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Anticancer activity of *Solanum khasianum* fruit extracts against Dalton's lymphoma tumor cells *in vitro* and *in vivo*

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ABSTRACT

Medicinal plants have played an important role in the discovery of drugs for the treatment of cancer and other diseases. The aim of present study was to investigate the anticancer potential of Solanum khasianum fruit extracts using Dalton's lymphoma tumor model in vitro and in vivo. The aqueous (SKF-Aq) and methanol (SKF-MeOH) extracts of S. khasianum fruit showed potent cytotoxic activity in a time and dose-dependent manner against Dalton's lymphoma cells in vitro. 800µg/ml dose produced percentage cytotoxicity of 91.8% and 43.5% respectively for SKF-Aq and SKF-MeOH extracts with an IC_{50} value of 307 and 1044µg/ml respectively after 48 h of treatment. SKF-Aq extract also induced more than 70% apoptotic cells after 48 h of incubation at 800µg/ml dose while SKF-MeOH extract induced less than 35% apoptotic cells. SKF-Aq extract dose-dependently increased the survivability of tumor-bearing mice. 400mg/kg/day showed comparatively better antitumor activity (%ILS- 206) against ascites Dalton's lymphoma and forty percent tumor-bearing mice survived more than 60 days. The phytochemical screening of aqueous extract of Solanum khasianum fruit revealed the presence of alkaloids and saponins in larger amount and flavonoids in small amount. The results of present study indicate the scope of developing anticancer drugs from Solanum khasianum fruit. However, further detailed study is necessary to identify and isolate the active principle(s), to find out the mechanism(s) involved in its anticancer activity.

Key Words: *Solanum khasianum, Dalton's lymphoma, Cytotoxicity, Apoptosis, Phytochemicals.*

INTRODUCTION

Natural plant products have been used for the treatment of various diseases for thousands of years and a large number of modern drugs have been developed from them. The first written records on the medicinal plants appeared in about 2600 BC [1]. Over the past decade, herbal medicine makes an impact on world health and international trade. Medicinal plants continue to play an important role in the healthcare system of the world's population [2]. Herbal medicines has a long history of use in the developing countries and continuous usage of herbal medicine 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 and causes serious health problems in both developed and developing countries. Every year, millions of people are diagnosed with cancer, leading to death in a majority of the cases [3]. Cancer is the diseases characterized by the deregulated proliferation of abnormal cells that invade and disrupt their surrounding tissues [4]. Therefore, investigations for finding new anticancer compounds are essential and the interest of various researchers around the world [5]. Drug discovery from natural plant products has played a vital role in the treatment of cancer and over the last century most of the plant metabolites and their derivatives have been used to combat cancer diseases [6, 7].

Chemotherapy, a major treatment modality used for the control of advanced stages of malignancies, exhibits severe side effects on the hosts [8]. 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 diseases, including cancer with minimal side effects. More than 50% of all modern drugs in clinical use are of natural products and many of which have the ability to control cancer cell growth [9]. It was also reported that more than 60% of cancer patients use vitamins or herbal products as therapy [8, 10]. Thus, medicinal plants continue to play an important role in the healthcare system of the world's population.

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 [11]. Solasodine, nitrogen containing steroidal glycoalkaloid has been reported to be obtained from different parts of the genus *Solanum* (Solanaceae). It has different biological activities including antifungal, insect growth regulation and enzyme inhibition property [12] it heals herpes skin lesions [13] inhibits proliferation of murine spleen cell cultures to mobilize gucocorticoids from the adrenals of rats and exhibit an antihepatotoxic effect on mice. It inhibits mouse lymphocyte proliferation induced by phytohemagglutinin. Thus, solasodine appears to have immunosuppressive activity. Solasodine also has antiurolithiatic and natriuretic activity [14]. It shows cytotoxic activities in vitro against variety of cancer cell lines [15] and antiproliferative activities against human colon (HT29) and liver (HepG2) cancer cells.

Solanum khasianum Clarke (Mizo name: Rulpuk), belonging to the Family: Solanaceae is generally distributed throughout Mizoram state and other parts of North-east parts of India [16, 17]. 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. Our preliminary investigation through literature search and consultation of local herbal practitioners revealed that *Solanum khasianum* Clarke is one of the most commonly used medicinal plants in Mizoram state, India. The fruit and seeds of this plant have been extensively used by the local people (Mizo) of Mizoram as a traditional home medicine for the treatment of toothache, cancer suspected diseases, skin diseases, non-specific cough and rheumatism etc. However, the details on the evaluation and establishment of the anticancer activity of this plant through scientific study have not been investigated. Thus, considering the importance of this plant with the probable anticancer medicinal value, particularly in the life of the people of Mizoram and other people in general, the present study was undertaken.

