exhibited that the ethanolic crude extract has shown potential anticancer activity through critical cytotoxic activity against human T-47D and HeLa cell lines with LC_{50} of 50µg/mL (Figure 3) (p<0.05), which showed higher cytotoxicity in comparison with the positive control.

As the control HCC1937 BL cells showed high proliferation which has been taken as 100% of viable cells regardless of the crude extract concentration, cell viability of the BT-474, as well as HeLa cell lines, were found to be suppressed significantly over 60% and 70% at a concentration 50µg/mL p<0.006, respectively (Figure 3a and 3b) when compared to those of the HEp-2 and HCC1937 BL cell lines that showed high proliferation. Hence the activity was more selective to BT-474 and HeLa cell lines when compared with HEp-2 and normal cell (HCC1937 BL) with spatial-selectivity indices >0.0 indicating higher activity. These results were further analyzed and confirmed by the following assays.

To affirm the anticancer activity further, LDH effusion were estimated in the treatment condition. The treatment with crude extract significantly stifled the release of LDH at 57.5 ± 3.1% and 61.4 ± 2.3% for 50µg/mL in T-47D and HeLa cell lines, respectively, and lightened the anticancer property and selectivity (Figure 4a and b) in contrast with those of the HEp-2 and HCC1937 BL cell lines. The

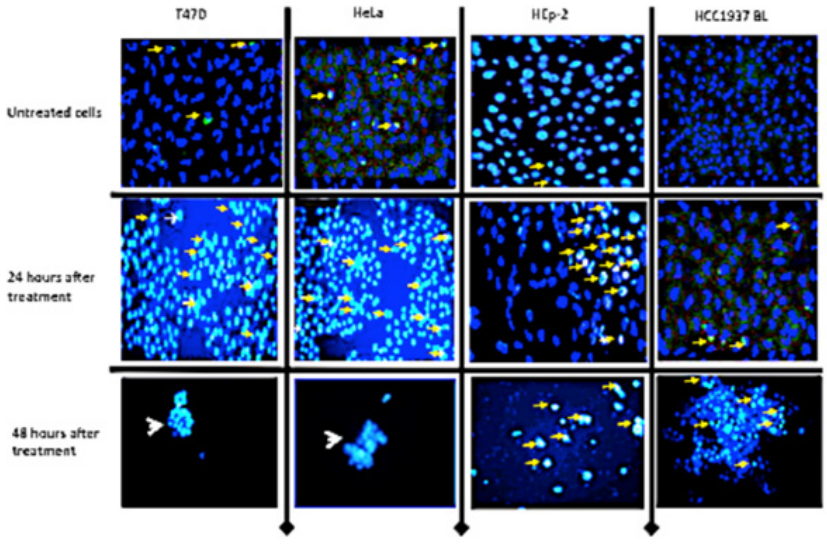

Figure 2. Influence of ALD on Apoptosis of Using Hoechst Stains (a) T-474D and (b) HeLa Cell Proliferation in Contrast with (c) HEp-2 and (d) HCC1937 BL Cell Lines as Observed Under Fluorescence Microscope after 24hrs and 48hrs Following the Addition of the ALD Extract.

