

## RESEARCH COMMUNICATION

# Apoptosis-Induced Cell Death due to Oleanolic Acid in HaCaT Keratinocyte Cells -a Proof-of-Principle Approach for Chemopreventive Drug Development

V Cijo George<sup>1</sup>, DR Naveen Kumar<sup>1</sup>, PK Suresh<sup>1</sup>, R Ashok Kumar<sup>2\*</sup>

### Abstract

Oleanolic acid (OA) is a naturally occurring triterpenoid in food materials and is a component of the leaves and roots of *Olea europaea*, *Viscum album* L., *Aralia chinensis* L. and more than 120 other plant species. There are several reports validating its antitumor activity against different cancer cells apart from its hepatoprotective activity. However, antitumor activity against skin cancer has not been studied well thus far. Hence the present study of effects of OA against HaCaT (immortalized keratinocyte) cells - a cell-based epithelial model system for toxicity/ethnopharmacology-based studies - was conducted. Radical scavenging activity (DPPH•) and FRAP were determined spectrophotometrically. Proliferation was assessed by XTT assay at 24, 48 and 72 hrs with exposure to various concentrations (12.5-200  $\mu$ M) of OA. Apoptotic induction potential of OA was demonstrated using a cellular DNA fragmentation ELISA method. Morphological studies were also carried out to elucidate its antitumor potential. The results revealed that OA induces apoptosis by altering cellular morphology as well as DNA integrity in HaCaT cells in a dose-dependent manner, with comparatively low cytotoxicity. The moderate toxicity observed in HaCaT cells, with induction of apoptosis, possibly suggests greater involvement of programmed-cell death-mediated mechanisms. We conclude that OA has relatively low toxicity and has the potential to induce apoptosis in HaCaT cells and hence provides a substantial and sound scientific basis for further validation studies.

**Keywords:** Triterpenoid - Oleanolic acid- HaCaT - XTT - DNA fragmentation ELISA

*Asian Pacific J Cancer Prev*, 13, 2015-2020

### Introduction

Naturally occurring potent compounds isolated from medicinal plants has opened an era for the treatment of various diseases with moderate side effects than chemically synthesised ones. So far, cancer chemotherapy has employed various classes of natural products including flavonoids, flavones, isoterpenoids, alkaloids, etc., which shows promising effects both in *in vitro* and *in vivo* studies (Patel, 2011). The side effects possessed by chemotherapeutic drugs continue to be a serious issue in current treatment and a cause for concern, which needs to be addressed. One of the threats in chemotherapy is the destruction of normal cells when undergoing treatment. Most of the anticancer drugs are highly cytotoxic in nature (Stephen & David, 2005) and hence may influence the normal cell turn over mechanisms. Hence researchers now focussed more on non/less-cytotoxic drugs of natural origin which can be developed as an anticancer agent.

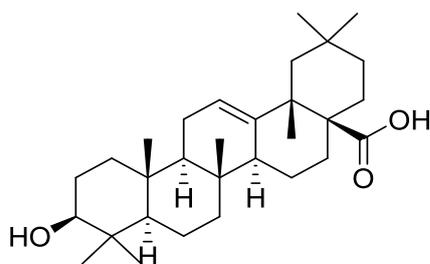
Apoptosis is a natural mechanism which controls cellular division and progression in human body. Uncontrolled cell division can result in cancer progression,

in sites, which lack normal apoptotic mechanism. These cells can be triggered by the use of natural products, thereby prompting the cells to die. Apoptotic induction has been a new target for innovative mechanisms-based drug discovery (Senderowicz et al., 2004; Rajkumar et al., 2011; Huang et al., 2012). Various plant compounds were reported to induce apoptosis and thus can target cancer cells towards death (Fulda, 2008; Ali et al., 2011; Deng et al., 2011). Specifically, pentacyclic triterpenoids exert their antitumor activity through different mechanisms, one of which is apoptosis induction (Masaaki et al., 2008; Bernard & Olayinka, 2010). Hence its evident that, such naturally occurring plant compounds plays a vital role in cancer prevention.

