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Tylophora Indica Extracts Down Regulates Bcl2 and Induces Apoptosis in Melanoma Cells

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ABSTRACT

Melanoma is the least common but most dangerous skin cancer with the highest morbidity rate. Owing to increased toxicity towards normal cells, cancer chemo therapy is found to be great challenge with chemo sensitive agents. Phytochemicals derived anticancer agents is a field of great application. The unexplored plants remain the lead for novel therapeutic agents. *Tylophoraindica*attracts research interest since little scientific data is available regarding the anticancer potential of the plant. In the present study purified ethanolic fraction showed the presence of compounds like alkaloids and quinines and the derivatives were confirmed by GC-MS analysis. Antiproliferative property of the alcoholic fraction was confirmed on malignant skin melanoma cell line SKMEL-28 by performing MTT assay, Neutral red uptake and LDH leakage assays respectively. The relative genotoxic effect of *Tylophoraindica* showed little damaging effects to genomic DNA when determined by comet assay and DNA fragmentation analysis. The apoptotic changes in nucleus were confirmed by fluorescent microscopic analysis by acridine orange/ethidium bromide staining. The molecular mechanisms of anticancer activity were mainly found to be due to the inhibition of BCL-2 gene expression by RT-PCR analysis. This study confirms anticancer activity of alcoholic fraction of *Tylophoraindica*, drawing new insights in their application for therapeutic regimens.

Keywords: *Tylophoraindica,* Bcl-2 gene regulation, SKMEL-28, Antiproliferative effects

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INTRODUCTION

Cancer is the second leading cause of death all over the world[1]. It is a multifactorial, multifaceted and multi-mechanistic disease requiring a multidimensional approach for treatment, control and prevention. The major causes of cancer are smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation[2]. Amongst the so many types of cancer reported on the basis of causative factors and locations of occurrence, melanoma was the least common but most dangerous skin cancer with the highest morbidity rate. It arises from the malignant transformation of melanocytesthat metastasizes into internal organs[3].Epidemological studies shows an increasing incidence of melanoma when compairing with other cancer types in world wide [45]. Melanoma is often characterized by resistance to cytotoxic agents which contributes to the high morbidity and mortality rates in patients.

Several chemopreventive agents are used to treat cancer, but most of them cause toxicity that prevents their long term usage[6].Therefore the new sources of anti-cancer agents that exert cytotoxicity activity against melanoma cells are in need. In this context, the natural products derived from medicinal plants have gained significance for treating cancer. Various phyto constituents that possess multiple biological and synergistic effects can be used to treat different ailments or enhance the effect of drugs. Certain natural products like alkaloids, polypropenoids and terpenoidshave been applied for chemoprevention of carcinogenesis and to suppress the malignancy [78].

Tylophoraindica(Family: *Asclepidaceae*) commonly known as Antmul is a twining perennial plant distributed throughout southern and eastern part of India. The leaf of *Tylophoraindica*extract, acts as an anti-tumour agent in ethnic medicine but little scientific evidence is available regarding its activity[9]. Even though many studies have reported on the antifungal activity [10]and anti-bacterial activity [11] of the plant extract, studies on the mechanism contributing its anti-tumour activity is limited. Many anticancer molecules has shown growth inhibition and/or apoptotic cell death of cancer cells by modulating the cell-cycle regulatory molecules[12]. Both the cell proliferation and apoptotic cell death are important determinants of growth of a tumour[13]. A balance between the two is critical in maintaining tissue homeostasis and normal development. As many chemotherapeutic agents have been identified to induce apoptosis in cancer cells[14], the apoptosis has been targeted for the treatment of cancer. Reports suggest that *Tylophoraindica* induces apoptosis in K562 cells as evidenced by nuclear condensation, apoptotic body formation, flipping of membrane phosphatidylserine, activation of

caspase-3 and release of mitochondrial cytochrome c, which are the characteristic features of apoptosis [15].

