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CYTOTOXIC POTENTIALS OF ALKALOID EXTRACT OF *SOLANUM KHASIANUM* BERRIES AGAINST DALTON'S LYMPHOMA, MCF-7 AND HELA CELL LINES *IN VITRO*

Gabriel Rosangkima* and Ganesh Chandra Jagetia

Department of Zoology, Mizoram University, Aizawl, Mizoram, India – 796 004.

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***Correspondence for Author**
Dr. Gabriel Rosangkima
Department of Zoology,
Mizoram University,
Aizawl, Mizoram, India.

ABSTRACT

In this study the cytotoxic potential of alkaloids extracted from *Solanum khasianum* berries was investigated against Dalton's lymphoma, MCF-7 and HeLa cells *in vitro*. Cytotoxicity was determined by MTT assay and also by trypan blue dye exclusion method. The result of present study showed that one of the alkaloid extract (SKF-Alk-2) exhibited concentration-dependent cytotoxicity in all the cell types *in vitro* with IC_{50} values of 19.76 µg/ml, 32.46 µg/ml and 36.82 µg/ml against DL, MCF-7 and HeLa cells respectively. The alkaloid extract also induced a significant increase in cancer cell apoptosis at a concentration of 20 µg/ml and above. Thin layer chromatography of SKF-Alk-2 showed two distinct spots on TLC plate with the R_f value of 0.81 and 0.91. However, further fractionation of SKF-Alk-2 by open column chromatography did not resulted to produce fractions with potent cytotoxicity suggesting the synergistic effect of SKF-Alk-2.

KEYWORDS: *Solanum khasianum*, alkaloids, cytotoxicity, apoptosis, synergistic effect.

INTRODUCTION

Natural products of plant origin have been used against various diseases and a large number of modern drugs have been developed from natural plant products. Medicinal plants continue to play an important role in the healthcare system of the world's population.[1] Ethno-medicinal practice has a long history in the developing countries and its continuous practice in the developing countries is mainly due to the high cost of western pharmaceuticals and healthcare. Cancer is one of the most life- threatening diseases all over the world. Millions of

people suffered from cancer every year, leading to death in a majority of the cases.[2] Therefore, research studies on finding new anticancer compounds are necessary and the interest of researchers around the world.[3] Plants have been used for the treatment of various human diseases since time immemorial. They maintain the health and vitality of individuals, and also cure a variety of diseases, including cancers with minimal side effects. More than 50% of all modern drugs in clinical use were derived from natural products and many of them were used to control cancer cell growth.[4] It was also reported that more than 60% of cancer patients use vitamins or herbal products as therapy.[5]

Solanaceae is a large plant family containing more than two thousand species and nearly half of them belong to a genus, *Solanum*. This family includes a large number of species known for the presence of a variety of natural products of medicinal importance.[6] Genus *Solanum* is rich in steroidal glycoalkaloids and flavonoids, an important group of plant secondary metabolites. These compounds are used as starting material for the synthesis of steroidal drugs. Various species of *Solanum* like *S. khasianum, S. lyratum, S. xanthocarpum, S. nigrum, S. gracile, S. tuberosum, S. laciniatum* are being extensively used for the treatment of various ailments like asthma, liver diseases and inflammation in the traditional system of medicine.[7] *Solanum khasianum* Clarke, belonging to the Family: Solanaceae is generally distributed throughout Mizoram state and other parts of North-East India.[8,9] It is a stout, branched and herbaceous weed commonly growing perennial. The stem and leaves has spines, the flowers are hermaphrodite and white. The fruit/berries are yellowish when ripe. The seeds are small, brown in colour and embedded in sticky mucilage. It has profound use in folklore medicine. There is a number of antifungal, antioxidant and cytotoxic compounds isolated from some *Solanum* species.[10,11,12,13] We have also reported previously the anticancer activity of aqueous and methanolic extracts of *S. khasianum* berries *in vitro*.[14] However, the details on the evaluation and establishment of the anticancer activity of alkaloidal extracts from this plant through scientific study have not been investigated. Therefore, present study was undertaken to evaluate the cytotoxic potentials of alkaloidal extract of *Solanum khasianum* berries on cancer cells *in vitro*.