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MATERIALS & METHODS

Animals and tumor model:

Inbred Swiss albino mice were maintained under conventional laboratory conditions at room temperature (20 ± 2°C) with free access to standard food pellets and water *ad libitum*. Ascites Dalton's lymphoma tumor is being maintained *in vivo* in 10 - 12 weeks old mice by serial intraperitoneal (i.p.) transplantations of $1x10^6$ viable tumor cells per animal. Tumor-transplanted hosts usually survived for 16 - 18 days. The use of animals in the present study is as per the ethical norms and has been cleared by institutional ethical committee of Mizoram University, Aizawl.

Plant materials and extract preparation:

S. khasianum fruits were obtained from the outskirt of Kawlkulh village, Mizoram, India, during the month of February. The plant specimen was authenticated by Dr. R. Lalfakzuala, Department of Botany, Mizoram University, Aizawl and the herbarium specimen (Voucher No. GRS-SKH 001) was deposited in the Department of Zoology, Mizoram University, Aizawl. The fruits were washed with tap water immediately after collection, shade dried and taken to the laboratory. The fruits were then cut into pieces, removed the seeds, and the remaining peel and flesh were dried in the oven under 50°C, and then blended into powders. For cold extraction, three different solvents were used in ascending order of polarity i.e. hexane, chloroform and methanol followed by hot water extraction. Briefly, the fruit powder was macerated (200g of fruit powder in 1 L solvents) for 48 hours at room temperature. The extract was filtered through Whatman No. 1 filter paper and the filtrate was evaporated to dryness at 50°C. However, in hot water extraction, residue of methanol extraction was boiled in double distilled water for two hours, allowed to cool and then filtration and drying was done at 50°C. All the four different fraction extracts were stored at -20°C. Out of four different extracts prepared, only hot water and cold methanol extracts were used in the present studies due to inconvenience in the solubility of hexane and chloroform extracts in the treatment vehicles.

Short-term cytotoxicity studies:

Short-term *in vitro* cytotoxicity of *S. khasianum* fruit extracts were assessed by the trypan blue exclusion method [18]. Briefly, Dalton's lymphoma cells aspirated from the mice's peritoneal cavities were washed three times with culture medium (MEM) to remove traces of blood. Ten thousand cells in 200µl MEM supplemented with gentamycin were seeded in 96 well plates and incubated for 24 h in CO_2 incubator (37°C, 5% CO_2). Thereafter, different concentrations of aqueous and methanol extracts of *S. khasianum* fruit (50, 100, 200, 400 and 800µg/ml) dissolved in 10µl of MEM were added to each well. Cells were harvested after 1, 2, 3, 24 and 48 h of extract treatment and processed for viability assay. Percentage cytotoxicity was calculated using the formula:

$$\% \text{ cytotoxicity} = 100 \times (T_{dead} - C_{dead})/T_{tot}$$

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_{tot} is the total number of dead and live cells in treated group.

MTT assay:

The MTT assay, based on the conversion of the yellow tetrazolium salt-MTT to purple-formazan crystals by metabolically active cells, provides a quantitative determination of viable cells. Cells are plated on to 96 well plates at a cell density of $1x10^4$ per well in 200 µL of MEM supplemented with gentamycin and allowed to grow in CO_2 incubator for 24 h (37°C, 5% CO_2). Then, different concentrations of *S. khasianum* extracts (in 10µl MEM) were added and incubated for an additional 48 h. 20µl MTT ([3- (4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide]) stock solution (5mg/ml in PBS) was added to each well and incubated for 5 h. The formazan produced by the viable cells was solubilized by addition of 20µl DMSO and incubated for 2 h. The absorbance was recorded at 560nm using microplate reader (iMark Microplate Reader). The percentage cytotoxicity was calculated with respect to vehicle control using the formula:

$$\% \text{ cytotoxicity} = \{(\text{Control absorbance} - \text{Test absorbance})/\text{Control absorbance}\}x100.$$

Apoptosis analysis:

For apoptosis analysis, cells ($1x10^5$/ml MEM supplemented with gentamycin) were seeded in 12-well plates and incubated in CO_2 incubator. After 24 hours of incubation, cells were treated with different concentrations of *S. khasianum* extracts (50, 100, 200, 400 and 800µg/ml) and incubated for an additional 48 hours. Thereafter, 40µl of acridine orange/ethidium bromide dye (100µg each in 1 ml PBS) was added to each well. After staining, cells were visualised immediately under a fluorescence microscope (Leica Microsystems CMS GmbH).