Oleanolic acid (OA) is a naturally occurring triterpenoid with chemical name of 3  $\beta$ -hydroxy-olea-12-en-28-oic acid (Figure 1) which are found in various plants including the leaves and roots of *Olea europaea*, *Viscum album* L., *Aralia chinensis* L., *Ligustrum lucidum* Ait., in various fruits and herbs (Perez-Camino et al., 1999; Liang et al., 2011). OA have been found to be active in various stages of tumor development, including inhibition

<sup>1</sup>School of Bio Sciences and Technology, VIT University, Vellore, <sup>2</sup>Department of Zoology, Government Arts College, Dharmapuri, India

\*For correspondence: ashoku\_2000@yahoo.com



**Figure 1. The Structure of 3  $\beta$ -hydroxy-olea-12-en-28-oic Acid (Oleanolic Acid)**

of tumor promotion, invasion and metastasis (Shishodia et al., 2003; Miao et al., 2007). Further, its anticancer effects has been reported in several cancer cell lines (Li et al., 1999; Chiang et al., 2005; Pui et al., 2009; Biswas et al., 2010), suggested its antitumor activity. However, being such a promising anticancer metabolite, its activity against epidermal keratinocytes was not being studied thus far to the best of our knowledge. Hence in this present study, we investigated the potency of OA on HaCaT cells for its possible antitumor effects by specifically targeting apoptosis pathway, by its ability to induce DNA fragmentation as measured by ELISA.

## Materials and Methods

### Chemicals and reagents

Ascorbic acid (AA), phenazine methosulphate (PMS) (also known as N-methylphenazonium methosulfate), Dulbecco's Modified Eagle Medium (DMEM) with (4.5 g/l of glucose and L-glutamine), Nutrient mixture F-12 Ham (with L-glutamine), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's phosphate buffered saline (PBS) ( $\text{Ca}^{2+}/\text{Mg}^{2+}$  free) were purchased from Himedia Laboratories Pvt. Ltd. (India). XTT {2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} was obtained from Sigma Chemical Co. (St. Louis, MO, USA). HaCaT (human immortalized keratinocyte) cells were obtained from National Centre for Cell Science (NCCS, Pune, India). Apoptotic kit, Cellular DNA fragmentation ELISA (# 11 585 045 001) -Roche Diagnostics, Germany. The remaining chemicals and solvents used were of standard analytical grade.

### Preparation of drug

Stock solutions of OA were prepared at 25.77 mM in 100% dimethyl sulfoxide (DMSO). Final concentrations of DMSO, prepared by serial dilution, never exceeded 1% (v/v). Freshly prepared solutions were sterilized using 0.22  $\mu\text{m}$  syringe filter and used for each experiment.

### DPPH• assay

The radical scavenging activities of OA were measured by DPPH• assay as described previously (Xi et al., 2009). 900  $\mu\text{l}$  of 0.1 mM DPPH radical solution was prepared in ethanol, and then mixed with 100  $\mu\text{l}$  of the sample dissolved in DMSO and kept in the dark for 30 min. Absorbance was recorded at 517 nm using a Cary 50 UV-Vis spectrophotometer (Varian, Inc., CA, USA). Ascorbic acid (expressed in  $\mu\text{g}$ ) was used as a positive

control. The reduction in the absorbance of the DPPH• solution indicated the free radical scavenging activities of OA. DMSO without the sample was employed as a control. The level of percentage scavenging of DPPH• by the extracts was calculated according to the following formula:

$$\% \text{ of Scavenging} = \frac{[\text{Abs (Control)} - \text{Abs (Sample)}]}{\text{Abs (Control)}} \times 100.$$

### FRAP assay

FRAP assay was carried out by the protocol of Benzie and Strain (1996) with some modifications. The stock solutions prepared were 300 mM acetate buffer (3.1 g  $\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$  and 16.8 ml  $\text{C}_2\text{H}_4\text{O}_2$ ; pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl) and 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution. Working FRAP solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml TPTZ solution and 2.5 ml of  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution. The mixture is then warmed at 37  $^\circ\text{C}$ . 150  $\mu\text{l}$  of different concentrations of OA (12.5, 25, 50, 100 and 200  $\mu\text{M}$ ) were allowed to mix with 2.85 ml of FRAP solution. The mixture is further incubated at dark for 30 min. Absorbance was read at 593 nm. Percentage  $\text{Fe}^{3+}$  reduction (to  $\text{Fe}^{2+}$ ) were calculated by a  $\text{FeSO}_4$  standard calibration curve. Percentage scavenging was also evaluated with respect to ascorbic acid equivalence (AAE) in  $\mu\text{g}$ .

