

# Cytotoxic and Apoptotic Effect of Commercial Tinctures of *Scutellaria baicalensis* on Lung Cancer Cell Lines

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## ABSTRACT

The cytotoxic and apoptotic potentials of four commercial tinctures of *S. baicalensis* (SB1, SB2, SB3 and SB4) were investigated on three human lung cancer cells SK-MES-1, SK-LU-1 and A549. The number of cells that survived treatment with different concentrations of the tincture in 24, 48 and 72 hours was quantified by the colorimetric MTT assay. Acridine orange/ Propidium iodide dye exclusion assay was used to test for apoptosis in 24 and 48 hours at 200 µg/ml concentration of the tinctures. All the tinctures except SB4 showed a dose and time-dependent effect on the cancer cells. The minimum cytotoxic concentration of SB1, SB2, and SB3 sample tinctures on the SK-MES-1 and SK-LU-1 cells was approximately 100 µg/ml in 48 hours. 100 µg/ml of SB1 and SB2 tinctures have significant ( $p < 0.05$ ) cytotoxic activity on the SK-MES-1 cells in 72 hours. The A549 cells were the least sensitive to toxicity as significant effect on growth inhibition was not seen until 300 µg/ml. Of all the tinctures, only the SB1 showed significant ( $p < 0.05$ ) cytotoxic activity on nontransforming normal dividing FS5 cells, pointing to the possibility of a non-specific side effect at 400 µg/ml. Estimating duration of effect from the time taken to reverse cell growth inhibition, SB1 tinctures had the longest effect of up to 72 hours on the SK-MES-1 cells. Other tinctures showed significant ( $p < 0.05$ ) time-dependent inhibition of cell growth up to at least 48 hours. Typical apoptotic morphological changes were observed with SB1, SB2 and SB3 tinctures in 24 hours but were more pronounced after 48 hours. The tinctures showed variable characteristics in terms of their dose response, duration of action and potential side effects. There is therefore an urgent need to standardize and regulate the manufacture of herbal formulations and establish a scientific evidence base on the use of commercial formulations of *S. baicalensis*.

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## INTRODUCTION

Cancer is a major public health burden in both developed and developing countries. It affects people of all ages but the risks for more severe forms increase with age (WHO, 2006; Cancer Research UK, 2007). Plants have long been used in the treatment of cancer (Hartwell, 1982). In Chinese traditional medicine, *Scutellaria baicalensis* root decoction is used in the treatment of cancer. In the West, tinctures (hydroalcoholic formulations) of *S. baicalensis* are preferred due to ease of storage, dosing and administration. Different manufacturers of *S. baicalensis* tincture use different drug - to - extraction solvent ratios and this usually results in non-uniformity in the flavonoid biomarker content of *S. baicalensis* tincture (Gao *et al.*, 2008).

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Despite Popular use of these tinctures, no uniform exists about their relative effectiveness. This study evaluated the cytotoxicity and apoptotic effects of available commercial tinctures of *S. baicalensis* on lung cancer cell lines (SK-LU-1, SK-MES-1 and A549).

## MATERIALS AND METHODS

### Tinctures of *S. baicalensis*

Different sample tinctures of *S. baicalensis* were provided by Dr Oliva Corcoran of the school of health and Biosciences of the university of east London. The samples were labelled SB1 (70%, 1:5), SB2 (45%, 1:1), SB3 (25%, 1:3) and SB4 (45%, 1:3), noting their concentration of alcohol and drug to extraction ratio.

### Preparation of working solutions

For the cytotoxicity assay, a 1 ml volume of each sample tincture was concentrated by evaporation of the alcohol content in a

fume chamber overnight. Each extract was subsequently dissolved in 100% DMSO to make 50mg/ml stock. A serial fold dilutions of the stock solution in Hank's Balanced salt solution (HBss, Sigma cell culture, Poole, Dorset, England) was done to obtain 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml. for the apoptotic assay.

#### Cell and cell culture conditions

Three human lung cancer cell lines, SK-MES-1, SK-LU-1 and A549 and a non tumorigenic normal dividing human foreskin fibroblast, FS5, were provided by the cell culture laboratory of the University of East London. All the cells were anchorage dependent cells. The cells were cultured in Dulbecco's modified Eagle medium in a class II Laminar flow hood (Galarre laboratories, BSB4A), followed by incubation in a 37°C, 5% CO<sub>2</sub>, humidified incubator for optimal cell growth. Each cell type in culture formed a monolayer that was passaged at least twice every week on the average. Cells were subcultured at a semi-confluent stage (70-80% confluence) when they were still in the log phase of growth.

#### Cell viability, count and inoculation density

Viable and cell count were determined by Trypan Blue dye exclusion assay after which cell suspensions were diluted to 1.5 X 10<sup>5</sup> and 0.5 X 10<sup>4</sup> viable cells per ml with plating medium for the cytotoxicity and apoptotic assay respectively. A volume of 100 µl of cell suspension in three replicates was transferred to each well of a collagen-coated 96- well plate to give a cell density of 1.5 X 10<sup>4</sup> cells per well, while 3 ml was transferred to 96 well plates containing already laid sterile coverslip giving the same density of cell for the apoptotic assay. Plates were incubated in a 37°C, 5% CO<sub>2</sub>, humidified incubator to allow the cells to attach.

#### Application of extracts

10 µl aliquote of each dosing solution was added to 100 µl of cell suspension to obtain 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml with the final concentration of DMSO in each concentration being less than 1%. For drug effect over 24 and 72 hours, six concentration points were used ranging from 3.125 – 100 µg/ml. while for effect over 48 hours, 12.5 µg/ml to 400 µg/ml were used. Control cells were treated with 10µl of medium alone.

