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ANTICANCER ACTIVITY OF SHIVANARVEMBU KUZHI THAILAM: AN *IN-VITRO* STUDY

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ABSTRACT

ShivanarVembuKuzhiThailam (SVKT) is a poly herbal siddha drug used for the skin care ailments. The aim of the current study is to evaluate the anticancer activity of SVKT in the skin cancer cell lines. The cytotoxic effect of SVKT was studied on the two skin cancer cell lines namely human squamous carcinoma cell line (A431) and murine melanoma cell line (B16F10). The results reveals that the SVKT is effective in killing the A431 cancer cells when compare to B16F10 cells. Further the gene expression studies were conducted in A431 cancer cells. Expression levels of genes such as Bcl-2, COX-2, p53 and MMP-9 were monitored. From the results, it can be inferred that the SVKT, upregulates the expression tumor suppressor gene p53 and down regulates the other regulatory genes such as Bcl-2, COX-2 and MMP-9.

KEY WORDS: ***Polyherbal, anticancer, cytotoxic, expression, genes.***

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INTRODUCTION

Skin's plays an important role in protecting the body from the external environment pollutants like chemicals and UV radiations. Thereby exerting a barrier function. Upon regular exposure to such toxic substances results in the cellular insults and imbalanced barrier function. The frequent insults faced by the skin cells make the irreversible damage to the cell and turns them cancerous.[1]Cancer is hyper proliferative disorder that involves transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis. Such cancer development can be in any type of the skin cell namely basal cells, squamous cells or melanocytes, thus resulting in the skin cancer. Even though the treatment like radiotherapy/ chemotherapy is available to address the issues, their side effects are enormous. Hence need for the hour is to have a holistic approach in the treatment.[2] Thus the aim of the current study is to evaluate a poly herbal drug Shivanar VembuKuzhi thailam (SVKT) and scientifically validate its anticancer potential in the skin cancer lines. SVKT is a polyherbal formulation containing three herbs namely *Indigoferra aspalathoides* (whole herb), *Corollocarpus epigeous* (tuber) and *Celestrus paniculatus* (Seeds). It's a Siddha herbal drug used for most of the skin care ailments and is prepared with the principle of destructive distillation process.[3] SVKT is prescribed as a carrier matrix also for many of the orally taken siddha drugs. In such case, the role of SVKT is to enhance the bioavailability of the drugs. *Indigoferra aspalathoides*is very well known for anti-inflammatory, anti- arthritic and anti-cancer properties.[4] The rhizome of *Corollocarpus epigeous*is known for treatment of syphilitic rheumatism, veneral disorders, chronic dysentery and as a Snake venom anecdote. The pharmacological properties such as analgesic, anti-pyretic and anti-inflammatory are well reported[5].The seeds and the oil of *Celestrus paniculatus* is bitter, acrid and thermogenic. The antibacterial, antimalarial, analgesic and antiarthritic property of the *Celestrus paniculatus* seeds are well documented in the scientific literature. The plant is commonly used as brain tonic, appetizer and for the treatment of skin disorders.[6]

MATERIALS & METHODS

The Shivanarvembu Kuzhithailam used in the current study was purchased from the Indian Medical Practitioners Co-operative Pharmacy and Stores Ltd (IMPCOPS), Coimbatore, India.

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), TPVG (0.2% Trypsin, 0.02% EDTA, 0.05% glucose in Phosphate buffer saline) solution and 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyltetrazolium bromide (MTT), were obtained from Sigma Aldrich Co, St Louis, USA. Antibiotics from Hi-Media Laboratories Ltd., Mumbai. Reverse transcriptase enzyme (200U/µL) and dNTP Mix from Thermo Scientific, Tri –Xtract from G – Bioscience and OligodT and Primers from Eurofins, Dimethyl Sulfoxide (DMSO) and propanol from E.Merck Ltd., Mumbai, India.

Cell lines and culture medium

To study the anti-skin cancer potential of the SVKT, the two cell lines namely B16F10 and A431 were procured from National Centre for Cell Sciences (NCCS), Pune, India. B16F10 and A431 stock cells were cultured in DMEM and MEM respectively. The media was supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates.