### Cell culture

HaCaT cultures were initiated and propagated in DMEM with Nutrient mixture F-12 Ham in 1:1 ratio respectively. The cells were supplemented with 10% fetal bovine serum in a humidified atmosphere with 5%  $\text{CO}_2$  at 37  $^\circ\text{C}$ . Cells were grown in polystyrene-coated T25 (25  $\text{cm}^2$ ) cell culture flasks, and were harvested in the logarithmic phase of growth. The cells were maintained at the above-mentioned culture conditions for all experiments.

### Proliferation assay: XTT

Cell proliferation assay-XTT was performed on HaCaT cells as described by Weislow et al. (1989) with minor modifications.  $1 \times 10^4$  cells, in 200  $\mu\text{l}$  of medium, were seeded in each well of a 96-well plate and incubated at 37  $^\circ\text{C}$  for a period of 24 hrs. The media were then replaced with 200  $\mu\text{l}$  of fresh media containing varying concentrations of OA (12.5, 25, 50, 100 and 200  $\mu\text{M}$ ). The plate was then re-incubated, maintaining the same conditions, for 24, 48 and 74 hrs after which, medium containing the drug was substituted by 200  $\mu\text{l}$  of fresh medium. 50  $\mu\text{l}$  of XTT reagent prepared in medium (0.6 mg/ml) containing 25  $\mu\text{M}$  of PMS was then added to all the wells. The plate was incubated in dark humid conditions at 37  $^\circ\text{C}$  for 4 hrs. After incubation, the orange colored complex formed was read at 450 nm using a Dynex Opsys MR<sup>TM</sup> Microplate Reader (Dynex Technologies, VA, USA) with a 630 nm reference filter. Wells containing cells without the OA served as the control and wells containing only culture medium and XTT reagent served as the blank.

Percentage cytotoxicity of the extracts was calculated by using the formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{OD of control} - \text{OD of treated cells}) \times 100}{\text{OD of control}}$$

### Morphological studies

Inverted phase contrast microscopic studies: HaCaT cells ( $1 \times 10^5$ ) were seeded in 6-well plates and were then incubated overnight at 37 °C in CO<sub>2</sub> incubator until 70 % confluency. The cells were then exposed to highest concentration of OA (200 μM) as taken in this study for 24, 48 and 72 hrs respectively, to observe cellular morphological changes. Morphological and confluency changes in both control and treated groups were observed using an inverted phase contrast microscope at 20 X magnification.

### Apoptosis detection

Cellular DNA fragmentation ELISA: The potential of OA to induce apoptosis was studied using cellular DNA fragmentation ELISA kit as per the supplier's instructions. Briefly, HaCaT cells were labelled with 10 μM BrdU at  $1 \times 10^5$  cells/ml density. 100 μl of these BrdU-labelled cells in culture medium were treated with varying concentrations of OA for a period of 4 hrs. The cells were then lysed and the apoptotic fragments were obtained after centrifugation at 1500 rpm for 10 min and subjected for ELISA procedure. 100 μl of this obtained sample was transferred to anti-DNA coated 96-well, flat-bottom microplates (MTPs). The plates were incubated for 90 min at 15-25 °C. The DNA was then denatured by microwave irradiation (500 W for 5 min) followed by addition of 100 μl anti-BrdU-POD conjugate solution. The plates were further incubated for 90 min and were washed 3 times with wash buffer (1X). 100 μl substrate (TMB) solution was then added for colour development. The absorbance was read at 450 nm after addition of 25 μl of stop solution.

### Statistical analysis

All the analytical experiments were carried out in triplicates (n=3). Data were presented as mean ± standard deviation (SD). MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations. Significant differences between groups were determined at  $p < 0.05$ .