#### Cytotoxicity/ MTT Assay (Van de Loosdrecht et al, 1994)

After incubation for 24, 48 and 72 hours, 10 µl aliquots of MTT solutions (5 mg/ml in PBS) were added to each well and re-incubated for 4 hours at 37°C. this was followed by centrifugation at 2000 rpm for 5 minutes. Then, the 110 µl of supernatant culture medium were carefully aspirated and 100 µl aliquots of isopropanol added to each well to dissolve the formazan crystals, followed by incubation for at least 1 hour to dissolve air bubbles. The culture plates were placed on a multiplate reader (Thermo multiskan spectrum 1500) and the absorbance measured at 570 nm. Cell viability rate was calculated as the percentage of MTT absorbed as follows:

$$\% \text{ survival} = [\text{mean experimental absorbance} / \text{mean control absorbance}] \times 100$$

#### Apoptotic Assay (Willingham, 1999)

Only SB1, SB2 and SB3 extracts were used. 300 µl aliquots of each dosing solutions of extracts was added to 3 ml of cell suspension per well. 3 µM of Camptothecin was used as the positive control while 300 µl of medium alone served as the negative control. Drug effect over 24 and 48 hours was used to quantify apoptosis.

#### Acridine Orange/ Propidium Iodide Assay

After 24 and 48 hours incubation, cells were centrifuged at 2000 rpm for 5 minutes onto the already laid sterile coverslips, followed by careful aspiration of supernatant culture medium in all the wells. The cells were washed twice with 3 ml of PBS after which 50 µl of a 10 µg/ml solution each of Acridine orange and propidium Iodide were added and let to stand for 3 minutes. Another washing with an equivalent volume (50 µl) of PBS was done before cells were viewed with a Nikon Optishot fluorescent microscope at X 400 magnification.

#### Statistical Analysis

The percentages of cell survival were presented graphically using Microsoft Excell 2010. Statistical analysis was done using one way ANOVA for multiple samples and students t-test for comparing paired sample sets. P- values < 0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

Considering the hydroalcohol extract yield from 1 ml volume of each of the tinctures (Table 1), it was observed that SB2 sample tinctures gave the highest yield followed by SB4. The low yield of SB1 and SB3 can be attributed to the drug-to-extraction solvent ratio as both have the smallest ratio of 1:5 and 1:3 respectively.

**Table 1:** Hydroalcohol extract Yield.

Sample Tincture	Weight of Hydroalcohol Extract (g)
SB1	0.1179
SB2	0.2196
SB3	0.0985
SB4	0.1394

Working with the findings of Geo et al., 2008 that only three major flavonoids (Baicalin, Biacalein and Wogonin) are present in the hydroalcohol extracts, SB1 with an extract yield of 117.9 mg will contain about 58 mg of baicalin, 47mg of baicalein and 13 mg of wogonin. These values are derivations from Gao's assertion showing baicalin to be 4.5 times the amount of wogonin and biacalein 3.7 times wogonin. Applying the same assumption, biomarker content as per the 219.6 mg extract yield for SB2 gives approximately 8 mg of baicalin, 206 mg of baicalein, and 5 mg of wogonin. This means that baicalein in SB2 is about 26 times the proportion of baicalin and 41 times that of wogonin. Considering

the 98.5 mg extract yield from SB3 sample tincture, the relative amount of the biomarkers will be approximately 91 mg for baicalin, 6 mg for baicalein and 2 mg for wogonin. This is derived from the proportionality ratio of 40.5:2.5:1 for biomarkers baicalin, baicalein and wogonin respectively. The same assumption could not be applied for the SB4 sample tincture as their relatively high extract yield could not be reconciled with their documented low total flavonoids biomarker content, suggesting the contributions of other solutes aside flavonoids in them. It is therefore most likely that baicalin and baicalein contributes equally to the observed effect seen with the SB2 tincture while for SB3 is baicalin. However, no single biomarker can be solely implicated for any effect observed with the SB4 tinctures.

At 100 µg/ml for 24 hours treatment, only SB1 showed non significant ( $p>0.05$ ) cytotoxicity on the SK-MES-1 cells just above the maximum concentration (Fig 1). All the other tinctures showed identical non- cytotoxic effects on all the cell lines (Figs 2,3 and 4). At higher dose, no cytotoxicity was observed with the SB4 tinctures on the SK-MES-1, SK-LU-1 and A549 cell lines while SB1, SB2 and SB3 tinctures showed significant ( $p<0.05$ ) cytotoxicity on SK-MES-1 cell line from over 100 µg/ml (Fig 5 and 6). Significant ( $p<0.05$ ) cytotoxicity with SB1, SB2 and SB3 was also observed from A549 cell line above 300 µg/ml (Fig 7). Of all the tinctures, only SB1 showed significant cytotoxicity effect ( $p<0.05$ ) on the normal dividing FS5 cells at concentration over 400 µg/ml (Fig 8). This points to possibility of side effects of SB1 at concentration up to 400 µg/ml.

Extending the duration of exposure up to 72 hours, SB1 showed significant ( $p<0.05$ ) cytotoxic effect on SK-MES-1 cell line at 65 µg/ml (Fig 9), SB2 tincture also showed significant activity but at concentration above 100 µg/ml. all the other tincture did not produce any cytotoxic effect on the SK-LU-1, A549 and FS5 cells (Figs 10 - 12). Adopting the flavonoids biomarker proportionality, ratio of SB1 and SB2 from Geo *et al*; 2008, the baicalein content of SB2 is about 3.6 times its content in the SB1 tinctures while the SB1 content of baicalin is about 8.39 times that of SB2 suggesting that high content of baicalin in SB1 may have contributed in its activity against SK-MES-1 cell line at lower concentration.