Mostly the cancer therapeutics act by inducing stress signals that can activate the intrinsic mitochondrial pathway of apoptosis in tumour cells[16].Bcl-2 family consist of anti-apoptotic and pro-apoptotic members, and is characterized to be the protein family involved in the regulation of apoptotic cell death [17].The Bcl-2 family constitutes both agonists and antagonists of apoptosis that functions at least in part through protein-protein interactions between various members of the family. The final outcome depends on the relative ratio of death agonists and antagonists. *In vitro* and *in vivo* studies have established that Bcl-2 expression confers an antiapoptotic activity[18]. With this background, in the present study we intend to determine the involvement of Bcl-2 gene on the antitumour activity of *Tylophoraindica*.

MATERIALS AND METHOD

*Tylophoraindica*were collected from Thiruvananthapuram, Kerala, India was dried in shade, powdered and stored at room temperature in air tight dark containers for further studies. SKMEL-28 cell lines procured from NCCS, Pune. All other chemicals specified were of analytical grade, procured from MERCK, India.

Preparation of plant extracts

Crude alcoholic extract preparation was carried out by using soxhlet extraction. The extract obtained was dried, dissolved in DMSO and used for further studies. Further purification was carried out by column chromatography. The eluents where then subjected to thin layer chromatography using chloroform: methanol in the ratio 8.5: 1.5.The regions with fluorescent bands were scrapped and stored at room temperature. Gas chromatography mass spectrometry (GCMS) was carried out for compound identification. The peaks obtained from the graph were identified from standard reference library.

Phytochemical Analysis

Phytochemical analysis was performed for the identification of alkaloids, flavanoids, saponins, phenol, glycosides, tannins, thiols and steroids as per previously reported protocols[192021].

***In vitro* anti-cancer responses of *Tylophora* extracts**

Cell lines

SKMEL-28 cells were cultured in Dulbecco's Modified Eagles medium in with 1mM sodium pyruvate, nonessential amino acids and 10% fetal bovine serum. These cell lines are sub-cultured

on every 4-5 days and maintained at 37°C in a humidified atmosphere containing 5% CO_2.Crude extract of *Tylophoraindica(*250 µg/µl) was used for all the experiments.

Proliferation assay on SKMEL-28 cells

MTT assay

Proliferative capacity of SKMEL-28 cells upon treatment with the crude extract of *Tylophoraindica* was assessed by the methyl thiazoltetrazolium bromide (MTT) assay as described by Finosh *et al*[22]. Confluent cells were incubated with MTT solution for 3 h at 37°C. The formazan crystals formed were dissolved in DMSO and the absorbance was read at 540 nm in ErbaLisa reader (ERBA, Germany).

Neutral Red Assay

SKMEL-28 cells were treated with 250 µg/µl of the crude extract of *Tylophoraindica* and assessed for neutral red uptake after 3 hours incubation with the dye and the absorbance was read at 493nm and recorded using the ELISA reader (ERBA, Germany)[23].

Lactate Dehydrogenase (LDH) Leakage Assay

The LDH enzyme leaked fromSKMEL-28 cells due to the loss of membrane integrity was determined from the culture medium. Briefly after plant extract treatment the cells were allowed to proliferate for 24h. Then to the cell culture supernatant 2.7ml potassium phosphate buffer, 0.1ml 6m M NADH solution, 0.1ml sodium pyruvate solution was added. The decrease of OD was recorded at 340nm in spectrophotometer at 25^0C.The blank solution was prepared by adding enzyme dilution buffer instead of sample.

Apoptosis assay

Apoptosis was determined by staining the cells with Acridine Orange/Ethidium Bromide (AO/EB) [24].After treatment with the crude extract of *Tylophoraindica*, the cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with PBS and observed by a fluorescence microscope using blue filter (Olympus CKX41 with Optika Pro5 camera).

Comet Assay

The *Tylophoraindica*crude extract treatedcell suspensions (1×10^4/ 5-10µl) were mixed into 10µl of low melting point agarose(LMA) and placed layer of agarose NMA (normal melting point agarose) on a glass slide. Then a final layer of NMA was coated over the LMA layer. Slides were then placed in cold lysing solution (2.5 M NaCL, 100mM Na_2EDTA, 10 mMTris pH 10 and 1% SDS to which 10% DMSO and 1%Triton X 100 added immediately prior to use) for 1 hour. After lysis the slides were placed in electrophoresis buffer (300 mMNaOH and 1mM disodium

EDTA pH 13) for 20 minutes to allow unwinding of DNA. Electrophoresis was conducted in the same buffer with a current of 300 mA for 20 minutes. Finally slides were washed in neutralizing buffer (0.4 mMTris, pH 7.5) three times for five minutes and stained with50µl ethidium bromide(20µg/ml)for the detection of the oxidative DNA damage. The comets were observed under fluorescent microscopy (Olympus CKX 41) and captured comets were measured quantitatively using Tritek comet scoring software[25].

RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated using the RNA isolation kit according to the manufactures instructions (Chromous biotech, India). Reverse transcriptase PCR was performed according to the manufactures instruction (Genei, Banglore) with BCL-2 primer Sequence, Forward: 5'-CTGCACCTGACGCCCTTCACC-3';Reverse:5'CACATGACCCCACCGAACTCAAAGA-3'. GAPDH was used as housekeeping gene. The PCR products were analyzed in 1.5% (v/v) agarose gels. Densitometry was performed using Image J software. The data were recorded as the ratio of sample to internal standard.

Statistics

Statistical analysis was carried out using Graph Pad prism statistical software. Differences between variables were also compared using Student's t test. 'p'value of less than 0.05 was considered statistically significant

RESULTS AND DISCUSSION

Phytochemical analysis

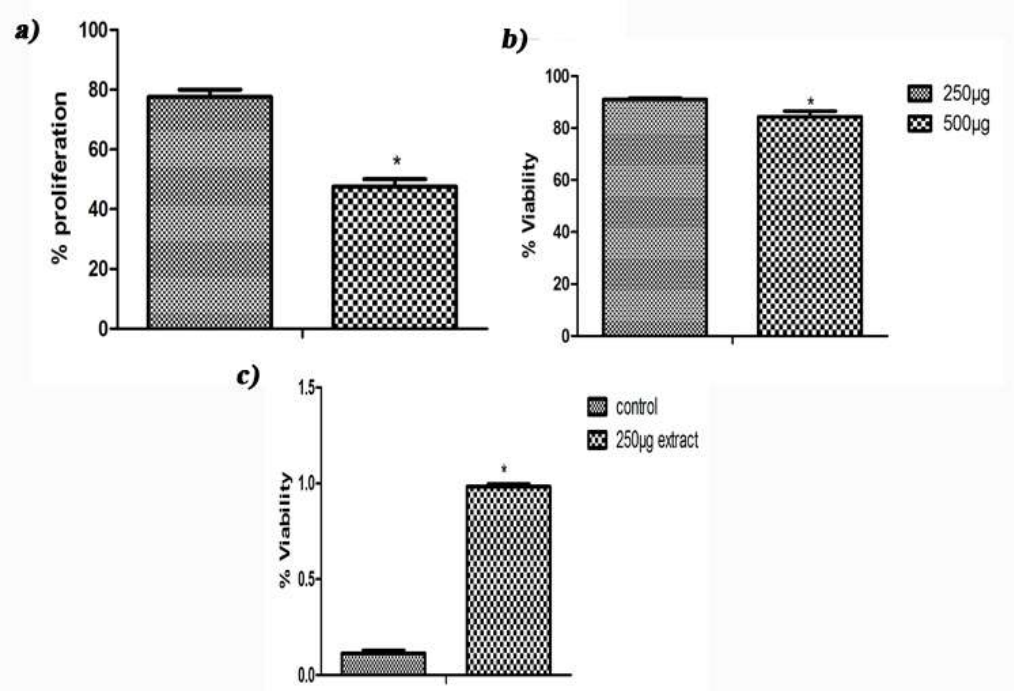
Preliminary phytochemical evaluations revealed that alkaloids are the major phytochemical present in the ethanolic extract of *Tylophoraindica.*(Table1). Further GC- MS analysis of crude extracts confirms the presence of alkaloids and quinones (Table 2).However more structural validation and elemental studies are required for identifying the major bioactive principle present in ethanolic extract of *Tylophoraindica*.

Among the most reported cancers, skin cancer is found to have a great impact on human population[26]. Many studies demonstrated that inhibition of cell invasion, migration and adhesion prevents metastasis[27]. Since, most anticancer drugs are not sufficiently tumor selective, the drugs which possessed anti-metastatic efficacy and low toxicity on normal tissues are required. Currently, it is well documented that natural compounds, especially plant extracts are one of the most important sources of potential anticancer drugs[28]. *Tylophoraindica* extract acts as an anti-tumour agent in ethnic medicine but little scientific evidence is available regarding its activity[9].