MATERIALS AND METHODS

Cell culture: Cell lines of different tissue origin such as MCF-7 (human breast tumour) and HeLa (human cervical cancer) obtained from the National Center for Cell Science, Pune, India. Dalton’s lymphoma cells (mouse ascites tumor) obtained from the Cell and Tumor

Lab., Department of Zoology, North Eastern Hill University, Shillong, Meghalaya, India. Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C in a CO_2 incubator.

Alkaloid extraction: The berries of *Solanum khasianum* were rinsed thoroughly with tap water, shade-dried, removed the seeds and powdered with an electric-grinder. Extraction of alkaloids from the berries of *Solanum khasianum* was done following the methods described earlier [15,16] with some modifications. Briefly, 200 g of dried powder of *Solanum khasianum* berries was transferred into the soxhlet apparatus and then defatted with petroleum ether to yield greenish yellow oil, which is rejected as it is devoid of glycoalkaloid. Defatted material was then transferred into a round-bottom flask and gently shaken for overnight in 3 L of 2% aqueous oxalic acid in a magnetic stirrer apparatus at room temperature. The mixture was filtered through Whatman No. 1 filter paper and the clear filtrate (1700 ml) was collected. The filtrate was then gently heated at 75°C and alkalinized by adding 85ml of 60% NaOH. The mixture was kept cool at room temperature and then transferred into the refrigerator (15°C) and left overnight. Precipitate formed was collected by centrifugation at 20°C for 10 minutes at 3000 rpm. The supernatant was evaporated to yield a dry mass extract (SKF-Alk-1). Part of the SKF-Alk-1 was refluxed in 450 ml of 0.5 M HCl for 90 minutes at 100°C. The mixture was cooled and again alkalinized by adding 85 ml of 60% NaOH and then heated at 100°C for 15 minutes. After cooling, the mixture was centrifuged as above and the brownish pellet was collected and dried at 70°C. The dried pellet was then dissolved in hot absolute methanol and the colored impurities were removed by treating with activated charcoal. The resultant yellowish white extract (SKF-Akl-2) was evaporated to dryness. The two extracts, SKF-Alk-1 and SKF-Alk-2 were tested for the presence of alkaloids and its cytotoxic activity *in vitro*.

Cytotoxicity assay: Cytotoxic activity of the extracts was determined by MTT assay [17] and the trypan blue exclusion method. Briefly, $1x10^4$ cells were inoculated in 96 well plates containing 200 µL of MEM and allowed to grow in CO_2 incubator for 24 h (37°C, 5% CO_2). Thereafter, different concentrations of plant extracts (SKF-Alk-1 and SKF-Alk-2) were added and incubated for an additional 24 and 48 h followed by the addition of 20µl MTT ([3- (4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide]) stock solution (5mg/ml in PBS) and incubation for 5 h. The formazan produced by the viable cells was solubilized by addition of

20 µl DMSO and incubated for 2 h. The absorbance was recorded at 560 nm using a microplate reader (iMark Biorad Microplate Reader). The percentage cytotoxicity was calculated with respect to vehicle control using the following formula
% cytotoxicity = {(Control absorbance – Test absorbance)/Control absorbance} x 100.

Another set of cell culture was prepared where cells were harvested after 24 and 48 h of different extract treatment and processed for trypan blue dye exclusion assay. Percentage cytotoxicity was calculated using the following formula
% cytotoxicity = 100 x $(T_{dead} – C_{dead})/T_{total}$
where, T_{dead} is the number of dead cells in treated group, C_{dead} is the number of dead cells in control group and T_{total} is the number of dead and live cells in treated group.

Apoptosis analysis: Apoptosis analysis was carried out following standard method. Cells ($1x10^5$/ml MEM) were seeded in 12-well plates and incubated in CO_2 incubator. After 24 h of incubation, cells were treated with different concentrations of extracts and incubated for an additional 48 h. Thereafter, 40 µl of acridine orange/ethidium bromide dye (100 µg each in 1 ml PBS) was added to each well. After staining, cells were visualized immediately under a fluorescence microscope (Leica Microsystems, GmbH, Wetzlar, Germany).