It has also been shown that baicalin is more potent than baicalein on human hepatoma cells (Yano *et al.*, 2004). Estimating duration of effect from the time it takes to reverse cell growth inhibition, SB1 tinctures had the longest duration of up to 72 hours on the SK-MES-1 cells. SK-LU-1 cells showed a time dependent effect up to 48 hours after which there was a significant increase in cell number compared to control. This suggests that the observed effect up to 48 hours may only be cytostatic. Inhibition of cell growth reversed after 24 hours in A549 cells and a growth stimulatory effect was observed in the FS5 cells in 24 hours (Fig 13). For the SB2 tincture, 24 hours treatment did not produce much effect on the SK-MES-1 cells. However, there was a significant drop ( $p<0.05$ ) in cell survival to 64.87% in 48 hours that start to reverse in 72 hours (Fig 14). There is therefore a possibility of a 48 hours cytostatic effect. The same trend was observed with the SK-LU-1 and A549 cell lines only that their 48 hours treatment was not significant ( $p>0.05$ ). effect of SB2 on the FS5 cells was most likely cytostatic and maximum effect as a result of receptor saturation in 24 hours can be suggested which was maintained up to 48 hours. However, there was a significant ( $p<0.05$ ) growth stimulating effect by the SB2 tinctures on the FS5 cell after 48 hours (Fig 14).

The possibility of a cytostatic effect up to 48 hours on the SK-MES-1 cells could be seen with the SB3 sample tinctures. It took only 24 hours for the growth inhibitory effect of the SB3 tinctures on SK-LU-1 cells to be reversed (Fig 15) pointing to possibility of 24 hours duration of cytostatic effect. Non-cytotoxic effect was seen in the A549 cells over 72 hours while the growth stimulatory effect on the FS5 cells was only observed after 48 hours. For the SB4 tincture, the same non-cytotoxic effect was seen in all the cell lines in 72 hours.

The acridine orange/propidium iodide Apoptosis assay show that the growth inhibition on the cancer cells after exposure to 200 µg/ml of the tinctures of *S. baicalensis* occurred by apoptosis. This was demonstrated by the appearance of condensed and fragmented nuclei in apoptotic cells compared to the negative control (Figs 17 and 18). It is therefore most likely that different commercial tinctures produce their different antiproliferative effects by apoptosis on SK-MES-1, SK-LU-1 and A549 cells.

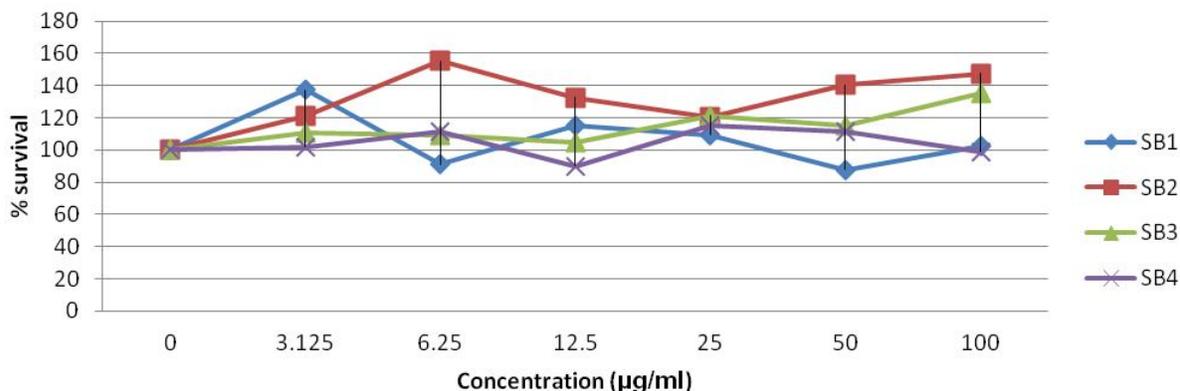


Fig. 1: Effect of 24 hours treatment of *Scutellaria baicalensis* on SK-MES-1 cells.

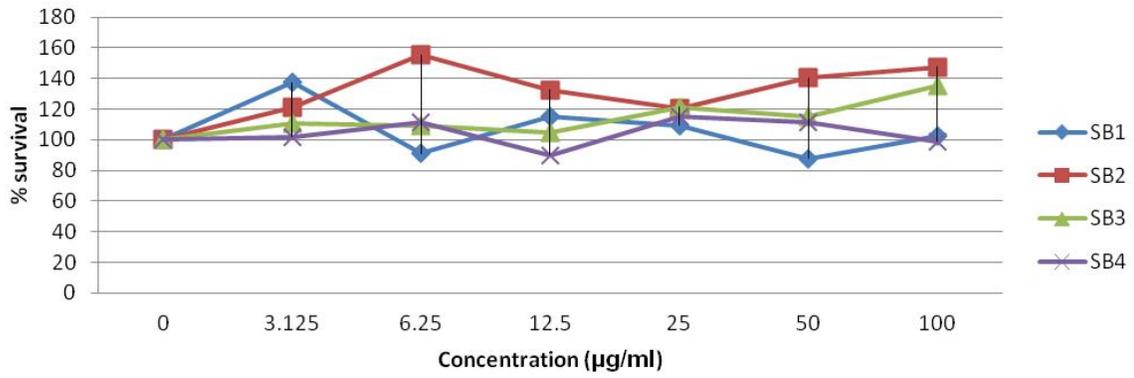


Fig. 2: Effect of 24 hours treatment of *Scutellaria baicalensis* on SK-LU-1 cells.