Cell viability assay

Sample preparation: The SVKT drug was dissolved in DMSO and MEM supplemented with 2% inactivated FBS was added to the solution to obtain a stock solution of 1 mg/ml concentration of SVKT. This solution was filter sterilized. The sample was serially diluted to obtain the test concentrations such as 1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml and 62.5µg/ml. The monolayer cell culture (B16F10 and A431) was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM and MEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed with medium once and 100 µl of different test concentrations of SVKT were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37° C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the SVKT solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. These plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at wavelength 540 nm. The percentage growth inhibition was calculated at each concentration and concentration of SVKT needed to inhibit cell growth by 50% (CTC_{50}) values were generated from the dose-response curves for each cell line.[7,8]

Gene expression analysis

The mRNA expression levels of Bcl-2, COX-2, p53 and MMP-9 were carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The A431 cells were cultured in 60 mm petridish and maintained in MEM medium for 48 hrs. The MEM medium was supplemented with FBS and amphotericin. The standard drug doxorubicin (10µg/ml) and SVKT at the concentration of 700 µg/ml and 350 µg/ml was added to two culture dishes containing A431 cells and incubated for 24 hr. Total cellular RNA was isolated from the untreated (control) cells, cells treated with the standard drug doxorubicin and the cells treated with SVKT using Tri Reagent. cDNA was synthesized from total isolated RNA by using reverse transcriptase kit. Then 50µl of the reaction mixture was subjected to PCR

for the amplification of Bcl-2, COX-2, p53 and MMP-9 gene using specifically designed primers procured from Eurofins India, as an internal control. The house keeping gene GAPDH was co-amplified with each reaction.

Gene transcript amplification conditions

Probes were incubated at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds and extension at 72°C for 1 min. This was followed by final extension at 72°C for 10 min.

Primers used in RT- PCR

For 1st strand synthesis: oligodT primer

For II nd strand synthesis: sequence details are as follows for the respective genes.

Table 1
Primer sequence used for the gene expression study

Gene	Primersequennce
Bcl-2	Forward 5'CTGCACCTGACGCCCTTCACC 3' Reverse 5' CACATGACCCCACCGAACTCA3'
COX – 2	Forward 5' TTCAAATGAGATTGTGGAAAAAT 3' Reverse 5' AGATCATCTCTGCCTGAGTATCTT 3
p53	Forward 5' CTGAGGTTGGCTCTGACTGTACCACCATCC 3' Reverse 5' CTCATTCAGCTCTCGGAACATCTCGAAGCG 3'
MMP-9	Forward 5' GTGCTGGGCTGCTGCTTTGCTG 3' Reverse 5' GTCGCCCTCAAAGGTTTGGAAT 3'

RESULTS & DISCUSSION

Cell viability assay

Skin is an organ with cells of varying morphology in different layers. Upon exposure to a chemical carcinogen or a stimulant, the response and susceptibility is different for each of the cell type.[9] Transformation of even a single (from any cell type) cell will result in the skin cancer.[10] Anticancer potential of any drug molecule must be evaluated in the maximum number of cell types, in order to understand the sensitivity of the drug.[11] In the current study, the cell viability of the cancer cells such as B16F10 and A431 was studied for the SVKT treatment in a dose dependent manner using MTT assay. The B16F10 and A431 cells were treated with SVKT at the concentration of 1000µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml. The percentage cytotoxicity exerted by B16F10 cells are 47.76±2.9, 31.26±0.3, 28.3±1.9, 25.5±2.3 and 6.86±2.3% to the respective SVKT treatment concentration. The percentage cytotoxicity exerted by A431 cells are 88.89±0.2, 26.57±1.0, 18.89±1.6, 11.8±1.7 and 8.83±1.1% to the respective SVKT treatment concentration. The cytotoxicity exerted by SVKT on B16F10 cells and A431 cells are depicted in the Figure 1. The concentration of the SVKT required to inhibit the 50% of the cell growth (CTC50) for B16F10 cells are >1000 µg/ml; where as for A431 cells is 707.19 µg/ml. From the results it can be inferred that the SVKT possess more anticancer activity on A431 cells than B16F10 cells.