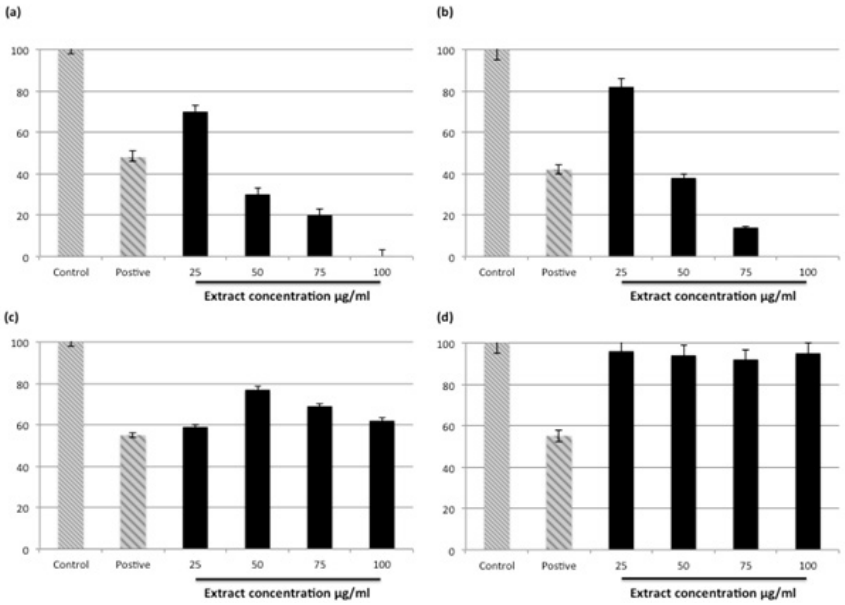

Figure 3. Effect of ALD on (a) T-474D and (b) HeLa Cell Proliferation in Contrast with (c) HEp-2 and (d) HCC1937 BL Using MTT Assay. Cells were seeded and incubated with various concentrations of the ALD extract for 24 hrs. Subsequently, cell proliferation was assessed using MTT assay. Values reflected as % in contrast with the number of cells at 0µg/mL concentration (Mean _+SEM, N=2.5). *p<0.005 and **p < 0.001 compared to the control HCC1937 BL. Values at 0µg/mL concentration was taken as 100% cell proliferation. HCC1937 BL is non-cancer cells, refer to Table 1.

positive control of Cyclophosphamide 5µg/ml lessened the release of LDH up to 43.27 ± 2.2% (Figure 4). The increased NO production (44.56 ± 2.4 µM) was seen in the positive control cells (Figure 5a and b), in any case, the NO level was diminished to 18.32 ± 2.1 µM of nitrate in the negative control. The extract showed critical hindrance variation in the NO production in a dosage subordinate way in all cancerous cell lines in contrast to the negative or normal cell line. Subsequently, the crude extract decreased the NO production in the cells (Figure 5a-c.).

Assessment of apoptosis was further carried out by determining the DNA laddering as a result of DNA fragmentation, characteristic of the late phase of apoptosis (Elmore, 2007) (Figure 6). T-47D, HeLa and HEp-2 cell lines treated with the extracts of ALD ethanolic extract demonstrated characteristics of DNA laddering from samples collected at 36 hours.