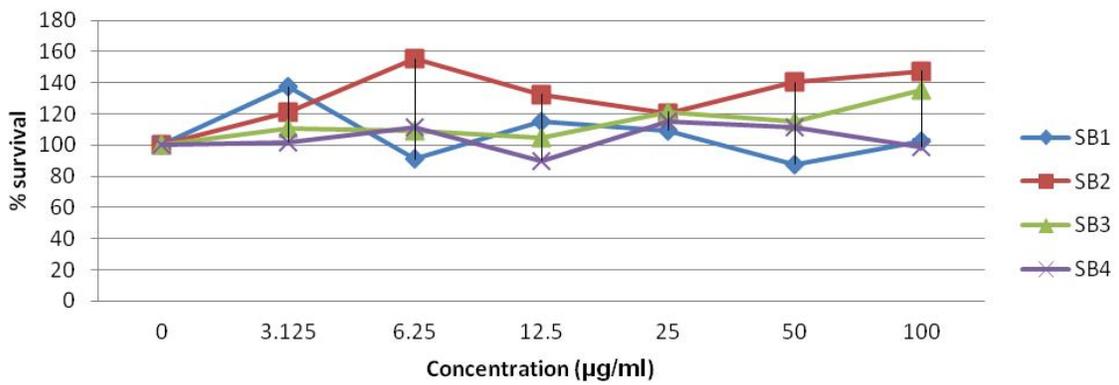


Fig. 3: Effect of 24 hours treatment of *Scutellaria baicalensis* on A549 cells.

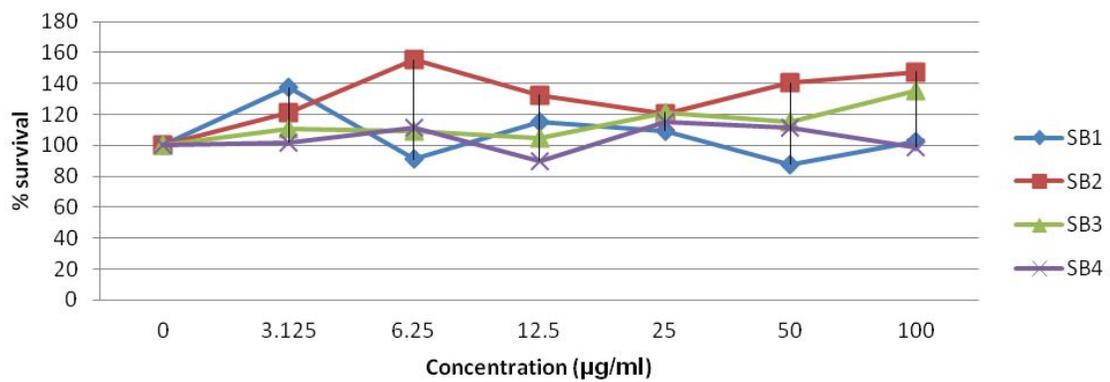


Fig. 4: Effect of 24 hours treatment of *Scutellaria baicalensis* on FS5 cells.

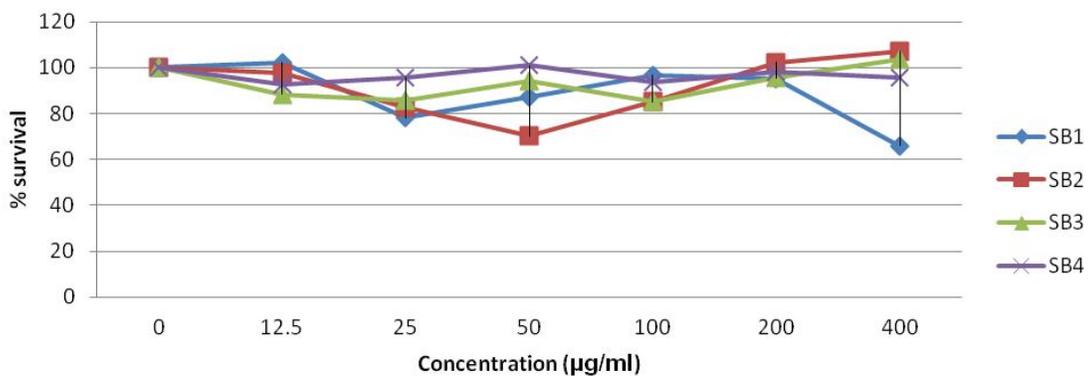


Fig. 5: Effect of 48 hours treatment of *Scutellaria baicalensis* on SK-MES-1 cells.

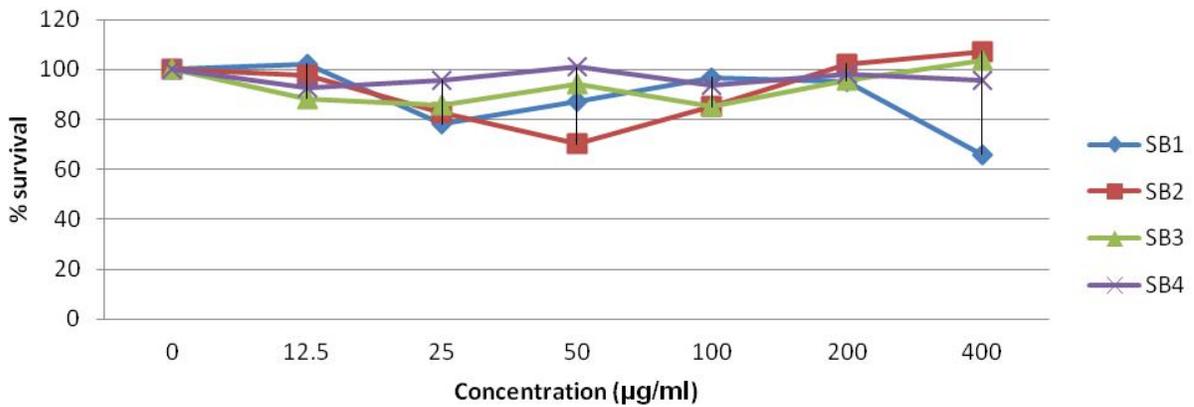


Fig. 6: Effect of 48 hours treatment of *Scutellaria baicalensis* on SK-LU-1 cells.