***In vivo* antitumor activity studies:**

In vivo antitumor activity of aqueous extract of *S. khasianum* fruit was studied in mice using Dalton's lymphoma (DL) tumor model [19]. One million viable DL cells (in 0.25ml PBS, 7.4 pH) were transplanted intraperitoneally in 10-12 weeks old Swiss albino mice. Tumor transplantation day was designated as day 0. Different doses of the extract (in 0.25ml PBS) were administered intraperitoneally for seven consecutive days starting from day one of tumor transplantation. Control group of mice received 0.25ml of PBS alone. Control and treated mice (50 to 800mg/kg/day) consisted of 10 mice each. The antitumor efficacy was reported in percentage of average increase in life span (%ILS) calculated using the formula (T/C x 100) – 100, where, T and C are the mean survival days of treated and control groups of mice respectively. The pattern of changes in body weight and food consumption of experimental mice from control and treated at a dose of 400mg/kg/day showing maximum %ILS were also recorded.

Phytochemical screening:

Qualitative phytochemical analysis of aqueous extract of *S. khasianum* fruit was conducted following the standard procedures [20,21]. The tests for phytomchemical screening include: tests for alkaloids, flavonoids, terpenoids, phenols, saponins, tannins, carbohydrates, cardiac glycosides and triterpenoids.

Statistical analysi:

Results were expressed as mean ± S.D. Student's *t*-test for analysis of statistical significance was performed using statistical software OriginPro 8 SRO v8.0724 (B724), Northampton, MA, USA. A value of $p \leq 0.05$ was considered to be significant for comparison between data sets.

RESULTS

Short-term cytotoxicity studies:

The aqueous (SKF-Aq) and methanol (SKF-MeOH) extracts of *S. khasianum* fruit showed a time and dose dependent cytotoxic activity against DL cells *in vitro* at a dose range of 50 – 800µg/ml during 1 – 48 h of treatment. The percentage cytotoxicity, performed by trypan blue dye exclusion method, corresponding to each time and dose level is shown in Table 1. The higher dose, 800µg/ml produced percentage cytotoxicity of 91.8% and 43.5% respectively for SKF-Aq and SKF-MeOH extracts with an IC_{50} value of 307 and 1044µg/ml respectively after 48 h of treatment.

MTT assay:

Cytotoxicity studies of the plant extracts in DL cells using MTT assay also exhibited similar pattern of percentage cytotoxicity during 48 h of incubation as observed by trypan blue dye exclusion method showing maximum percentage cytotoxicity at 800µg/ml dose for both SKF-Aq (91.5%) and SKF-MeOH (42.8%) extracts as well as IC_{50} value of 313µg/ml and 1075µg/ml respectively (Table 2). The present results revealed a significantly higher *in vitro* cytotoxic potential of aqueous extract of *S. khasianum* fruit as compared to methanol extract.

Apoptosis analysis:

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* fruit extracts, DL 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 [22]. Five different concentrations were chosen (Figure 1 & 2). The result of present study shows that SKF-Aq extract induced more than 70% apoptotic cells after 48 h of incubation at 800µg/ml dose whereas all other doses induced less than 35% apoptotic cells

(Figure 1). As compared to SKF-Aq extract, SKF-MeOH extract exhibited lesser apoptotic index showing less than 35% apoptotic cells for all the doses after 48 h of incubation (Figure 2).

In vivo antitumor activity studies:

In the antitumor activity studies, ascites Dalton's lymphoma has been commonly used as an important murine experimental tumor model [23,24]. For the *in vivo* antitumor activity studies, SKF-Aq extract showing significantly higher cytotoxic activity was selected. Different doses of SKF-Aq extract and their effects on the survivability of the hosts have been described in Figure 3. The deaths of mice, if any, were recorded daily and the survival pattern of mice in different treatment groups was determined.