Clonogenic assay

The cytotoxic effect of plant extracts was also further confirmed by clonogenic assay following standard method, where 300 cells were incubated into 25 cm^2 petridishes and allowed to attach for 16 h. Thereafter, cells were treated with various concentrations of plant extracts and left undisturbed for 10 days for the formation of cell clones. The cultures were then removed and stained using gentian violet. The total number of colonies/plate was counted. Plating efficiency (PE) and surviving fraction (SF) were calculated as mean ± SD by the following formula
PE = (No. of colonies formed/No. of cells seeded) x 100.
SF = (No. of colonies formed after treatment/No. of cells seeded x PE) x 100.

Cell growth inhibition assay

Cell growth inhibition assay was performed according to the method described by Harikumar et al (2009) [18]. Briefly, Cells (1 x 10^4/well) were plated in 96-well plates for 4 h and then treated with different concentrations of the extracts and incubation was continued for 48 h.

Cell growth inhibition was assayed using MTT assay as well as trypan blue exclusion method. The percentage of growth inhibition was calculated using the formula

% Inhibition = [{Control Abs. – Test Abs.} / Control Abs.] x 100

Thin Layer Chromatography: TLC of the potent alkaloid extract (SKF-Akl-2) was performed following standard protocol using TLC Silica gel 60 F_{254}. A mobile phase with the composition of chloroform:methanol 95:5 was prepared. The chamber of TLC was filled with the mobile phase ± 1 cm depth and allowed to be saturated with the vapour. The solution of substance (SKF-Akl) was then spotted on TLC plate using glass capillary tube. After being developed, the TLC plate was sprayed with Draggendorff's reagent. Positive result was shown by a brown spot coloration. The R_f value of test alkaloid was compared with the R_f values of alkaloid from literatures. The formula used for the calculation of R_f value is given as

$$R_f \text{ value} = \frac{\text{Distance moved by the sample}}{\text{Distance moved by the solvent front.}}$$

Fractionation of SKF-Alk-2 by column chromatography: The potent alkaloid fraction of *S. khasianum* berries (SKF-Alk-2) was subjected to open column chromatography on normal phase silica gel (60G) and eluted with a solvent mixture of hexane/ethyl acetate (10:0 – 0:10, v/v) and finally eluted with methanol affording 7 fractions (500 ml each). All the fractions were evaporated to dryness under reduced pressure and tested for their cytotoxic activity against DL and MCF-7 cells *in vitro*.

Statistical analysis: All statistical analysis was done using statistical software 'OriginPro 8 SRO v8.0724 (B724), Northampton, MA, USA'.

RESULTS

The two alkaloid fractions extracted from *S. khasianum* berries were designated as SKF-Alk-1 and SKF-Alk-2. The cytotoxicity tests using MTT assay and trypan blue exclusion method revealed that one of the alkaloid fraction, SHF-Alk-1, did not exhibit cytotoxic potential against the three cell lines *in vitro*. However, the other alkaloid fraction, SKF-Alk-2, showed potential cytotoxic effects against DL, MCF-7 and HeLa cells in a concentration-dependent manner (Table 1). The results also showed that SKF-Alk-2 exhibited higher cytotoxic effect against DL cells during 48 h of incubation as compared to other cells with IC_{50} values of

19.76 µg/ml, 32.46 µg/ml and 36.82 µg/ml against DL, MCF-7 and HeLa cells respectively (Table 1).

Table 1. *In vitro* cytotoxic activity of alkaloid extracts of *Solanum khasianum* fruit on different cell lines.