GC-MS analysis of purified fractions gave a peak value at 42.93 comparing with library search suggests the presence of alkaloids and quinones compounds. These compounds are reported to exhibit antitumor activity but the proper mechanism is not yet revealed[29].Induction of apoptosis is reported to be a main gate way of mechanisms involved in the anti-tumour capacity of a molecule[30]. In the present study SKMEL-28 cell lines was undertaken to study the effect of *Tylophoraindica*as an anti-tumour agent through a Bcl2 dependent mechanism.

Inhibitory effects of crude extract on the proliferation ofSKMEL-28 cells

Unlimited cell proliferation is a hall mark of cancer cells. We found that ethanolic plant extracts have anti proliferative effects on SKMEL-28 cells and the proliferative capacity decreases with increasing concentration of the extract (Figure 1a). We further confirmed the anti-proliferative effects of *Tylophoraindica*inSKMEL-28cells by neutral red uptake assay (Figure 1b) and LDH leakage assay (Figure 1c)and similar results were obtained.



Figuew 1a.Inhibition of cell proliferation in SKMEL-28 cells by *Tylophoraindica.* Cells were treated with the crude extract of *Tylophoraindica*at different concentrations of 250µg and 500µg and the cell proliferation was assessed using MTT assay and neutral red uptake assay(Figure 1b).LDH activity in A5SKMEL-28 cells (Figure 1c).

Crude extract induced apoptosis in SKMEL-28 cells

Apoptotic effects of *Tylophoraindica*extract in SKMEL-28 cells were performed by double staining method using acridine orange and ethidium bromide. The results depict (Figure 2) that

the extracts induced an increase in apoptotic cell death. This revealed the anti-cancer effects of the extracts.

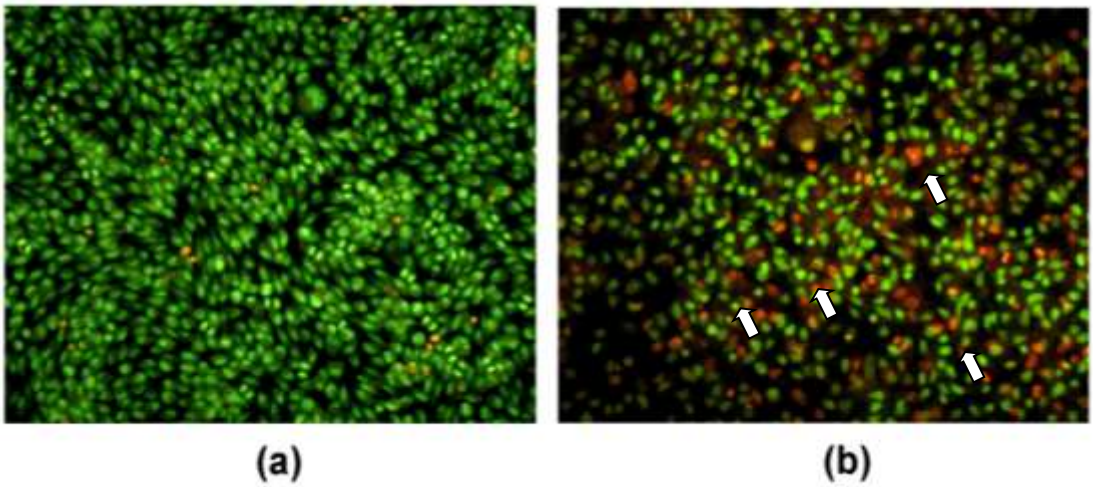


Figure 2: **Apoptotic cell death induced by *Tylophoraindica* extract. Arrows indicates cells undergoing apoptosis.**

Comet assay

The genotoxic potential of the crude extract was further confirmed by comet assay after treating the cells with the extracts. The decrease in the DNA intensity on the head regions of the comets confirmed that the extracts had elicited nuclear damage leading to apoptosis (Figure 3).