SKF-Aq extract dose-dependently increased the survivability of tumor-bearing mice. Among five different dose used, 400mg/kg/day showed comparatively better antitumor activity (%ILS- 206) against ascites Dalton's lymphoma (Figure 3). The comparative survival patterns of tumor-bearing mice treated with different doses of SKH-Aq extract were shown in Figure 4. Hundred percent survivors were noted till 48 days with 400mg/kg/day dose and forty percent mice survived more than 60 days.

During tumor growth progression, there was a rapid increase in the body weight of control tumor-bearing mice reaching 32g on the 16th day of tumor growth. Treatment with SKF-Aq extract at 400mg/kg/day dose significantly decreases the body weight of tumor-bearing mice after 12th day of tumor growth (Figure 5). In control tumor-bearing mice, there is a gradual decrease in their food consumption. However, the extract treatment also caused a significant increase in the food consumption of tumor-bearing mice after 12th day of tumor growth (Figure 5).

Phytochemical screening:

The phytochemical screening of aqueous extract of *Solanum khasianum* fruit (SKF-Aq) revealed that alkaloids and saponins were present in larger amount with flavonoids in small quantities. However, the extract tested negative for the presence of terpenoids, phenols, tannins, carbohydrates, cardiac glycoside and triterpenoids (Table 3).

Table No. 1: *In vitro* cytotoxic activity of *S. khasianum* extracts on DL cells at different time intervals of incubation by Trypan blue exclusion method

Treatment	Dose (µg/ml)	% cytotoxicity at different times of incubation (mean±S.D.)				
		1 hour	2 hour	3 hour	24 hour	48 hour
SKF-Aq	50	8.6±1.24	10.2±1.43	12.5±1.73	8.4±0.97	9.8±0.89
	100	14.1±2.04	17.9±2.27	21.3±2.08	15.0±1.74	19.2±1.33
	200	25.5±1.85	27.0±2.05	36.0±2.24	31.1±1.89	33.6±2.53
	400	33.0±1.79	36.2±1.77	51.6±1.84	46.4±2.07	50.5±2.71
	800	39.1±2.06	43.9±1.93	67.1±2.19	85.2±2.11	91.8±2.88
			IC_{50}			**307.9**
SKF-MeOH	50	1.9±0.46	4.2±0.52	2.5±0.65	4.1±0.72	4.5±0.78
	100	6.5±0.86	6.9±1.12	6.3±0.94	7.2±1.16	8.8±1.33
	200	6.9±1.17	10.0±1.62	10.6±1.06	14.2±1.43	15.1±1.50
	400	10.3±1.83	19.7±2.08	19.3±1.87	22.4±2.19	26.7±2.01
	800	13.1±2.01	21.2±2.13	19.8±2.05	28.1±2.06	43.5±2.27
			IC_{50}			**1044**

Table No. 2: *In vitro* cytotoxic activity of *S. khasianum* extracts on DL cells after 48 hours of incubation by MTT assay

Treatment	Dose (µg/ml)	% cytotoxicity (mean±S.D.)
SKF-Aq	50	9.9±2.18
	100	18.6±2.25
	200	33.2±2.09
	400	49.5±2.23
	800	91.5±2.87
	IC_{50}	**313.9**
SKF-MeOH	50	4.2±1.94
	100	9.1±2.02
	200	14.7±2.56
	400	26.4±2.08
	800	42.8±2.26
	IC_{50}	**1075**

Table No. 3: Preliminary phytochemical analysis of aqueous extract of *S. khasianum* (SKF-Aq)

Test	Alkaloids	Flavonoids	Terpenoids	Phenols	Saponins	Tannins	Carbohydrates	Cardiac glycoside	Triterpenoids
	++	+	-	-	+++	-	-	-	-

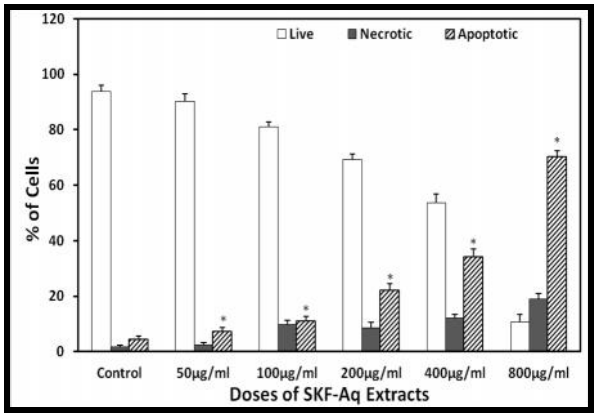

Fig. 1: Graph showing percentage of cell death via necrosis and apoptosis after 48 hours of treatment with aqueous extract of *S. khasianum* fruit (SKF-Aq). Results are expressed as mean±S.D. Student's t-test, N = 5. p Y 0.05 represents the statistically significant difference between the control and treated groups.