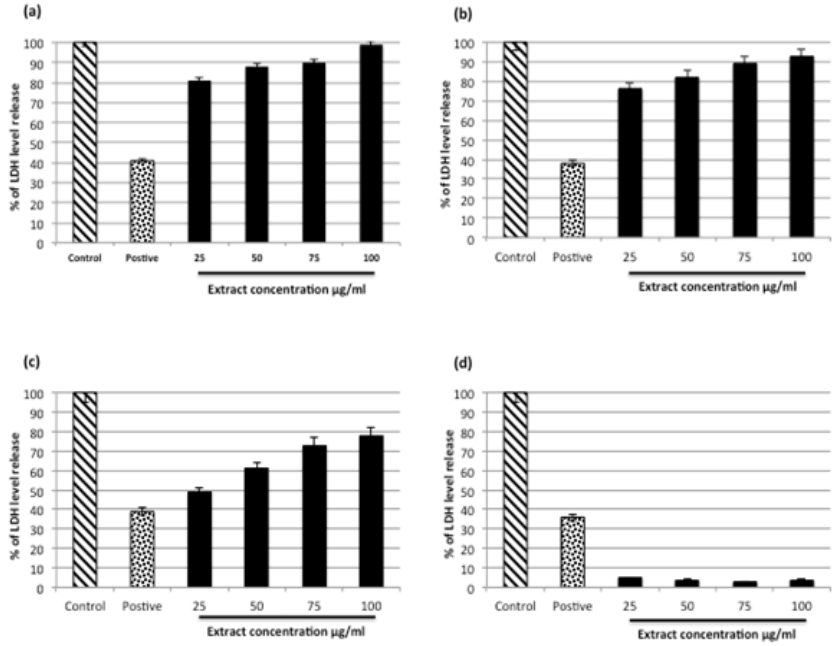

Figure 4. Shows LDH Enzyme Activity for LDH Leakage Test in Percentage (a) T-474D and (b) HeLa Cell Proliferation in Contrast with (c) HEp-2 and (d) HCC1937 BL.

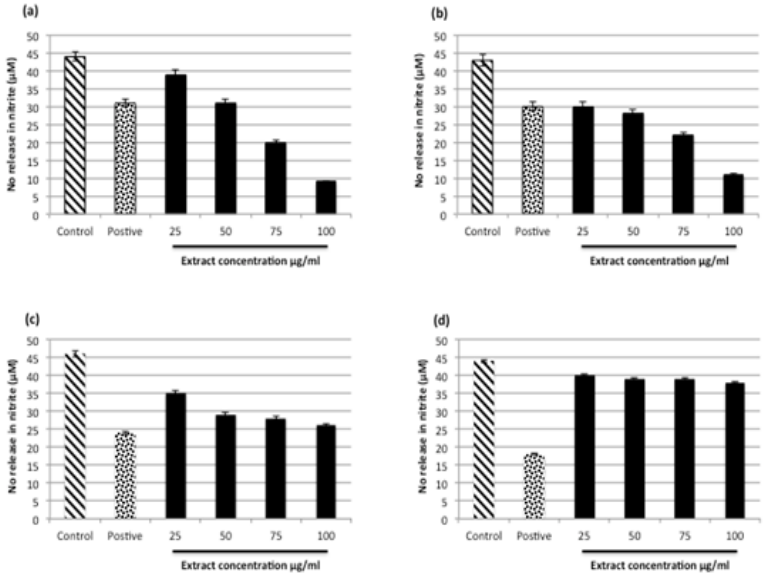

Figure 5. Shows NO Release in Nitrite "µM" Concentration Test in Percentage for (a) T-474D and (b) HeLa Cell Proliferation in Contrast with (c) HEp-2 and (d) HCC1937 BL.

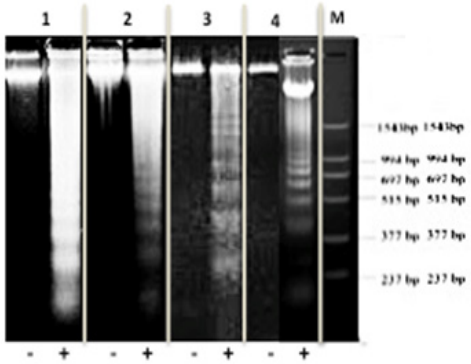

Figure 6. DNA Laddering in (1) T-47D, (2) HeLa, (3) HEp-2 and (4) HCC1937 BL Cell Lines. DNA laddering was visualized in the cell lines following after treatment with of the crude extracts for 36 hours. The + of the HCC1937 BL is related to the addition of a Cyclophosphamide reagent (5µg/mL).

Discussion

In modern medicine, no satisfactory effective chemotherapeutic agent is available to cure cancer in general and breast and cervical cancer in particular. Plant and animal products continue to be viable sources of drugs for the treatment of many diseases. The first step to replace the current chemotherapeutic agents with plant and animal-based agents is the in-vitro anticancer activity assays.