Plant extracts	Dose (µg/ml)	Percentage cytotoxicity (Mean ± S.D.)					
		DL cells		MCF-7		HeLa	
		24 h	48 h	24 h	48 h	24 h	48 h
SKF-Alk-1	5	1.4±0.4	1.3±0.7	1.5±0.7	1.5±0.7	1.2±0.7	1.5±0.7
	10	1.7±0.6	1.7±0.8	1.7±0.4	1.6±0.5	1.5±0.8	1.7±0.5
	20	1.7±0.5	1.9±0.9	1.8±0.4	1.9±0.8	1.5±0.6	1.7±0.5
	30	1.9±0.4	1.9±0.6	1.9±0.7	1.9±0.8	1.5±0.8	1.9±0.8
	40	1.8±0.3	2.1±1.7	1.9±0.6	2.2±0.9	1.8±0.8	1.9±0.7
	50	1.9±0.6	2.1±2.1	2.0±0.8	2.2±0.6	1.8±0.7	2.0±0.9
SKF-Alk-2	5	21.7±1.4	27.4±2.3	18.5±0.8	22.5±0.9	20.4±1.4	25.3±1.1
	10	28.5±2.3	29.4±1.8	25.5±0.9	28.2±0.8	24.7±1.2	28.8±1.5
	20	31.9±2.1	43.0±1.6	28.7±1.4	36.2±1.5	29.7±1.5	38.4±1.7
	30	43.0±1.7	55.6±1.8	35.6±1.8	49.6±1.2	33.6±0.9	46.2±1.2
	40	62.0±1.9	72.8±1.2	47.2±1.3	62.1±1.3	47.2±1.4	57.7±1.2
	50	73.4±1.2	80.7±1.5	61.3±0.9	69.4±1.3	54.9±1.6	63.9±1.4
IC_{50}			19.76		32.46		36.82

Results are mean ± S.D. N = 6.

In the apoptosis analysis, five different concentrations of extract such as 10, 20, 30, 50 and 100 µg/ml were used. The result of present study shows that SKF-Alk-2 extract induces apoptosis in all the cell types in a dose-dependent manner. As compared to the respective controls, the significant increase in the percentage of apoptosis was seen at a dose of 20 µg/ml and above in all the cell types. It induced 76%, 43% and 51% apoptosis in DL, MCF-7 and HeLa cells respectively at a concentration of 100 µg/ml (Fig. 1).

Cytotoxic potential of SKF-Alk-2 was also investigated through clonal culture of cancer cell lines. It was observed that SKF-Alk-2 produced cytotoxicity on the cell lines in a concentration-dependent fashion. Treatment with different concentrations of SKF-Alk-2 resulted in a significant decline in the clonogenicity of DL, MCF-7 and HeLa cells (Table 2). Maximum decline in reproductive capacity of all cell types was observed at 100 µg/ml. Analysis of survival fractions also suggest that SKF-Alk-2 extract showed more significant decline in the number of colonies of DL cells as compared to MCF-7 and HeLa cells.

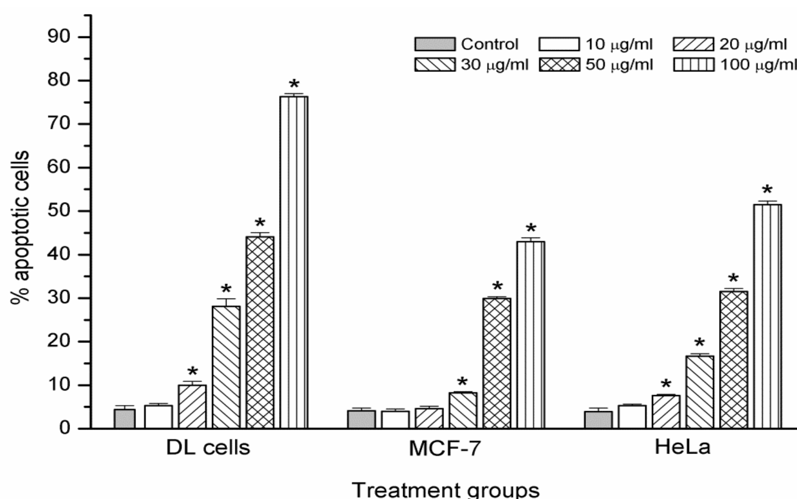

Figure 1. Percentage apoptotic cells induced by SKF-Alk-2 *in vitro*. Results are mean ± S.D. Student's t-test, N = 6, p < 0.05.

Table 2. Survival analysis of cancer cells treated with SKF-Alk-2 extract *in vitro*.