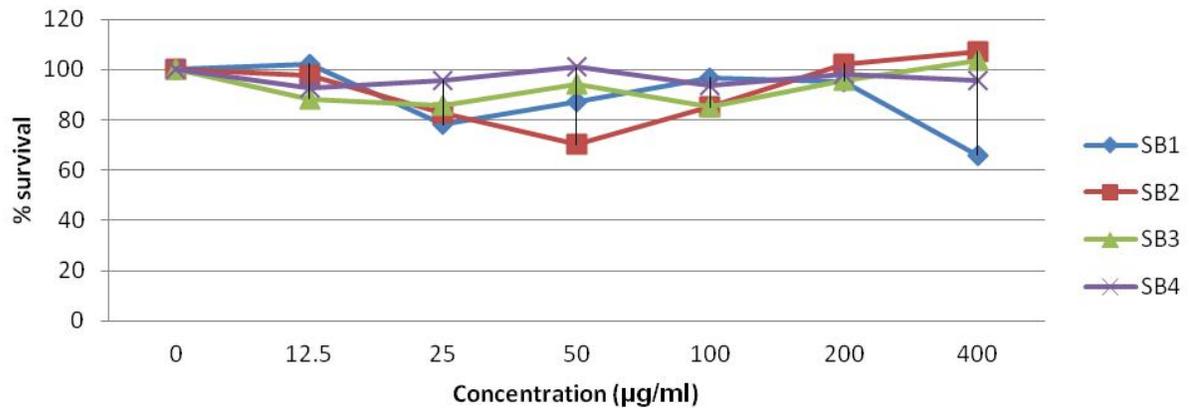


Fig. 7: Effect of 48 hours treatment of *Scutellaria baicalensis* on A549 cells.

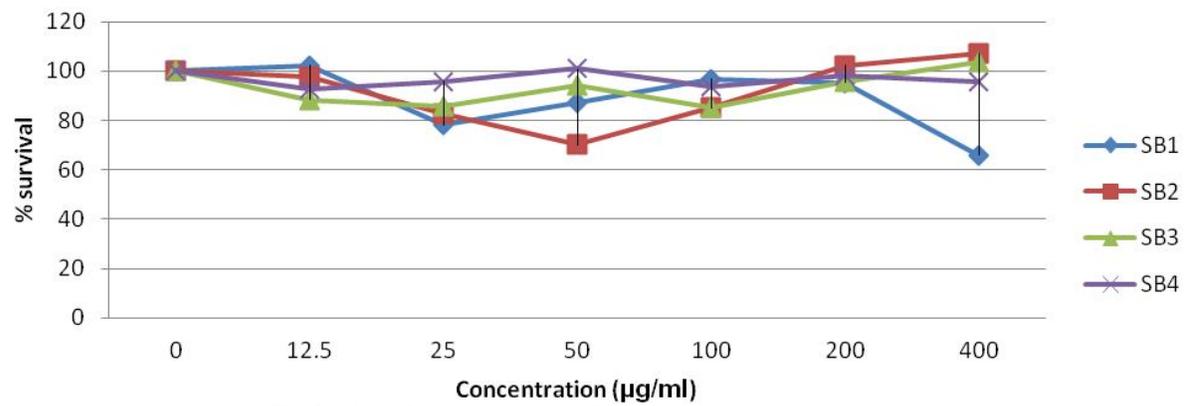


Fig. 8: Effect of 48 hours treatment of *Scutellaria baicalensis* on FS5 cells.

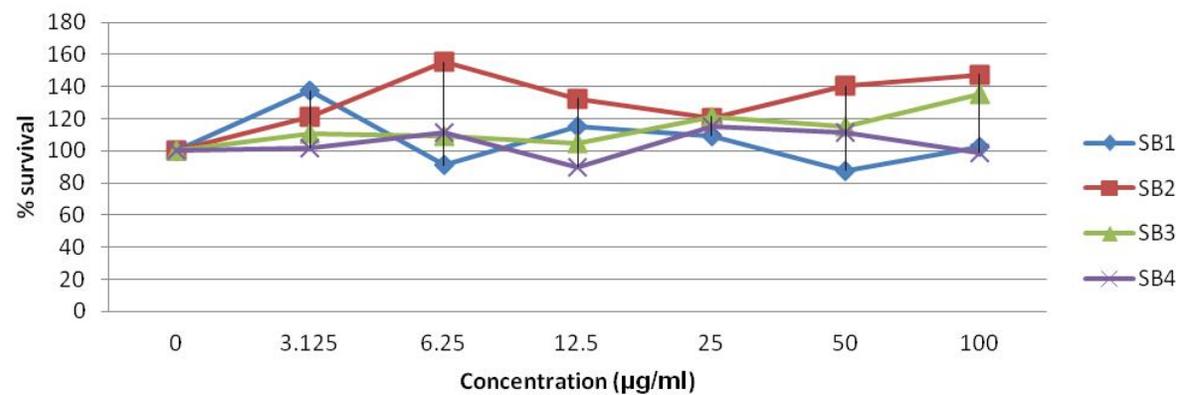


Fig. 9: Effect of 72 hours treatment of *Scutellaria baicalensis* on SK-MES-1 cells.

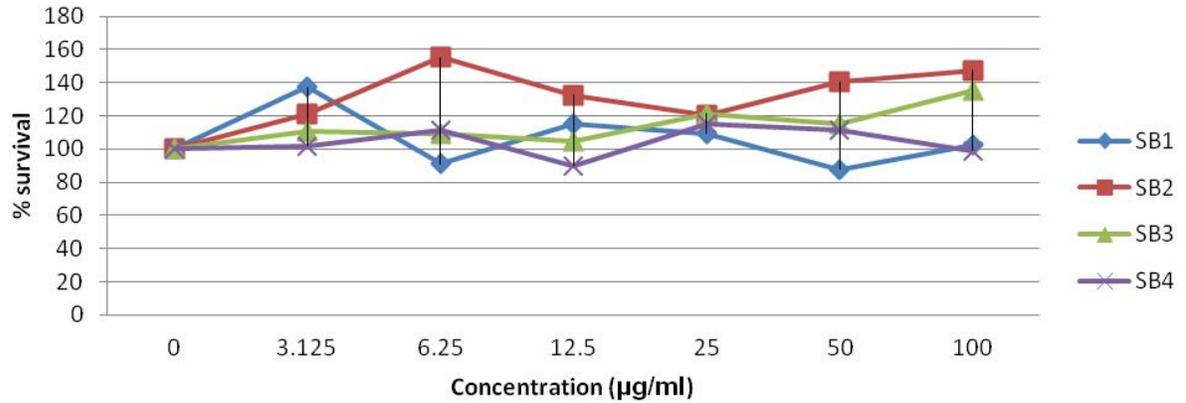


Fig. 10: Effect of 72 hours treatment of *Scutellaria baicalensis* on SK-LU-1 cells.