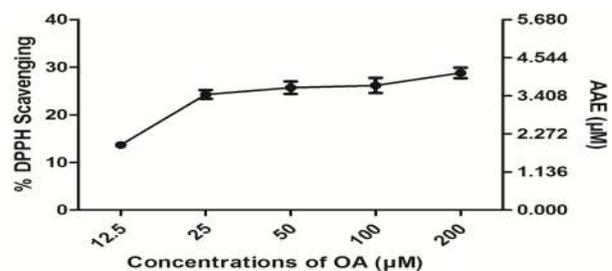
## Results

### DPPH assay

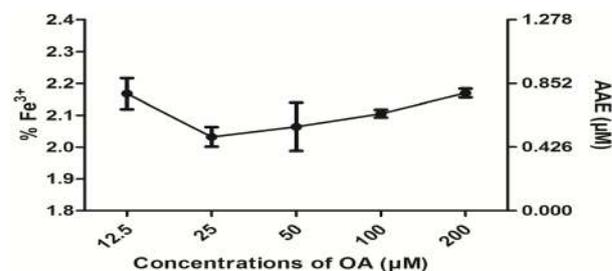
Radical scavenging ability was determined by DPPH• assay read at 517 nm. DPPH is a kind of stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity (Blois, 1958). Qualitative data obtained showed that OA has moderate radical scavenging potential with 200 μM resulted 28.82% of ROS scavenging which is equal to 7.4 μg of AAE in μg used as positive control.

### FRAP assay

The ability of OA to reduce ferric (III) iron to ferrous



**Figure 2. OA Shows Moderate Scavenging Potentials of Free Radicals in a Dose-Dependent Manner with AAE as Standard.** Values given are as mean ± SD (n=3,  $p < 0.05$ ).



**Figure 3. Percentage Fe<sup>3+</sup> Reducing Potential of OA along with AAE (μg).** Data expressed as mean ± SD of n = 3 samples ( $p < 0.05$ ).

(II) iron was determined by FRAP reagent. Figure 3 shows the % Fe<sup>3+</sup> reduction by OA along with ascorbic acid equivalence in μg.

### XTT assay

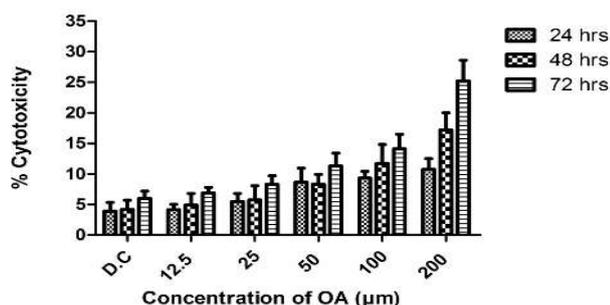
Cell viability was measured at 450 nm for different concentrations of OA after 24, 48 and 72 hrs. There is a moderate cytotoxicity possessed by this triterpenoid in HaCaT cells. However, there is a slight increase in toxicity with higher duration of exposure. At the higher concentration of 200 μM, 26.2 % of toxicity was observed after 72 hrs of incubation which infers that the drug is moderately cytotoxic in nature to HaCaT cells.

### Morphological studies

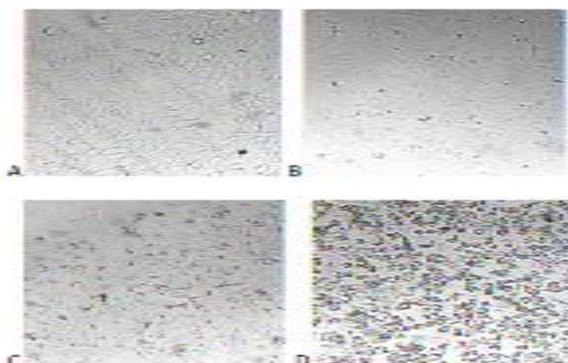
Cell death is characterised by changes in morphology which includes cell membrane disruption, chromosome condensation and apoptotic bodies formation (Susan, 2007; Engin et al., 2011). The highest concentration (200 μM) of OA was exposed to HaCaT cells for 24, 48 and 72 hrs to study its effect on cell membrane with respect to the control. Morphological changes in the cells, at various exposure times and a decline in cell number, were observed under 20X magnification (inverted phase contrast microscope).

### Apoptosis detection

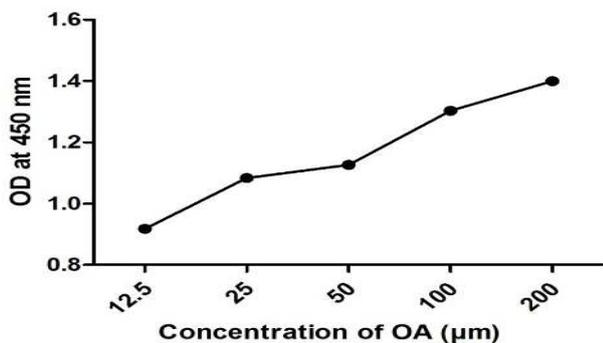
Apoptosis is a physiological cell suicide program that helps to maintain homeostasis, in which cell death naturally occurs during tissue turnover (Samali et al., 1996; Andreas et al., 2011). Cells undergoing apoptosis usually show several cellular changes, including formation of plasma membrane blebs, reduction in cell volume, chromatin condensation, and DNA fragmentation, but cells retain their membrane and organelle integrity (Yu-Fang and Yue-Hwa, 2004; Oliver et al., 2011). DNA