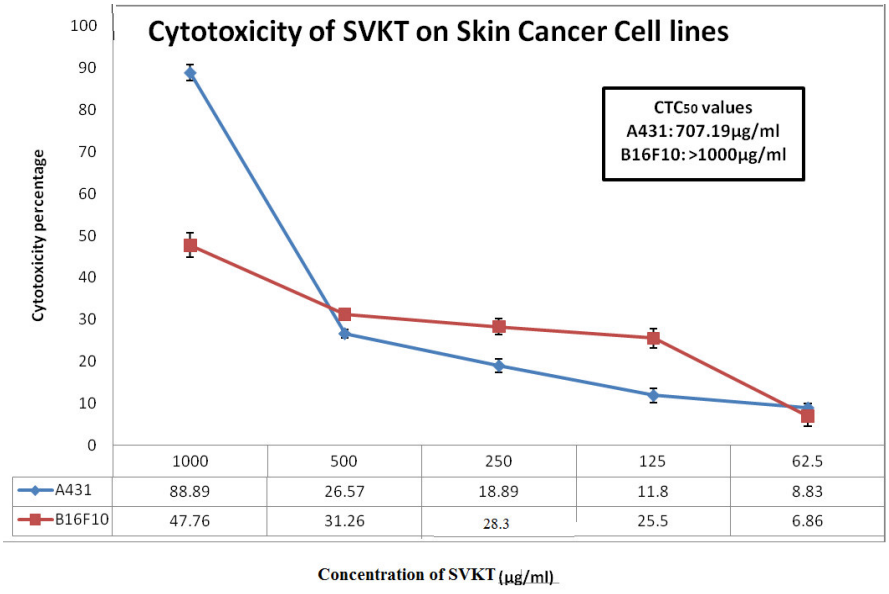


Figure1
Dose dependent cytotoxic effect of SVKT on the A431 and B16F10 cell lines.