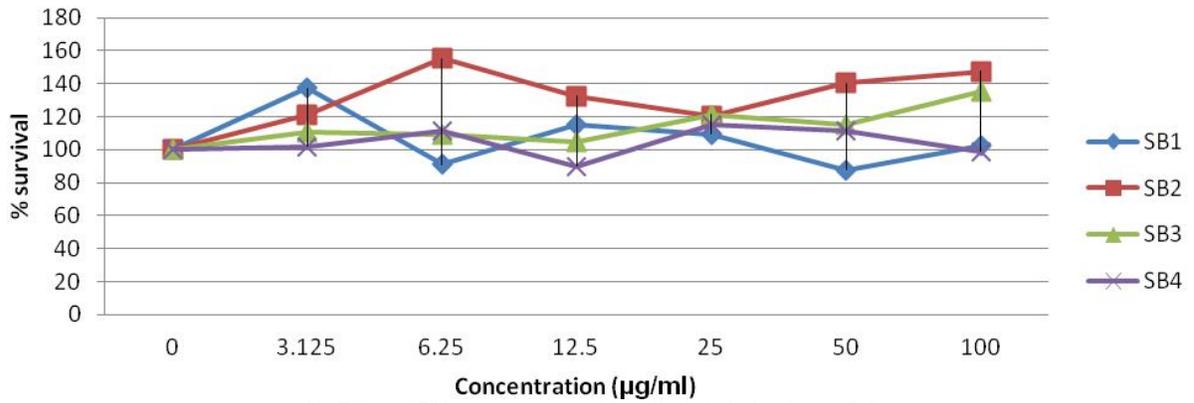


Fig. 11: Effect of 72 hours treatment of *Scutellaria baicalensis* on A549 cells.

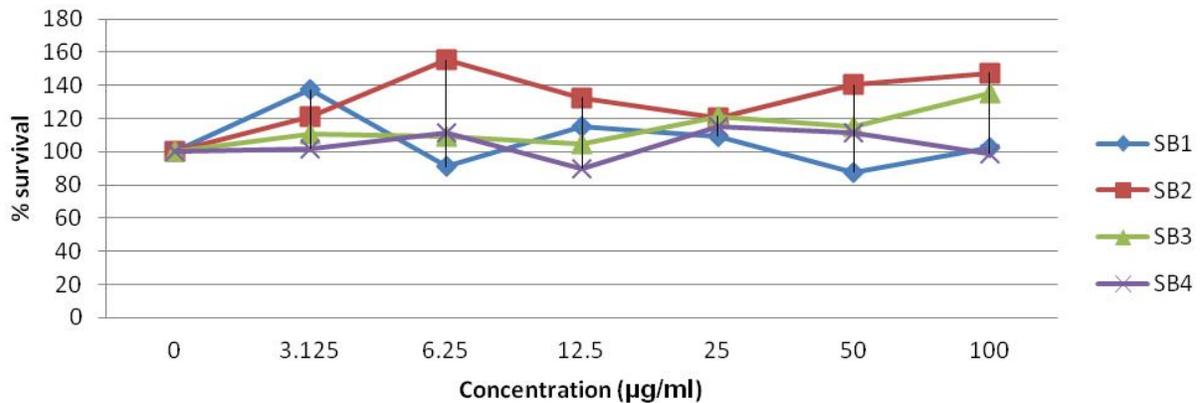


Fig. 12: Effect of 72 hours treatment of *Scutellaria baicalensis* on FS5 cells.

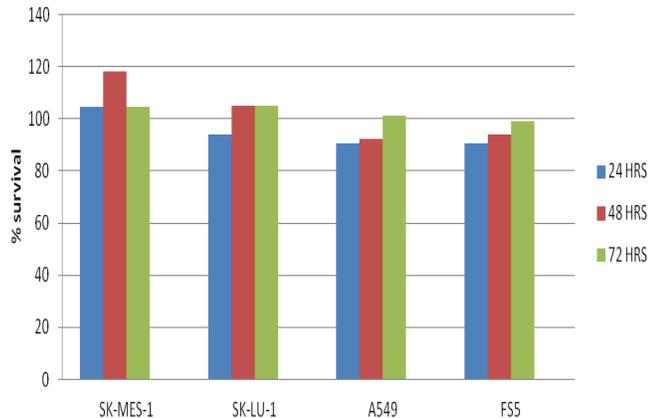


Fig. 13: Effect of 100µg/ml of SB1 on cells over 24, 48 and 72 hours.