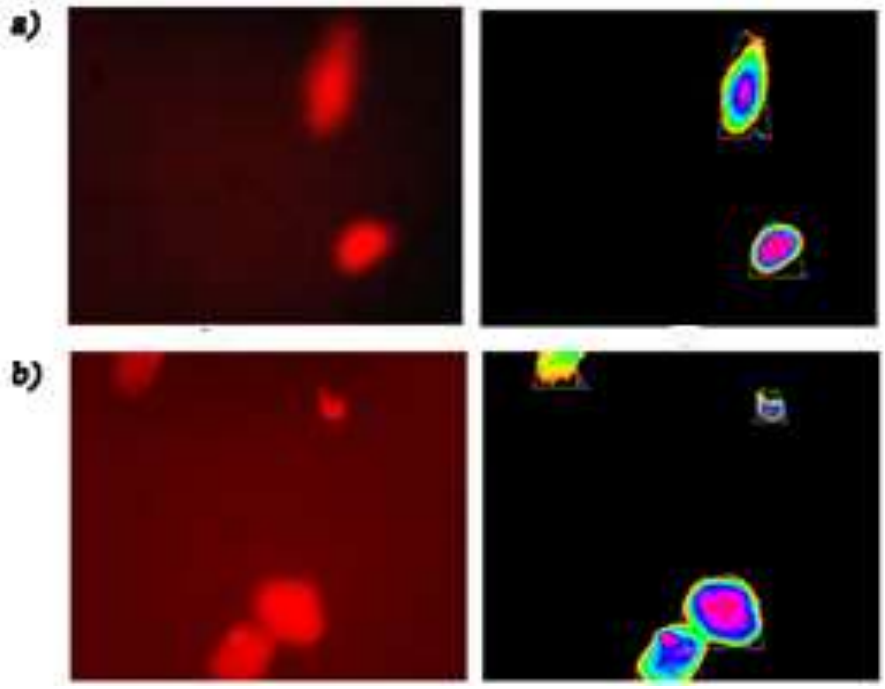


Figure 3: **Increase in comet area and comet length was observed in cells treated with *Tylophoraindica* extracts confirming induction of apoptosis.**

RT PCR analysis reveals decreased expression of Bcl-2

RT-PCR data with regards to effect of *Tylophoraindica*extract in SKMEL-28 cells revealed a significantly decreased ($p<0.05$) expression of Bcl-2 gene compared to control. Further, Image analysis using IMAGE J software showed 59.08 % reduction in Bcl -2 expression which, can also be considered to be significant (Figure 4).

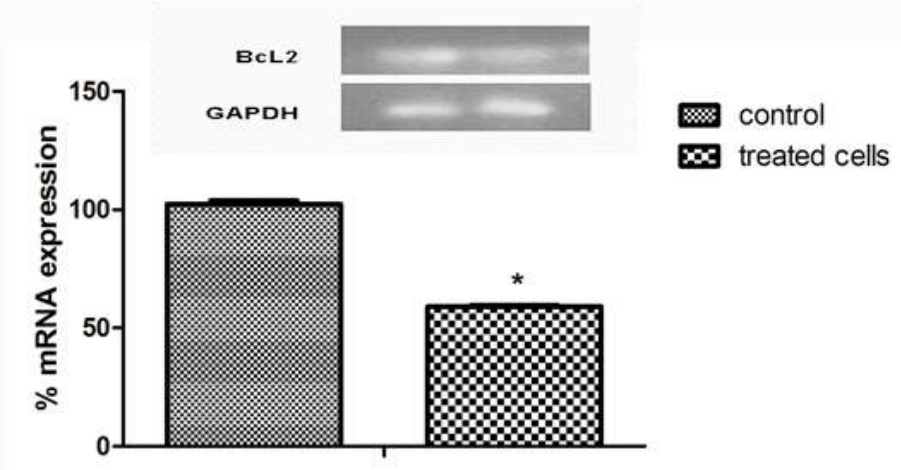


Figure 4: **RT-PCR analysis of Bcl-2 gene.Values are significant ($p < 0.05$) when compared to control**