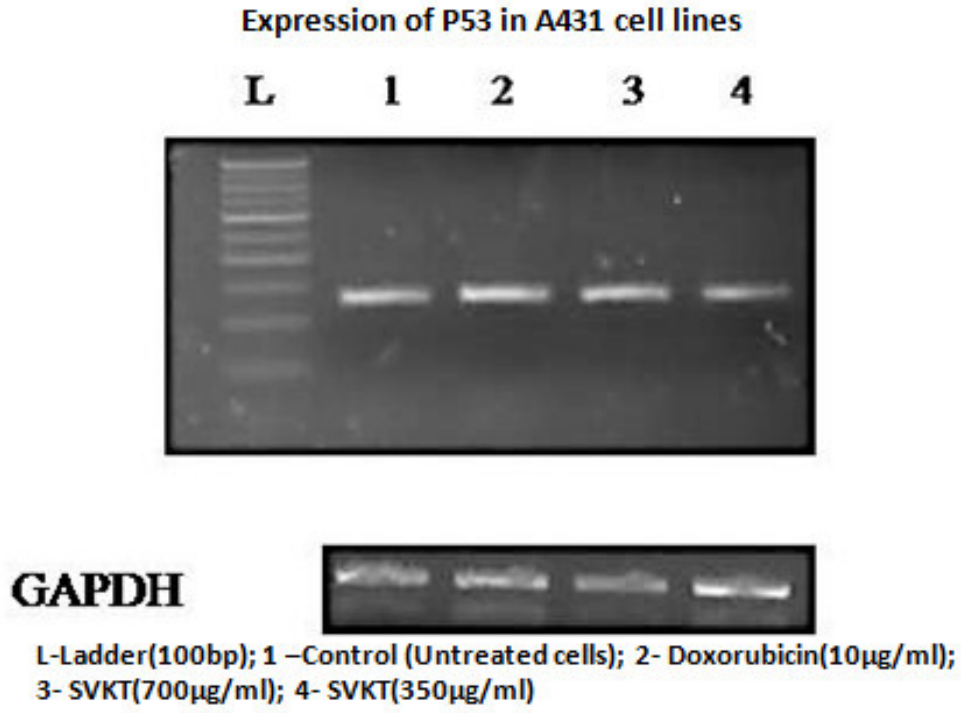
Gene expression results

Understanding the cell viability results, the study was narrowed to A431 cell lines to study the expression profiles Bcl-2, COX-2, p53 and MMP-9 genes. The cells treated with the standard drug doxorubicin (10µg/ml) serves as the positive control.

p53

p53 is one of the well-studied tumor suppressor gene. Upon exposure to the stress conditions like UV radiation or carcinogen, there will be a high levels of reactive oxygen species in the cell, which ultimately damages the macromolecules of the cells, especially DNA and causes enhanced expression of p53 in the cells.[12] The level of expression is directly proportional to the extent of cellular insult. Enhanced p53 expression induces the reversible cell cycle arrest to stop the multiplication of the damaged cells and apoptosis is induced to clear the damaged cells from the healthy tissue.[13] In the current study, the expression of p53 (w.r.t control cells) is increased by 0.1 fold and 0.07 fold with the treatment of SVKT at the concentration of 700µg/ml and 350µg/ml respectively, whereas the standard drug doxorubicin induces the expression by 0.17 fold. The gene expression profile of the p53 gene is shown in Figure 2 and the level of upregulation of p53 is depicted in Figure 6.

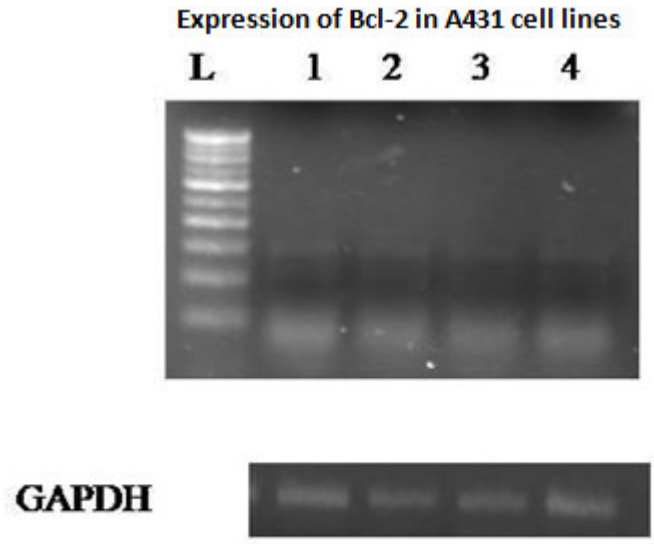
Figure 2
RT-PCR profile of p53 gene amplified from A431 cells treated with SVKT



Bcl-2

Bcl-2 is a family of proteins involved in regulating the anti-apoptotic and pro-apoptotic events in the cells.[14] These proteins play an important role in protecting the cells from the cytotoxicity. In cancer cells, the overexpression of Bcl-2 decreases the probability of cell death.[15] The drugs are targeted to the gene machinery to suppress the expression of Bcl-2, via p53 dependent or independent mechanism.[16] In the current study, the expression of Bcl-2 (w.r.t control cells) is decreased by 0.09 fold and 0.02 fold with the treatment of SVKT at the concentration of 700µg/ml and 350µg/ml respectively, whereas the standard drug doxorubicin suppresses the expression by 0.15 fold. The gene expression profile of the Bcl-2 gene is shown in Figure 3, and the extent of down regulation of Bcl-2 is depicted in Figure 6. It can be inferred from the study results, that the SVKT treatment down regulates the expression Bcl-2, an anti-apoptotic factor and thereby increases the probability of apoptotic induction in the A431 cells.