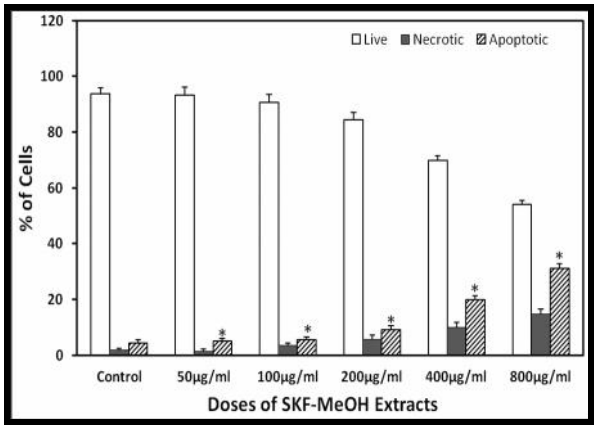

Fig. 2: Graph showing percentage of cell death via necrosis and apoptosis after 48 hours of treatment with methanol extract of *S. khasianum* fruit (SKF-MeOH). Results are expressed as mean±S.D. Student's t-test, N = 5. p Y 0.05 represents the statistically significant difference between the control and treated groups

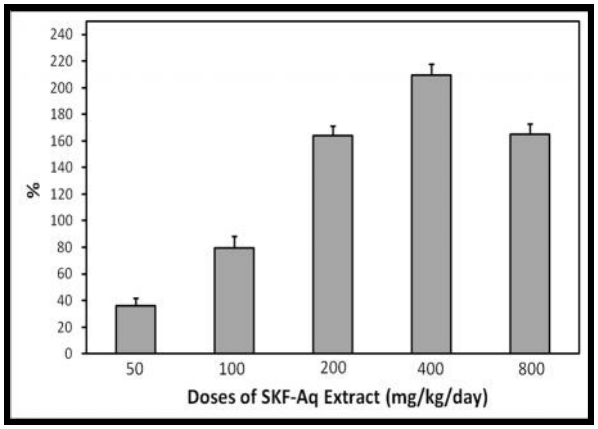

Fig. 3: Graph showing percentage increase in life span (%ILS) of tumor-bearing mice after treatment with different doses of aqueous extract of *S. khasianum* fruit (SKF-Aq). Results are expressed as mean±S.D. Student's t-test, N = 10. *p Y 0.05 represents the statistically significant difference between the control and treated groups.

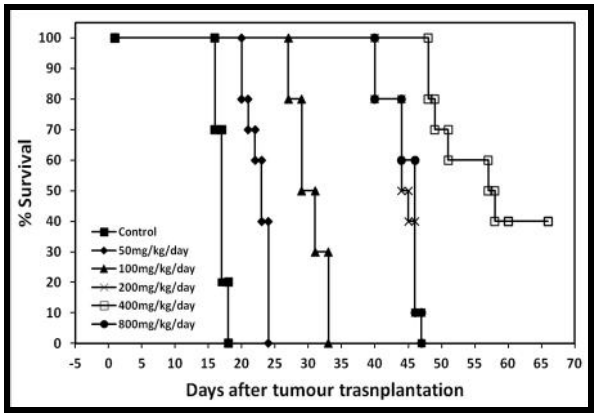

Fig. 4: Graph showing percentage survival of tumor-bearing mice after treatment with different doses of aqueous extract of *S. khasianum* fruit (SKF-Aq)