Tumour migration which involves invasion and metastasis is an important process during melanoma progression[31].In the present study, the ethanolic extract of *Tylophoraindica* inhibits SKMEL-28 cell proliferation which signified its anti-tumour activity. Anti-proliferative effects of *Tylophoraindica* might be due to the presence of alkaloids present in the crude fraction[32]. Further, it was found that crude extract induces apoptosis in SKMEL-28 cell lines. The induction of apoptosis in target cells is a key mechanism for most anti-tumor therapies[30]. These results were further confirmed using comet assay and the significant tail length observed can be considered as induction of apoptosis, but the detailed molecular mechanisms of inducing apoptosis in these cells are still remains unclear. However the differential regulation of proapoptotic and antiapoptotic Bcl-2 family members appears to be a key event in the execution of apoptosis[33]. Gene expression analysis has shown that Bcl-2 expression was found to be decreased on treatment with the crude extract. In parallel with this results accumulating evidence suggest that reduced expression of Bcl-2 results in induction of apoptosis and inhibition of drug resistance[34].Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli[35]. The significance of the study relies on the fact that crude extracts can offer multi targeted responses in cancer cells rather than pure compounds thereby overruling adverse side effects and drug efflux pumps.

CONCLUSION

As per the experimental findings we conclude that ethanolic extract of *Tylophoraindica* has marked anti-tumor activities through its ability to induce apoptosis. However large-scale randomized, double blind placebo or positive drug parallel controlled studies were required to confirm the efficacy and apoptosis-inducing potential of *Tylophoraindica* extract in various cancers in the clinical setting. The present study promotes the use of *Tylophoraindica* extract as an alternative therapeutics for the management of cancer.

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REFERENCES

1. Madhusudan S, Middleton MR. The emerging role of DNA repair proteins as predictive, prognostic and therapeutic targets in cancer. Cancer Treat Rev. 2005;31(8):603-617. doi:10.1016/j.ctrv.2005.09.006.
2. Ames BN, Gold LS, Willett WC. The causes and prevention of cancer. Proc Natl Acad Sci U S A. 1995;92(12):5258-5265.
3. Looi CY, Moharram B, Paydar M, et al. Induction of apoptosis in melanoma A375 cells by a chloroform fraction of Centratherum anthelminticum (L.) seeds involves NF-kappaB, p53 and Bcl-2-controlled mitochondrial signaling pathways. BMC Complement Altern Med. 2013;13:166. doi:10.1186/1472-6882-13-166.
4. MacKie RM, Hauschild A, Eggermont AMM. Epidemiology of invasive cutaneous melanoma. Ann Oncol. 2009;20(suppl 6):vi1-vi7. doi:10.1093/annonc/mdp252.
5. Erdei E, Torres SM. A new understanding in the epidemiology of melanoma. Expert Rev Anticancer Ther. 2010;10(11):1811-1823. doi:10.1586/era.10.170.
6. BHATIA S, TYKODI SS, THOMPSON JA. Treatment of Metastatic Melanoma: An Overview. Oncol Williston Park N. 2009;23(6):488-496.
7. Talib WH. Antiproliferative Activity of Plant Extracts Used Against Cancer in Traditional Medicine. Sci Pharm. 2010;78(1):33-45. doi:10.3797/scipharm.0912-11.
8. Bhakuni DS, Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN. Screening of Indian plants for biological activity. II. Indian J Exp Biol. 1969;7(4):250-262.
9. Chitnis MP, Khandalekar DD, Adwankar MK, Sahasrabudhe MB. Anti-cancer activity of the extracts of stem and leaf of Tylophora indica. Indian J Med Res. 1972;60(3):359-362.
10. Ahmad S, Jahan N, Khatoon R, Shahzad A, Shahid M. Antimicrobial activity of in vitro raised callus of Tylophora indica Merr. against resistant bacteria harbouring bla genes and comparison with its parent plant. Med Plants - Int J Phytomedicines Relat Ind.

2013;5(4):187. doi:10.5958/j.0975-6892.5.4.030.