Figure 3
RT-PCR profile of Bcl-2 gene amplified from A431 cells treated with SVKT

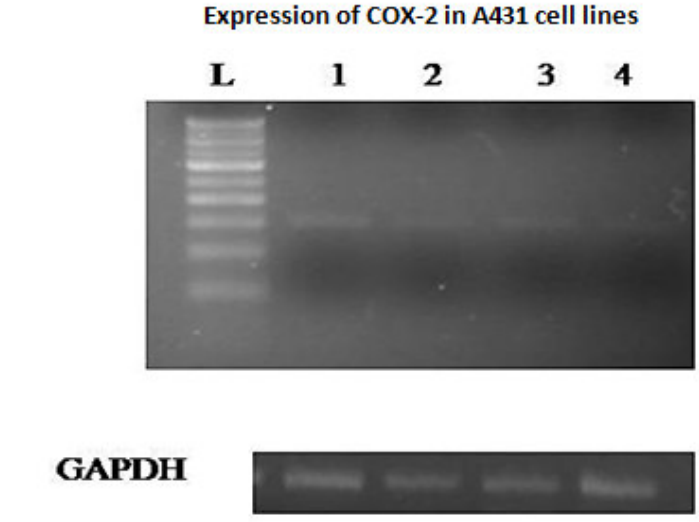


L-Ladder(100bp); 1 –Control (Untreated cells); 2- Doxorubicin(10µg/ml); 3- SVKT(700µg/ml); 4- SVKT(350µg/ml)

COX-2

COX-2 is a gene that expresses the enzyme cyclooxygenase that catalysis the rate limiting step for the synthesis of prostaglandins.[17] The up regulation of the COX-2 gene in the cancer cells is very well reported along with the fact that the use of Non-Steroidal Anti-Inflammatory Drugs like aspirin reduces the risk of cancer.[18] The underlying mechanism being the up regulation COX-2 increases the production of prostaglandins, which in turn act through the auto or paracrine signaling mechanism thereby, enhances the angiogenesis. The angiogenesis is an initial phase of tumor metastasis.[19] In the current study, the expression of COX-2 (w.r.t control cells) is decreased by 0.02 fold and 0.13 fold with the treatment of SVKT at the concentration of 700µg/ml and 350µg/ml respectively, whereas the standard drug doxorubicin suppresses the expression by 0.18 fold. The gene expression profile of the COX-2 gene is shown in Figure 4 and the extent of down regulation of COX-2 is depicted in Figure 6. It can be inferred from the study results, that the SVKT treatment down regulates the expression COX-2, factor responsible for the angiogenesis by inducing prostaglandins.

Figure 4
RT-PCR profile of COX-2 gene amplified from A431 cells treated with SVKT



L-Ladder(100bp); 1 –Control (Untreated cells); 2- Doxorubicin(10µg/ml); 3- SVKT(700µg/ml); 4- SVKT(350µg/ml)

MMP-9

MMP-9 is a matrix metalloprotease enzyme involved in the extracellular matrix degradation function.[20] MMPs play a key critical role in influencing the bioavailability or functionality of various factors that are involved in disrupting the balance between growth and antigrowth signals in the tumor microenvironment.[21]MMP-9 mainly regulates the bioavailability of Vascular Endothelial Growth Factor (VEGF), thereby induces the tumor angiogenesis and is implicated in vasculogenesis.[22]In the current study, the expression of MMP-9 (w.r.t control cells) is decreased by 0.07 fold and 0.04 fold with the treatment of SVKT at the concentration of 700µg/ml and 350µg/ml respectively, whereas the standard drug doxorubicin suppresses the expression by 0.14 fold. The gene expression profile of the MMP-9 gene is shown in Figure 5 and the extent of down regulation of MMP-9 is depicted in Figure 6. Results of this study indicate that the SVKT reduces the expression of vasculogenesis inducing factor MMP-9.