Cancer has turned into the main source of death in the world (Saravanan et al., 2012). In particular, the chronic liver disease has turned into a worldwide health problem; it causes around 20,000 deaths every year (Shakya 2012). Apoptosis, cytolytic cell demise, enhanced ROS generation in mitochondria, effusion of ROS, activation of Kupffer cells and collagen production by stellate cells are the major indications of the pathophysiology of the liver disease condition (Center, 2004) that prompts to liver cancer. Serious antagonistic impacts and limited efficacy of the conventional therapies have prompted to an increase in the dependence on complementary and alternative therapies for the management of liver diseases (Verma and Paul, 2007).

In the present study, we aimed to screen potentiality of the ALD extract of the compoistae family plant species for its anticancer efficacy and growth of human T-47D and HeLa cell lines in comparison with the control HEp-2 and HCC1937 BL cell lines (Table 1). In spite of the fact that the dynamic components of ALD extract involved in apoptosis were not investigated in this study, a few past studies have found that ALD extract contains significantly high amounts of antioxidant and anticancer components (Saraf, 2010). Therefore, it may be accountable for the cytotoxicity effect exerted by the crude extract. The anticancer activity of the ALD against Hepatocellular Carcinoma HepG2 Cells and bladder cancer cells were proven in studies performed by Nair et al., (2014) and Rasul et al., (2013), respectively. Induction of apoptosis by costunolide (a naturally occurring sesquiterpene lactone, first isolated in Saussurea costus roots in 1960) in bladder cancer cells is mediated through ROS generation and mitochondrial dysfunction. However, no studies have been conducted so far to demonstrate its antitumor activity against T-47D and HeLa cancer in parallel with the non-cancerous cell line. The T47D and HeLa cancer cell lines are widely used due to its relatively high steady-state functioning of the free radical production and antioxidant defenses; therefore, variations of responses at different conditions are easily detected (Alia et al., 2006).

The current study shows that the crude ethanolic extract caused potential cytotoxicity effect against the T-47D and HeLa cell lines after 24hrs (Figure 3). Such toxicity also was found to be against the HEp-2 cell line but at 48hrs ($p< 0.05$). This may be explained by estrogen receptor expression differences between the three cell lines. Since estrogen receptor is expressed by T-47D and HeLa cell lines but not by HEp-2. These findings were found in agreement of what has been found with the camel milk (Hasson et al., 2015). The explanation may speculate

that the main target for the ALD to initiate apoptosis in T-47D and HeLa cell lines is the estrogen receptor whereas an alternative non-estrogen receptors pathway may have mediated apoptosis in HEp-2 cell line. This was confirmed further by the low in cell density observed using the Hoechst 33342 DNA Staining assay (Figure 2).

Excessive generation of the radical oxygen scavenger (ROS) and other radicals can damage proteins, carbohydrates, polyunsaturated fatty acids and DNA and lead to oxidative stress for certain cancers (Ebenharder and Grünhage, 2003). Provoke cytotoxic effects in a time- and subordinate way and elevated reactive oxygen species (ROS) level in many human cancer cells such as colon cancer 'colo 205' cells following exposure to capsaicin were reported (Lu, 2010). Nitric oxide at adequate levels is involved in the normal function of organs whereas disturbance of its release can cause a destruction of target tissue amid inflammation and septic shock (Saravanan et al., 2012). The ethanolic extract of ALD showed significant NO inhibitory effect (53.5%) in macrophages (Chen and Kang, 2013). LDH is a more reliable and precise marker of cytotoxicity n light of the fact that damaged cells are fragmented completely during the course of prolonged incubation with substances (Asirvatham et al., 2013). Malondialdehyde, being a major breakdown product of lipid peroxides, is a useful index of lipid peroxidation (Saravanan et al., 2013). Enhanced hepatic MDA, a major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acids in the cell membrane, reflects a causal role of lipid peroxidation in liver damage (You et al., 2010). Several studies showed that exposure of cancer cell lines to capsaicin induces cell cycle arrest at the G1 phase. Lee et al., (2000) demonstrated the apoptosis mechanism by capsaicin in A172 human glioblastoma cells. According to Hugan et al., (2009) capsaicin induces apoptosis through elevating the levels of intracellular ROS and Ca^{2+}, promoting the levels of Bax, GADD153, and GRP78, decreasing membrane potential, Bcl-2 XIAP and CIAPI. The situation is not different from that as we predict that the increasing caspase – 3 activations in both T-47D and HeLa cells speculate similar profile. ROS play a significant role in the induction of apoptosis in many cancerous cells like HepG2 cells by capsaicin. In our investigation, the ethanolic extract of ALD also induced apoptosis in T-47D, HeLa and HEp-2 cells with the lower extent of the latter cell line. Thus the study confirmed the anticancer efficacy of the ALD ethanolic extract, by modulating the free radicals release and also established anti-inflammatory potential.