**Figure 4. Cytotoxicity of OA on HaCaT Estimated by XTT Assay after 24, 48 and 72 hrs Incubation.** Values given are as mean ± SD (n=3, p < 0.05). D.C = DMSO control.



**Figure 5. A is the Control HaCaT Cells with 70% Confluency. B is the Treated Cells which Show Changes in Cell Structure at 24 hrs of Drug Exposure. C is the Cells Treated with OA for 48 hrs and D is the Treated Cells which Undergo Cell Death Vigorously at 72 hrs of Drug Exposure Through Cellular Destruction**



**Figure 6. Apoptosis Induction in HaCaT Cells by OA Demonstrated by Cellular DNA Fragmentation ELISA with Increasing Number of DNA Fragments**

fragmentation in HaCaT cells were measured by ELISA at 450 nm. OA shows a dose-dependent increase in the number of apoptotic fragments at various concentrations (Figure 6) there by confirming its ability to induce apoptosis.

**Discussion**

The need to develop more effective and less toxic anticancer drugs has prompted researchers to explore new sources of pharmacologically active compounds (Hasmah et al., 2010). Recent literature reports have indicated that many anticancer drugs /cancer chemo preventive agents

act through the induction of apoptosis to prevent tumor promotion/ progression and this method is successfully adopted in cancer chemotherapy. Naturally occurring triterpenoids have gained attention amongst drug discoverers, in recent years with its numerous potentials in anticancer research (Mark, 2008).

In the present study, the ability of OA in HaCaT cells was investigated by XTT assay at various time intervals. The radical scavenging ability of OA was evaluated prior to XTT by DPPH• and FRAP assay. A moderate free radical quenching shown by OA, (Figure 2 & Figure 3) at different concentrations, suggested its scavenging effect when compared to ascorbic acid (positive control). The stoichiometry of reactions of OA in DPPH• and Fe<sup>+3</sup> radicals were distinctively dissimilar, which may be referred to as a reason for the difference in scavenging potentials as observed in these assays (George et al., 2012). XTT result (Figure 4) showed only a moderate cytotoxicity on HaCaT cells after 24, 48 and 72 hrs of incubation. However, OA was found to be very cytotoxic in the studies conducted previously (Ko et al., 1999; Jie et al., 2002; Fu et al., 2005) in various cancer cell lines.

Pre-treatment with ursolic acid increases the activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in UVB-irradiated lymphocytes (Ramachandran & Rajendra, 2008). Similar result, was also reported in HaCaT cells, and correlated with an increase in glutathione levels (Hitoshi et al., 2008). However, in our studies, after exposure of 200 μM for 72 hrs, only 26.2% cytotoxicity was observed which suggests that the drug is only moderately cytotoxic in nature, probably due to the increase in antioxidant enzymes in human immortalized keratinocytes and/or modulation of cellular thiols. Further, our work is consistent with the empirical data reported by other *in vitro* studies using the same cell line (Liu, 2005). Being less cytotoxic in nature, OA may be a better candidate, when delivered at the site, for inducing cell death by apoptosis.

Morphological changes of apoptosis found in most cell types initially starts with a reduction in cell volume and condensation of the nucleus (Vinay et al., 2011). These changes have been reported to enable the intracellular organelles, such as mitochondria, to retain their normal morphology (Antonio et al., 2010; Bin et al., 2011). This change is followed by plasma membrane blebbing and nuclear fragmentation to form apoptotic bodies (Kataoka, 1996; Oliver et al., 2011). Hence the cell morphology was studied by using inverted microscope after exposure of 200 μM OA at various time intervals. A remarkable cellular refraction was observed during various periods of drug exposure which includes reduction in cell number and cell wall rupture. Cellular damage, at the DNA level, was also done using a ELISA-based kit which detects fragmentation of this biomolecule. These results showed a dose-dependent increase in DNA fragments with increasing concentration of OA, thereby providing evidence for its apoptotic potential. However, the precise mechanism/pathway by which OA induces apoptosis is still to be elucidated.