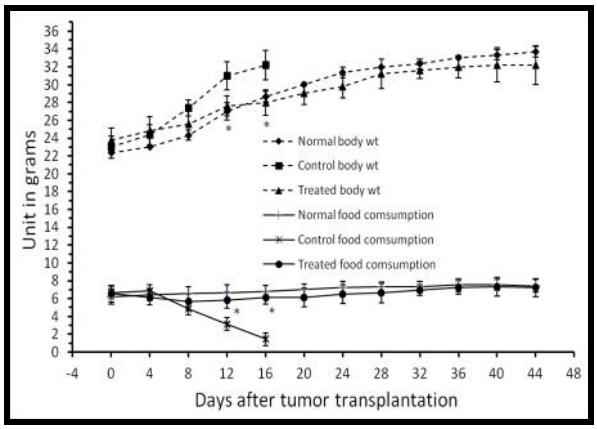

Fig. 5: Graph showing the pattern of changes in the body weight and food consumption of mice during tumor growth after treatment with aqueous extract of *S. khasianum* fruit (SKF-Aq) at a dose of 400mg/kg/day. Student's t-test, N = 10. p Y 0.05 represents the statistically significant difference between the control and treated groups

DISCUSSION

Cancer is the uncontrolled growth and spread of cells. The growth of cancer often invades the surrounding tissues and can metastasize to distant tissues and organs. Many cancers can always be prevented by avoiding exposure to common risk factors, such as tobacco smoke, diet and obesity. Chemotherapy is regarded as one of the most efficient cancer treatment approach. Faced with palliative care, many cancer patients seek alternative and complementary methods of treatment such as usage of phytomedicine [25]. Nowadays researchers are focusing towards the development of anticancer drugs from plant sources resulting in the discovery of chemotherapeutic agents such as paclitaxel, vincristine, camptothecin, podophyllotoxin etc. In the present study a common plant *Solanum khasianum* was selected and the methanol and aqueous extracts of its fruit were screened for their anticancer potential against sacites Dalton's lymphoma *in vitro* and *in vivo*.

Cytotoxic activity of the extracts was assessed by trypan blue exclusion and MTT methods. Both the tested extracts showed time and dose-dependent cytotoxic activities against Dalton's lymphoma cells. The most potent cytotoxic activity of aqueous and methanol extracts were observed at 800µg/ml dose during 48 h incubation with the IC_{50} value of 307 and 1044µg/ml respectively. Staining cells with fluorescent dyes, including acridine orange and ethidium bromide, is used in evaluating the nuclear morphology of apoptotic cells. To confirm that apoptosis has been induced by the extracts of *Solanum khasianum* fruit, Dalton's lymphoma cells were analysed 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 [22]. The result of present study also showed that using the AO/EB staining procedure, the apoptotic index were dose dependent, i.e., a higher apoptotic index was induced with higher concentrations of the extracts.

The merit of the anticancer drug can be judged by accessing the increase in life span of tumor-bearing animals [26]. Tumor-bearing mice possessed increased ascites fluid and tumor cell counts. Ascites fluid provides the essential nutrients for the growth of cancer cells hence increase in fluid volume directly correlated with tumor growth [25]. In the present study, antitumor activity of aqueous extract of *Solanum khasianum* fruit against Dalton's lymphoma tumor-bearing mice was assessed by the parameters such as tumor volume, food consumption, mean survival time and percentage increase in life span. The tumor volume was found to be significantly increased in tumor-bearing control animals and food consumption and mean survival time were significantly decline in tumor-bearing control animals when compared with normal animals. These results could suggest either a direct cytotoxic effect of aqueous extract of *Solanum khasianum* fruit on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition.

Throughout history, plant products and their modified analogs have been rich sources of clinically useful drugs, including anticancer agents. Such anticancer principles from plants include alkaloids, terpenes, lignans, flavones etc [27]. Alkaloids, isolated from medicinal plants, have a varied range of effects. It has potent anticancer activity against various cancers. They are chemically heterogeneous group of approximately 2500 basic nitrogen containing substances, found in more than 150 plant families, widely distributed in higher plants. It is abundantly found in the families such as Apocynaceae, papaveraceae, papilionaceae, ranunculaceae, rubiaceae, rutaceae and solanaceae [28]. The result of present studies revealed the presence of alkaloids in the aqueous extract of *S. khasianum* fruit which could be responsible for its medicinal effect particularly anticancer property.

CONCLUSION

Plant products have been played an important role in the development of anticancer agents such as camptothecin derivatives, topotecan, irinotecan, vinblastine, vincristine, etoposide and taxol [29]. Our present study indicates the scope of developing anticancer drugs from *Solanum khasianum* fruit. However, further investigation is required to identify the active principle(s), to find out the exact mechanism of its anticancer activity and to study its side effects in the host animals.

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