Induction of apoptosis was further confirmed by demonstration DNA Laddering. DNA fragmentation is a well-known biochemical feature of apoptosis. One of the numerous proteins cleaved by caspase 3 is DFF45. Upon cleavage, DFF45 releases and activates the endonuclease DFF40, which breaks DNA strands in the internucleosomal regions, producing fragments of various lengths; these fragments form a unique laddering pattern.

Interestingly, the HEp-2 cells treated with the extracts of ALD ethanolic extract, which had apoptotic cells higher than 28% in contrast to T-47D and HeLa, also showed DNA laddering (Figure 6 line 3). It should be noted that the extract of the ALD was very selective as possessed low cytotoxicity to HEp-2 cells (SI = 9.6) as compared to its cytotoxicity in the T-47D and HeLa cells. Hence, these findings indicating that ALD ethanolic extract-induced apoptotic DNA fragmentation (Figure 4d).

In conclusion, our findings clearly demonstrated that exposure of T-47D, HeLa and HEp-2 cells to ALD ethanolic extract, reduces cell viability. The extract significantly suppressed the release of LD and NO production in a dose-dependent manner. In summary, the present investigation suggests that ethanolic extract of ALD has a potential selectivity as an anticancer agent. Since this study have proven that the ALD plant has anti-cancer properties, perhaps next step would be to demonstrate its anti-tumorigenic properties in tumor-bearing mice in parallel with a sophisticated molecular prospect screening as well as analyzing caspase activation.

Conflict of interest statement

We declare that we have no conflict of interest.

Funding

This work was supported by SQU –Research Methodology internal Grant 2016/2017 Grant from College of Medicine and Health Sciences, Sultan Qaboos University. Sultanate of Oman.

Acknowledgments

We would like to thank SQU for their internal grant support as well as the animal house staff and immunology lab staff for their continuous and generous help. Special thanks to Ms. Khazina Al Harthy - Immunology Laboratory, SQU for her technical support.

References

Alia M, Mateos R, Ramos S, et al (2006). Influence of quercetin and rutin on growth and antioxidant defense system of a human hepatoma cell line (HepG2). *Eur J Nutr*, **45**, 19-28.

Asirvatham R, Christina AJ, Murali A (2013). In vitro antioxidant and anticancer activity studies on Drosera Indica L. (Droseraceae). *Adv Pharm Bull*, **3**, 115-20.

Center SA (2004). Metabolic, antioxidant, nutraceutical, probiotic, and herbal therapies relating to the management of hepatobiliary disorders. *Vet Clin Small Anim Pract*, **34**, 67–172.

Chen L, Kang YH (2013). Anti-inflammatory and antioxidant activities of red pepper (Capsicum annuum L.) stalk extracts: Comparison of pericarp and placenta extracts. *J Funct Foods*, **5**, 1724-31.

Chua HP, Aminah A (2013). Determination of antioxidant activities of dried kacangma (Leonurus sibiricus) extract in three bioassay systems. *JTAFS*, **41**, 221-9.

Domvri K, Zarogoulidis P, Darwiche K, et al (2013). Molecular targeted drugs and biomarkers in NSCLC, the evolving role of individualized therapy. *J Cancer*, **4**, 736–54.