Plant extract	Dose (µg/ml)	SF ± S.D.		
		DL cells	MCF-7	HeLa
SKF-Aq	0	0.971±0.114	0.985±0.123	0.973±0.104
	10	0.804±0.057	0.932±0.101	0.884±0.112
	20	0.451±0.057	0.754±0.065	0.548±0.083
	30	0.305±0.093	0.538±0.076	0.372±0.074
	50	0.092±0.109	0.244±0.053	0.186±0.059
	100	0.021±0.057	0.172±0.069	0.138±0.072

SF = Survival Fraction. Results are mean ± S.D. N = 6.

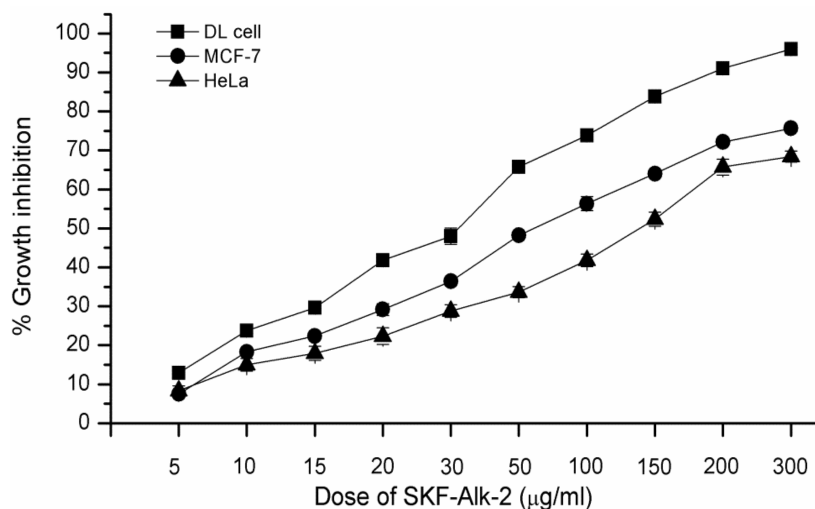

Figure 2. Graph showing the effect of SKF-Alk-2 on the *in vitro* proliferation of different cell lines (MTT Assay). Results are mean ± S.D. N = 6.

The results of cell growth inhibition studies also support the *in vitro* cytotoxicity and antiproliferative potentials of SKF-Alk-2 on cancer cells. The result of present study revealed that treatment of DL, MCF-7 and HeLa cells with SKF-Alk-2 caused a concentration-dependent increase in the cytotoxicity after 48 hours. Out of different doses used, maximum growth inhibition was observed at 300 µg/ml SKF-Alk-2. (Fig. 2).

After being sprayed with Draggendorff's reagent, thin layer chromatography of SKF-Alk-2 showed two brown spots on TLC plate with the R_f value of 0.81 and 0.91 (Fig. 3). Comparison of the results with literatures indicated that the two spots showed a very similar R_f values with an indole alkaloids, vindoline ($R_f = 0.81$) and ajmalicine ($R_f = 0.91$).

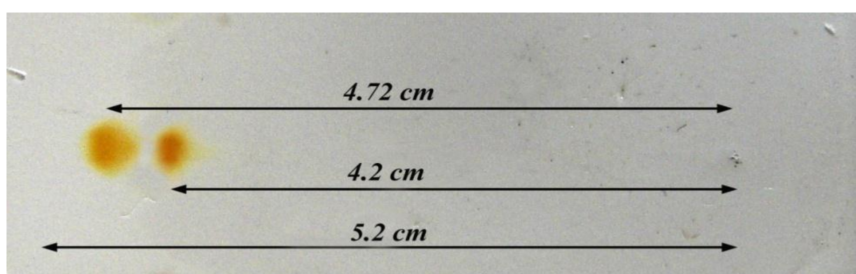

Figure 3. The TLC chromatogram of SKF-Alk-2 of *S. khasianum* berries showing alkaloids with R_f values 0.81 and 0.91.