Figure 5
RT-PCR profile of MMP-9 gene amplified from A431 cells treated with SVKT

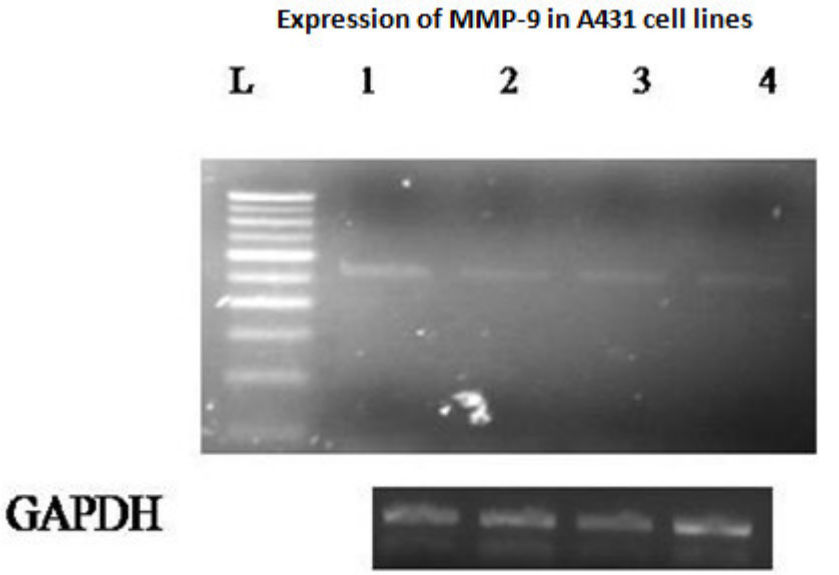
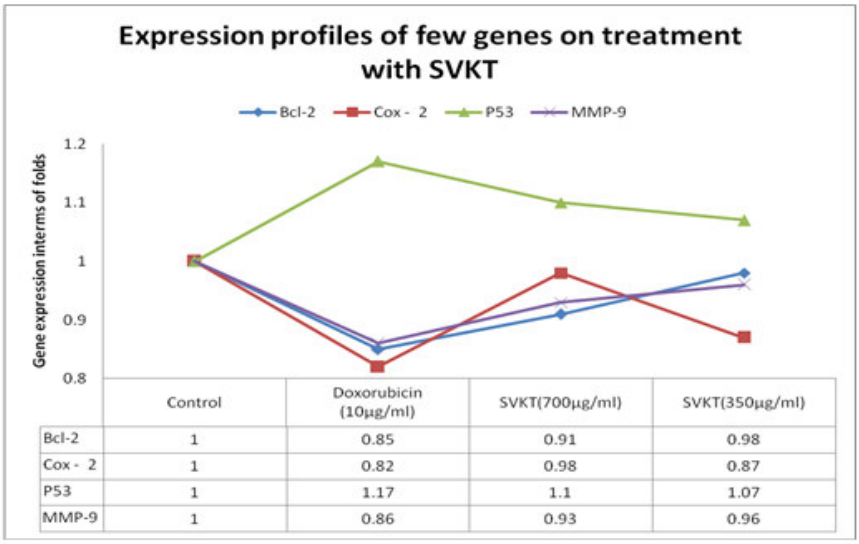


Figure 6
Dose dependent expression profile of few genes on treatment with SVKT VsStandard Doxorubicin



	Control	Doxorubicin (10µg/ml)	SVKT(700µg/ml)	SVKT(350µg/ml)
Bcl-2	1	0.85	0.91	0.98
Cox - 2	1	0.82	0.98	0.87
P53	1	1.17	1.1	1.07
MMP-9	1	0.86	0.93	0.96

CONCLUSION

Based on the current study, it can be concluded that the drug SVKT is effective against the human squamous cell carcinoma in comparison to mouse melanoma. SVKT induced cytotoxicity by upregulating of p53 and down regulating of Bcl-2, COX-2 and MMP-9. SVKT interact with multiple targets to exert its anti-cancer activity. The possible mechanisms could be induction of apoptosis in the cancer cells and inhibition of the cascading (such as inflammatory and growth factor) signals responsible for the vasculogenesis and tumor angiogenesis.

CONFLICT OF INTEREST

Conflict of interest declared none.

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