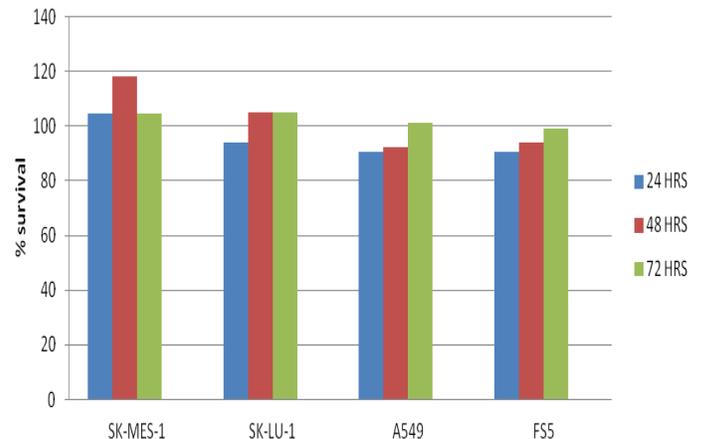
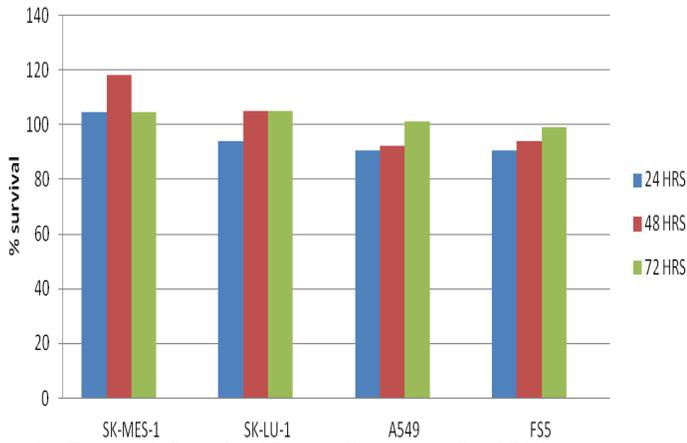
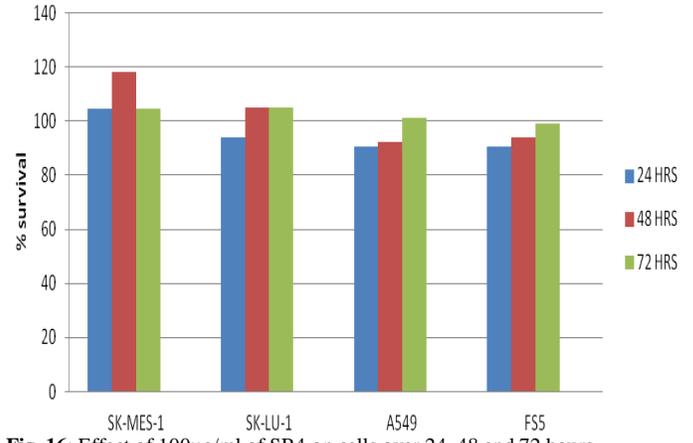


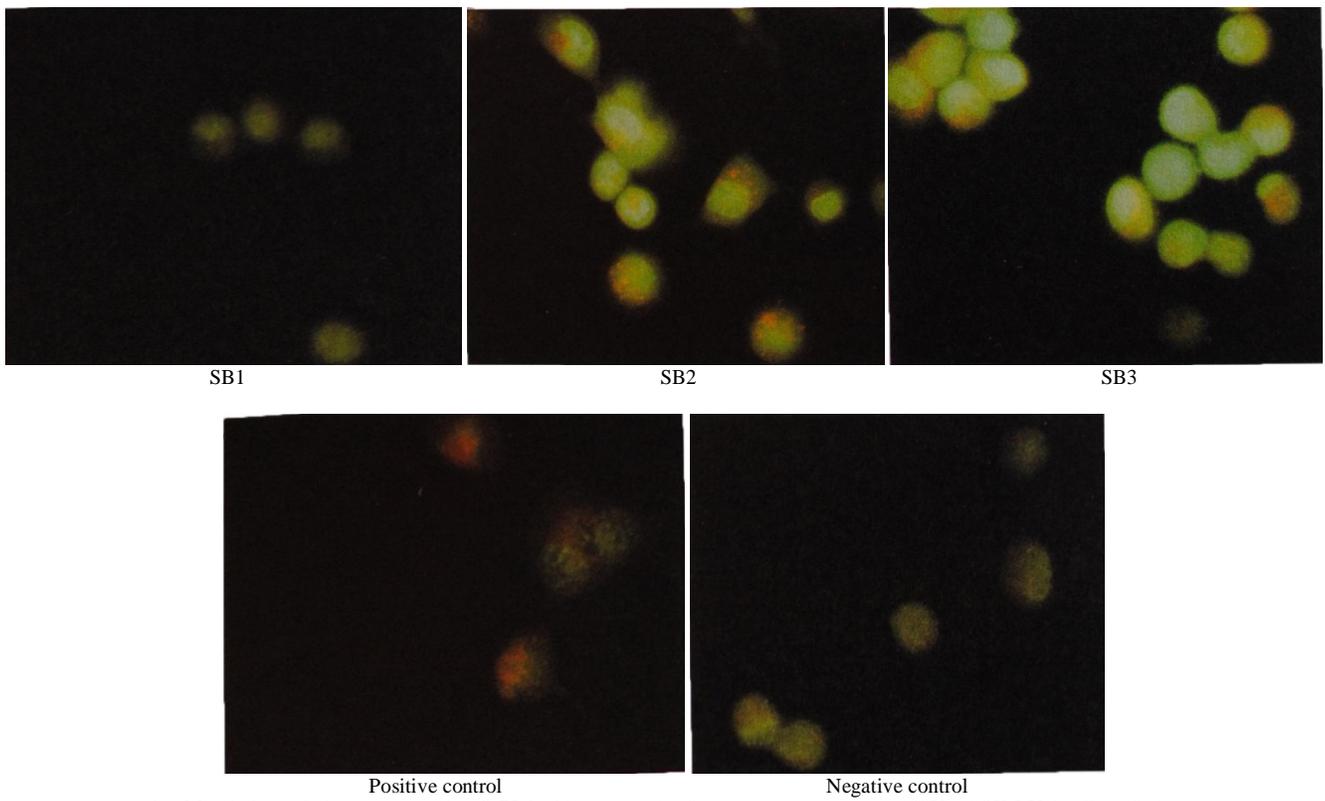
Fig. 14: Effect of 100µg/ml of SB2 on cells over 24, 48 and 72 hours.