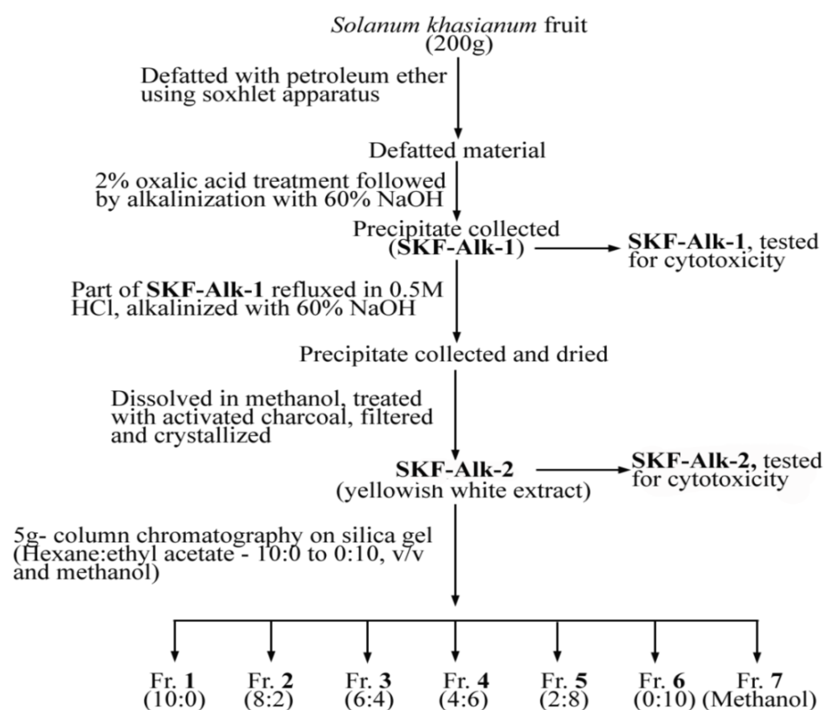

Figure 18. Schematic diagram representing extraction and fractionation of alkaloids from *S. khasianum* berries.

Fractionation of SKF-Alk-2 by column chromatography yielded seven different fractions (Fraction **1, 2, 3, 4, 5, 6, and 7**). Figure 4 schematically represents the extraction and fractionation procedure for these fractions. However, *in vitro* cytotoxicity studies of these fractions on DL and MCF-7 cells did not show potential cytotoxic effect of any of the extract (Table 3).

Table 3. ***In vitro*** **cytotoxic activity of different fractions of SKF-Alk-2 on DL and MCF-7 cells determined by MTT assay.**

Extract Fractions	Dose (μg/ml)	Percentage cytotoxicity (mean ± S.D.)			
		DL cells		MCF-7	
		24 h	48 h	24 h	48 h
1	10	1.3±0.5	1.9±0.6	1.1±0.5	1.8±0.8
	30	1.2±0.8	1.6±0.6	2.2±0.5	2.2±0.7
	50	1.8±0.7	2.4±0.8	2.1±0.7	2.7±0.7
2	10	2.1±0.5	2.6±0.5	1.4±0.4	2.0±0.6
	30	2.3±0.6	3.2±0.7	1.5±0.5	2.3±0.8
	50	2.8±0.4	3.3±0.5	1.9±0.4	2.3±0.7
3	10	1.6±0.7	2.4±0.6	2.1±0.4	2.6±0.7
	30	2.7±0.8	3.4±0.5	2.7±0.6	2.9±0.8
	50	2.4±0.5	3.5±0.5	3.0±0.6	3.1±0.7
4	10	1.5±0.6	1.8±0.7	2.2±0.5	3.0±0.8
	30	1.6±0.7	2.2±0.5	2.3±0.7	3.6±0.8
	50	2.2±0.7	2.7±0.8	2.7±0.5	3.6±0.5
5	10	2.7±0.6	3.1±0.6	2.6±0.6	2.7±0.6
	30	2.8±0.8	3.0±0.7	3.0±0.8	3.1±0.8
	50	3.3±0.8	3.5±0.5	3.3±0.8	3.4±0.6
6	10	2.1±0.7	2.8±0.5	2.1±0.5	2.8±0.6
	30	1.8±0.5	2.7±0.5	2.7±0.6	2.7±0.9
	50	2.4±0.5	3.6±0.7	2.6±0.7	3.1±0.7
7	10	3.2±0.6	3.3±0.6	4.2±0.5	5.1±0.8
	30	3.8±0.9	4.0±0.7	4.8±0.6	5.0±0.5
	50	4.1±0.7	4.2±0.7	5.5±0.5	5.8±0.6

Percentage cytotoxicity was calculated against control. Results are mean ± S.D. N = 6.