In conclusion, the study concludes that, OA has the

potential to induce DNA fragments in immortalized keratinocytes, thereby prompting cell death through apoptosis and thus can be subjected for further investigation.

## Acknowledgements

We are thankful to the management of VIT University, Vellore, Tamil Nadu, India, for providing the necessary infrastructure for successful accomplishment of this research project. Authors also thank Mr. K. R. Ethiraj, Assistant Professor, SAS, VIT University, for his contribution for drawing the structure of OA which is used in this study. The authors report no declarations of interest.

## References

- Ali AS, Azhar R, Muhammad K, et al (2011). Eupatilin: A flavonoid compound isolated from the artemisia plant, induces apoptosis and G2/M phase cell cycle arrest in human melanoma A375 cells. *Afr J Pharm Pharmacol*, **5**, 582-8.
- Andreas S, Suzanne C, Jerry MA (2011). Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *The EMBO J*, **30**, 3667-83.
- Antonio JR, Juan FA, Ewan TM, et al (2010). Apoptotic markers in protozoan parasites Parasites & Vectors. *Parasites and Vectors*, **3**, 1-15.
- Benzie IFF, Strain JJ (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem*, **239**, 70-6.
- Bernard SA, Olayinka OA (2010). Search for a novel antioxidant, antiinflammatory/ analgesic or antiproliferative drug: cucurbitacins hold the ace. *J Med Plants Res*, **4**, 2821-6.
- Bin Z, Lu L, Kai C, et al (2011). Mechanisms of TRAIL and Gemcitabine induction of pancreatic cancer cell apoptosis. *Asian Pac J Cancer Prev*, **12**, 2675-8.
- Biswas M, Ghosh AK, Haldar PK (2010). Anti-leishmanial and anti-cancer activities of a pentacyclic triterpenoid isolated from the leaves of Terminalia arjuna Combretaceae. *Trop J Pharma Res*, **9**, 135-40.
- Blois MS (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, **181**, 1199-200.
- Chiang YM, Chang JY, Kuo CC, et al (2005). Triterpenes from aerial roots of Ficus microcarpa. *Phytochemistry*, **66**, 495-501.
- George VC, Kumar DRN, Rajkumar V, et al (2012). Quantitative assessment of the relative antineoplastic potential of the n-butanolic leaf extract of Annona Muricata Linn. in normal and immortalized human cell lines. *Asian Pac J Cancer Prev*, **13**, 699-704.
- Deng F, Lu JJ, Liu HY, et al (2011). Synthesis and antitumor activity of novel salvicine analogues. *Chin Chem Lett*, **22**, 25-8.
- Engin U, Ceyda A, Yusuf Y (2011). Apoptosis: why and how does it occur in biology? *Cell Biochem Funct*, **29**, 468-80.
- Fu LW, Zhang SJ, Li N, et al (2005). Three new triterpenes from Nerium oleander and biological activity of the isolated compounds. *J Nat Prod*, **68**, 198-206.
- Fulda S (2008). Betulinic acid for cancer treatment and prevention. *Int J Mol Sci*, **9**, 1096-107.
- Hasmah A, Azimahtol HLP, Judit H, et al (2010). A natural compound from Hydnophytum formicarium induces apoptosis of MCF-7 cells via up-regulation of Bax. *Cancer Cell Int*, **10**, 1-6.
- Hitoshi M, Yuri O, Yasunobu O, et al (2002). alpha-tocopherol increases the intracellular glutathione level in HaCaT keratinocytes. *Free Radic Res*, **36**, 705-9.
- Huang T, Gong WH, Li XC, et al (2012). Induction of apoptosis by a combination of Paclitaxel and Carboplatin in the presence of hyperthermia. *Asian Pac J Cancer Prev*, **13**, 81-5.
- Jie L, Wei-Jian G, Qing-Yao Y (2002). Effects of ursolic acid and oleanolic acid on human colon carcinoma cell line HCT15. *World J Gastroenterol*, **8**, 493-5.
- Kataoka S, Tsuruo T (1996). Apoptosis. *Oncologist*, **1**, 399-401.
- Ko HH, Yen MH, Wu RR, et al (1999). Cytotoxic isoprenylated flavans of Broussonetia kazinoki. *J Nat Prod*, **62**, 164-6.