Ebenharder R, Grünhage D (2003). Free radical scavenging abilities of flavonoids as mechanism of protection against mutagenicity induced by tert-butyl hydroperoxide or cumene hydroperoxide in Salmonella typhimuriumTA10. *Mutat Res*, **540**, 1-18.

El Agamy, EI, Ruppanner R, Ismail A, Champagne CP, Assaf

R (1992). Antibacterial and antiviral activity of camel milk protective proteins. *J Dairy Res*, **59**, 169- 75.

Elmore S (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol*, **35**, 495–516.

Hasson SS, Al-Balushi MS, Said EA, et al (2012). Neutralisation of local haemorrhage induced by the saw-scaled viper *Echis carinatus sochureki* venom using ethanolic extract of Hibiscus aethiopicus L. *eCAM*, **2012**, 8.

Hasson SS, Al-Busaidi JZ, Al-Qarni ZA (2015). In vitro apoptosis triggering in the BT-474 human breast cancer cell line by lyophilised camel's milk. *Asian Pac J Cancer Prev*, **16**, 6651-61.

Hasson SS, Al-Jabri AA, Sallam TA, et al (2010). Antisnake venom activity of *hibiscus aethiopicus* L. against Echis ocellatus and Naja n. nigricollis. *J Toxicol*, **2010**, e837864.

Hasson SS, Mothana RA, Sallam TA (2010). Serine protease variants encoded by *Echis ocellatus* venom gland cDNA: cloning and sequencing analysis. *J Biomed Biotechnol*, **2010**, 1-12.

Hasson SSA, Al-Blushi MS, Alharthy K (2013). Evaluation of anti-resistant activity of *Auklandia (Saussurea lappa)* root against some human pathogens. *APJTB*, **3**, 557–62.

Hawkins JP (2007). Health care in Maya Guatemala: Confronting Medical Pluralism in a Developing country. University of Oklahoma Press, Medical, pp 268.

Hogan MV, Grant RE, Lee L (2009). Jr Analgesia for total hip and knee arthroplasty: a review of lumbar plexus, femoral, and sciatic nerve blocks. *Am J Orthop (Belle Mead NJ)*, **38**, 129–33.

Irshad S, Mahmood M, Perveen F (2012). In-vitro anti-bacterial activities of three medicinal plants using agar well diffusion method. *Res J Bio*, **2**, 1–8.

Ivanova D, Gerova D, Chervenkov T, Yankova T (2005) Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *J Ethnopharmacol*, **96**, 145–50.

Kittaneh M, Montero AJ, Glück S (2013). Molecular profiling for breast cancer: a comprehensive review. *Biomarkers Cancer*, **5**, 61–70.

Kontou N, Psaltopoulou T, Panagiotakos D, Dimopoulos MA, Linos A (2011). The mediterranean diet in cancer prevention: a review. *J Med Food*, **14**, 1065–78.

Lee YS, Nam DH, Kim JA (2000). Induction of apoptosis by capsaicin in A172 human glioblastoma cells. *Cancer Lett*, **161**, 121-30.

Levin B, Lieberman DA, McFarland B, et al (2008). Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology*, **134**, 1570-95.

Li LP, Zhang BG, Xiao PG, et al (2015). Native medicinal plant richness distribution patterns and environmental determinants of Xinjiang, Northwest China. *Chin Herbal Med*, **7**, 45–53.

Lu HF, Chen YL, Yang JS (2010). Antitumor activity of capsaicin on human colon cancer cells in vitro and colo 205 tumor xenografts in vivo. *J Agric Food Chem*, **58**, 12999–3005.

Lu J, Huang G, Li HP, et al (2014). Prediction of cancer drugs by chemical-chemical interactions. *PLoS One*, **9**, e87791.