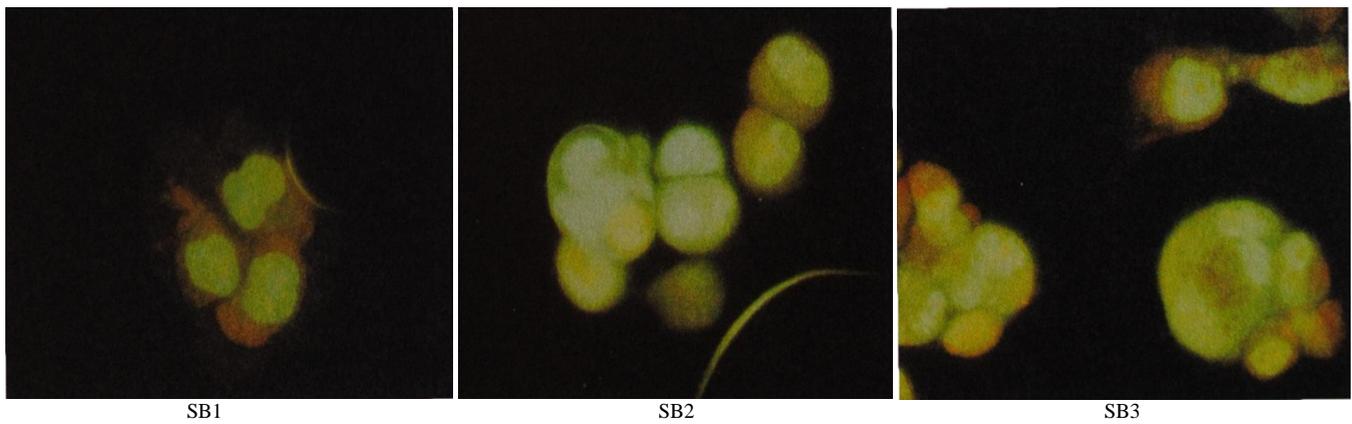
**Fig. 15:** Effect of 100µg/ml of SB3 on cells over 24, 48 and 72 hours.



**Fig. 16:** Effect of 100µg/ml of SB4 on cells over 24, 48 and 72 hours



**Fig. 17:** Morphological observation with AO/PI double staining by fluorescence microscope (400X) of SK-LU-1 cells in 24 hours.



**Fig. 18:** Continued.....

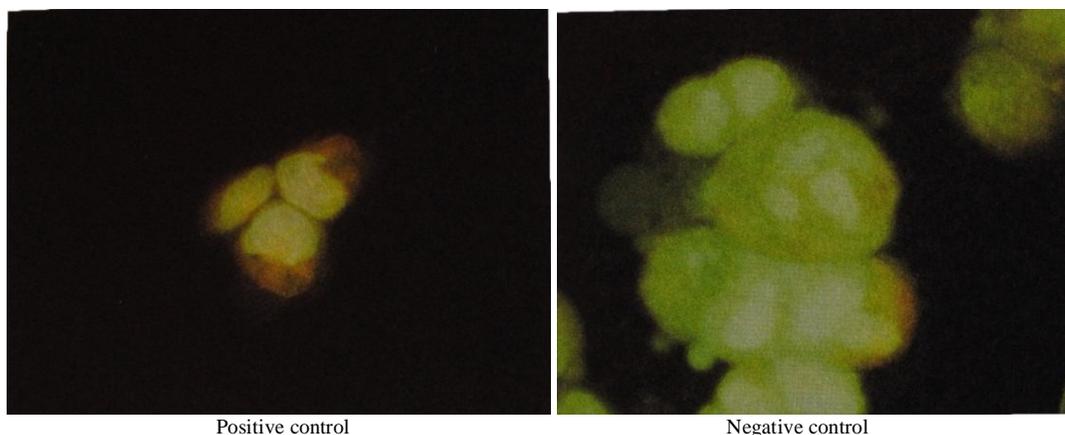


Fig. 18: Morphological observation with AO/PI double staining by fluorescence microscope (400X) of SK-MES-1 cells in 48 hours.

## CONCLUSION

The study showed appreciable quantitative difference in response by the different cell lines to *S. baicalensis* tinctures. The SB4 tinctures prepared from 45% ethanol and 1:3 drug-to-extraction solvent ratio did not show any potential for cytotoxicity on all the cell lines even up to 400 µg/ml in 24, 48 and 72 hours. The A549 cells were least sensitive to toxicity as significant effect was not seen until 300 µg/ml. All the tinctures except the SB4 thus exhibited a concentration-dependent effect on the cell lines. Of all the tinctures, only the SB1 showed significant ( $p < 0.05$ ) cytotoxicity on the normal dividing FS5 cells at concentration above 400 µg/ml suggesting that 400 µg/ml will most likely be the maximum tolerable dose (MTD) of the tincture in therapy.

The evidence of cell growth stimulation at 100 µg/ml with some tinctures may be attributed to a low-dose effect. A reverse of the cell growth inhibition suggest that the observed effect on cell survival may not be cytotoxic after all, but rather cytostatic effects that reversed after some time. Typical apoptotic morphological changes observed in 24 hours but that were of more evidence after 48 hours indicates that SB1, SB2 and SB3 sample tinctures inhibited SK-MES-1, SK-LU-1 and A549 cell proliferation through the apoptotic pathway. It is therefore proposed that though the SB1, SB2 and SB3 tinctures have potential as anticancer therapeutics, their equivalent doses have varied biological characteristics and therefore the need to standardize

and regulate the production of commercial herbal preparations of *Scutellaria baicalensis*.

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