- Liang F, Wai AY, You-Hua X, et al (2011). Oleanolic acid from Prunella Vulgaris L. induces SPC-A-1 cell line apoptosis via regulation of Bax, Bad and Bcl-2 expression. *Asian Pac J Cancer Prev*, **12**, 403-8.
- Li J, Xu LZ, Zhu WP, et al (1999). Effects of ursolic acid and oleanolic acid on Jurkat lymphoma cell line *in vitro*. *Chin Oncol*, **9**, 395-7.
- Liu J (2005). Oleanolic acid and ursolic acid: research perspectives. *J Ethnopharmacol*, **100**, 92-4.
- Mark SB (2008). Natural products to drugs: natural product derived compounds in clinical trials. *Nat Prod Rep*, **25**, 475-516.
- Masaaki N, Tatsuo T, Aimi U, et al (2008). Inotodiol, a lanostane triterpenoid, from Inonotus obliquus inhibits cell proliferation through caspase-3-dependent apoptosis. *Anti Can Res*, **28**, 2691-6.
- Miao YL, Henery WCL, Wen HY, et al (2007). Up-regulation of matrix metalloproteinase family gene involvement in ursolic acid-induced human lung non-small carcinoma cell apoptosis. *Anti Can Res*, **27**, 145-54.
- Oliver K, Lorenzo G, Marta L, et al (2011). Cell death assays for drug discovery. *Nature Rev Drug Discov*, **10**, 221-37.
- Patel JR, Choubisa B, Dholakiya BZ (2011). Plant Derived Compounds Having Activity against P388 and L1210 Leukemia Cells. *Chemical Sc J*, **33**, 1-16.
- Perez-Camino M, Cert A (1999). Quantitative determination of hydroxy pentacyclic triterpene acids in vegetable oils. *J. Agric. Food Chem*, **47**, 1558-62.
- Pui KW, William CST, Zhi-Tao L, et al (2009). Oleanolic acid isolated from Oldenlandia diffusa exhibits a unique growth inhibitory effect against ras-transformed fibroblasts. *Life Sci*, **85**, 113-21.
- Rajkumar V, Guha G, Kumar RA (2011). Apoptosis induction in MDA-MB-435S, Hep3B and PC-3 cell lines by Rheum emodi rhizome extracts. *Asian Pac J Cancer Prev*, **12**, 1197-200.
- Ramachandran S, Rajendra PN (2008). Effect of ursolic acid, a triterpenoid antioxidant, on ultraviolet-B radiation-induced cytotoxicity, lipid peroxidation and DNA damage in human lymphocytes. *Chemico Bio Intera*, **176**, 99-107.
- Samali A, Gorman AM, Cotter TG (1996). Apoptosis: the story so far. *Experientia*, **52**, 933-41.
- Senderowicz AM (2004). Targeting cell cycle and apoptosis for the treatment of human malignancies. *Curr Opin Cell Biol*, **16**, 670-8.
- Shishodia S, Majumdar S, Banerjee S (2003). Ursolic acid inhibits nuclear factor-kB activation induced by carcinogenic agents through suppression of Ikb kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. *Cancer Res*, **63**, 4375-83.
- Stephen N, David ET (2005). Chemical approaches to the discovery and development of cancer therapies. *Nature Reviews Cancer*, **5**, 285-96.
- Susan E (2007). Apoptosis: A review of programmed cell death.

V Cijo George et al

*Toxicol Pathol*, **35**, 495-516.

- Vinay D, Richa S, Showket H, et al (2011). Comparative anticancer potential of clove (*Syzygium aromaticum*) - an Indian spice - against cancer cell lines of various anatomical origin. *Asian Pac J Cancer Prev*, **12**, 1989-93.
- Weislow R, Kiser DL, Fine J, et al (1989). New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. *J Nat Cancer Inst*, **81**, 577-86.
- Xi M, Hai C, Tang H, et al (2009). Antioxidant and antiglycation properties of total saponins extracted from traditional chinese medicine used to treat diabetes mellitus. *Phytotherapy Res*, **22**, 228-37.
- Yu-Fang K, Yue-Hwa C (2004). Induction of apoptosis in a non-small cell human lung cancer cell line by isothiocyanates is associated with P53 and P21. *Food Chem Toxicol*, **42**, 1711-8.