Miles MA, Wink DA, Cook JC, Grisham MB (1996). Determination of nitric oxide using fluorescence spectroscopy. *Methods Enzymol*, **268**, 105–20.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunological Method*, **65**, 55-63.

Mothana RA, Alsaid MS, Hasson SS, et al (2012). Antimicrobial and antioxidant activities and gas chromatography mass spectrometry (GC/MS) analysis of the essential oils of Ajuga bracteosa Wall. ex Benth. and Lavandula dentata L. growing wild in Yemen. *J Med Plants Res*, **6**, 3066–71.

Nair SVG, Hettihewa M, Rupasinghe HP (2014). Apoptotic and inhibitory effects on cell proliferation of hepatocellular carcinoma HepG2 cells by methanol leaf extract of Costus speciosus. *Biomed Res Int*, **2014**, 637098.

Randhir R, Lin YT, Shetty K (2004). Phenolic, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asian Pac J Clin Nutr*, **13**, 295–7.

Rasul A, Bao R, Malhi M, et al (2013). Induction of apoptosis by costunolide in bladder cancer cells is mediated through ROS generation and mitochondrial dysfunction. *Molecules*, **18**, 1418–33.

Reed JC, Pellecchia M (2005). Apoptosis-based therapies for hematologic malignancies. *Blood*, **106**, 408–18.

Ryu NH, Park KR, Kim SM, et al (2012). A hexane fraction of Guava leaves (Psidium guajava L.) induces anticancer activity by suppressing AKT/Mammalian target of rapamycin/Ribosomal p70 S6 Kinase in human prostate cancer cells. *J Med Food*, **15**, 231–41.

Saraf A (2010). Phytochemical and antimicrobial studies of medicinal plant costus speciosus (Koen.). *Eur J Chem*, **7**, 405–13,

Saravanan S, Babu NP, Pandikumar P, et al (2012). Immunomodulatory potential of Enicostema axillare (Lam.) A. Raynal, a traditional medicinal plant. *J Ethnopharmacol*, **140**, 239–46.

Saravanan S, Hairul Islam VI, Gabriel Paulraj M, Ignacimuthu S (2011). Protective effect of Enicostemma axillere on mutagenicity of Salmonella typhimurium by increasing free radical scavenging abilities. *Pharmaceutical Biol*, **50**, 698-706.

Saravanan S, Pandikumar P, Pazhanivel N, Gabriel Paulraj M, Ignacimuthu S (2013). Hepatoprotective role of Abelmoschus esculentus (Linn.) Moench on carbon tetrachloride induced liver injury. *Toxicol Mech Methods*, **23**, 528–36.

Saxena S, Singh SK, Lakshmi MG, et al (2012). Proteomic analysis of zebrafish caudal fin regeneration. *Mol Cell Proteomics*, **11**, 014118.

Shakya AK, Sharma N, Saxena M, Shrivastava S, Shukla S (2012). Evaluation of the antioxidant and hepatoprotective effect of Majoon-e-Dabeed- ul-ward against carbon tetrachloride-induced liver injury. *Exp Toxicol Pathol*, **64**, 767–73.

Vashishtha SC, Nazarali AJ, Dimmock JR (1998). Application of Fluorescence Microscopy to Measure Apoptosis in Jurkat T Cells After Treatment with a New Investigational Anticancer Agent (N.C.1213). *Cell Mol Neurobiol*, **18**, 437-45.

Verma S, Thuluvath PJ (2007). Complementary and alternative medicine in hepatology: review of the evidence of efficacy. *Clin Gastroenterol Hepatol*, **5**, 408–16.

Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N (2014). Apoptosis and molecular targeting therapy in cancer. *Biomed Res Int*, **2014**, 150845.

You Y, Yoo S, Yoon HG, et al (2010). In vitro and in vivo hepatoprotective effects of the aqueous extract from Taraxacum officinale (dandelion) root against alcohol-induced oxidative stress. *Food Chem Toxicol*, **48**, 1632–37.