11. Gupta S, George P, Gupta V, Tandon VR, Sundaram KR. Tylophora indica in bronchial asthma--a double blind study. Indian J Med Res. 1979;69:981-989.
12. Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer. Nat Rev Cancer. 2002;2(5):331-341. doi:10.1038/nrc795.
13. Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis. 2000;21(3):485-495. doi:10.1093/carcin/21.3.485.
14. Liu J, Shen H-M, Ong C-N. Salvia miltiorrhiza inhibits cell growth and induces apoptosis in human hepatoma HepG2 cells. Cancer Lett. 2000;153(1–2):85-93. doi:10.1016/S0304-3835(00)00391-8.
15. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516. doi:10.1080/01926230701320337.
16. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004;116(2):205-219.
17. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999;13(15):1899-1911.
18. Chaudhary KS, Abel PD, Lalani EN. Role of the Bcl-2 gene family in prostate cancer progression and its implications for therapeutic intervention. Environ Health Perspect. 1999;107(Suppl 1):49-57.
19. Harborne JB. Phytochemical Methods A Guide to Modern Techniques of Plant Analysis. Springer Science & Business Media; 1998.
20. Evans WC. Trease and Evans' Pharmacognosy. Baillière Tindall; 1989.
21. Sofowora A. Medicinal Plants and Traditional Medicine in Africa. Wiley; 1982.
22. Gnanaprakasam Thankam F, Muthu J, Sankar V, Kozhiparambil Gopal R. Growth and survival of cells in biosynthetic poly vinyl alcohol–alginate IPN hydrogels for cardiac applications. Colloids Surf B Biointerfaces. 2013;107:137-145. doi:10.1016/j.colsurfb.2013.01.069.
23. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc. 2008;3(7):1125-1131. doi:10.1038/nprot.2008.75.
24. Gnanaprakasam Thankam F, Muthu J. Influence of plasma protein–hydrogel interaction moderated by absorption of water on long-term cell viability in amphiphilic biosynthetic hydrogels. RSC Adv. 2013;3(46):24509. doi:10.1039/c3ra43710h.
25. Thankam FG, Muthu J. Biosynthetic alginate–polyester hydrogels with inherent free radical scavenging activity promote cellular response. J Bioact Compat Polym. 2013;28(6):557-573. doi:10.1177/0883911513508670.
26. Stratigos A, Nikolaou V, Kedicoglou S, et al. Melanoma/skin cancer screening in a Mediterranean country: results of the Euromelanoma Screening Day Campaign in

Greece. J Eur Acad Dermatol Venereol JEADV. 2007;21(1):56-62. doi:10.1111/j.1468-3083.2006.01865.x.

27. Schlüter K, Gassmann P, Enns A, et al. Organ-specific metastatic tumor cell adhesion and extravasation of colon carcinoma cells with different metastatic potential. Am J Pathol. 2006;169(3):1064-1073. doi:10.2353/ajpath.2006.050566.
28. Surh Y-J. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer. 2003;3(10):768-780. doi:10.1038/nrc1189.
29. Gao D, Xu H, Philbert MA, Kopelman R. Bioeliminable Nanohydrogels for Drug Delivery. Nano Lett. 2008;8(10):3320-3324. doi:10.1021/nl8017274.
30. Yin P-H, Liu X, Qiu Y-Y, et al. Anti-tumor activity and apoptosis-regulation mechanisms of bufalin in various cancers: new hope for cancer patients. Asian Pac J Cancer Prev APJCP. 2012;13(11):5339-5343.
31. Pietraszek K, Brézillon S, Perreau C, Malicka-Błaszkiewicz M, Maquart F-X, Wegrowski Y. Lumican - derived peptides inhibit melanoma cell growth and migration. PloS One. 2013;8(10):e76232. doi:10.1371/journal.pone.0076232.
32. Hammerová J, Uldrijan S, Táborská E, Slaninová I. Benzo[c]phenanthridine alkaloids exhibit strong anti-proliferative activity in malignant melanoma cells regardless of their p53 status. J Dermatol Sci. 2011;62(1):22-35. doi:10.1016/j.jdermsci.2011.01.006.
33. Tzifi F, Economopoulou C, Gourgiotis D, Ardavanis A, Papageorgiou S, Scorilas A. The Role of BCL2 Family of Apoptosis Regulator Proteins in Acute and Chronic Leukemias. Adv Hematol. 2011;2012:e524308. doi:10.1155/2012/524308.
34. Rao J, Xu D-R, Zheng F-M, et al. Curcumin reduces expression of Bcl-2, leading to apoptosis in daunorubicin-insensitive CD34+ acute myeloid leukemia cell lines and primary sorted CD34+ acute myeloid leukemia cells. J Transl Med. 2011;9:71. doi:10.1186/1479-5876-9-71.
35. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 1997;275(5303):1129-1132.