DISCUSSION

In various anticancer studies, murine ascites Dalton's lymphoma, human breast tumor (MCF-7) and cervical cancer (HeLa) have been commonly used as an important experimental cancer models.[19,20,21] In this study, the antiproliferative properties of the alkaloid extracts were determined using trypan blue exclusion method and MTT assay. The principle of MTT assay is based on the reduction of a soluble tetrazolium salt, by mitochondrial dehydrogenase activity of viable tumor cells, into a soluble colored formazan product that can be measured spectrophotometrically after dissolution.[22] The IC_{50} value was used as a parameter for

cytotoxicity. The criterion for cytotoxicity for the crude extracts, as established by the National Cancer Institute (NCI), is an IC_{50} value lower than 30 µg/ml.[23] Alkaloids have a wide distribution in the plant kingdom and mainly exist in higher plants. They are important chemical compounds that serve as a rich reservoir for drug discovery. Several alkaloids isolated from natural herbs exhibit antiproliferation and antimetastasis effects on various types of cancers both *in vitro* and *in vivo*. Alkaloids, such as camptothecin and vinblastine, have already been successfully developed into anticancer drugs.[24,25] The range of alkaloid concentration necessary to elicit the anticancer effects is wide.[26,27,28] The needed concentration is relatively higher for most of the alkaloids to produce anticancer effects, compared with the widely used chemotherapeutic drugs such as taxol and vinblastine, although both are also naturally derived alkaloids.

Staining cells with acridine orange and ethidium bromide is used in evaluating the nuclear morphology of apoptotic cells. To confirm that apoptosis has been induced by *S. khasianum* berries extract (SKF-Alk-2), cells were analyzed in the presence of acridine orange and ethidium bromide staining (AO/EB staining). Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity.[29] The percentage of apoptotic cells after treatment increased in a concentration-dependent manner in all treated cells with less than 50% in MCF-7 and more than 50% in DL and HeLa cells, i.e., a stronger apoptosis signal was induced with higher concentrations of the extract. Thus, SKF-Aq-2 extract, through morphological analysis using AO/EB staining procedure, showed that the extract was able to trigger cell death through apoptosis. The observation using AO/EB staining also showed that SKF-Alk-2 was able to induce apoptotic activity at concentrations as low as 20 µg/ml.

Several studies indicates that combination therapy probably provides an optimal venue for the clinical application of compounds because most of the alkaloids exhibit synergistic or enhancement effects when combined with chemotherapeutic drugs in both *in vitro* and *in vivo* experiments.[30,31,32,33] In the present study, none of the compound(s) fractionated by open column chromatography from SKF-Alk-2 showed cytotoxic activity against cancer cells *in vitro*. However, a crude alkaloidal fraction of SKF-Alk-2 extract exhibited potent cytotoxicity suggesting the synergistic effect of the extract. Therefore, the exact anticancer mechanisms of alkaloids require further identification using new pharmacological technologies. Exploration of the effective combinational therapy methods may also be required. In addition to this,

further preclinical anticancer trials for these alkaloid extracts need to be performed. Since the only extract (SKF-Alk-2) with potent anticancer activity is a combination of multiple alkaloids, further isolation and identification of active principles is required. A comparison of thin layer chromatographic profile of SKF-Alk-2 with literatures suggested that an indole alkaloids, vindoline and ajmalicine, with anticancer and antioxidant properties,[34,35,36] may also be present in the SKF-Alk-2 extract.

CONCLUSION

Alkaloid extract of *Solanum khasianum* berries (SKF-Alk-2) showed a significant anticancer efficacy against DL, MCF-7 and HeLa cells in a concentration-dependent manner along with more than 60% cytotoxicity at a maximum dose of 50 µg/ml. None of the fractions isolated from SKF-Alk-2 exhibited cytotoxic activity against DL and MCF-7 cells *in vitro* suggesting the synergistic anticancer effect of the plant extract. So, it may be concluded that SKF-Alk-2 exerts the high anticancer activity against DL, MCF-7 and HeLa cells but further studies are required to establish its anticancer efficacy using more human cancer cell lines and also to observe any adverse effect in normal cells.

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