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RESEARCH ARTICLE

IN VITRO ANTICANCER SCREENING OF MEDICINAL PLANTS OF MIZORAM STATE, INDIA, AGAINST DALTON'S LYMPHOMA, MCF-7 AND HELA CELLS

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ABSTRACT

In this study the anticancer potentials of some traditional anticancer medicinal plants was investigated against Dalton's lymphoma (DL), MCF-7 and HeLa cells *in vitro*. Cytotoxicity of various plant extracts was determined by MTT assay. The result of present studies showed that out of 24 different extracts studied three extracts such as methanol extracts of *Solanum khasianum* fruit and *Dillenia pentagyna* stem bark as well as aqueous extracts of *Solanum khasianum* fruit showed potent anticancer activity on all the three cancer cells tested in a concentration-dependent manner. The most potent anticancer activity was observed with the aqueous extract of *S. khasianum* fruit (SKF-Aq) with IC_{50} values of 18.24 µg/ml, 23.65 µg/ml and 21.26 µg/ml on DL, MCF-7 and HeLa cells respectively. Phytochemical analyses revealed the presence of large amount of alkaloids and flavonoids in the potent plant extracts which may be suggested to play an important role in their anticancer activities.

INTRODUCTION

Since medieval times, plants have been the major source of medicines for the treatment of various diseases. Currently plants remain an integral part of the health care in different countries including developed countries. According to the report of WHO in the late 90's, a very high percentage of the world's population rely on plant based therapies to cover the needs of the primary health care (Dikshit *et al*, 2004). Medicinal plants possess an important position in the drug discovery and many modern drugs have their origin in traditional medicine of different cultures. Hence, regardless of the advantages of the synthetic and combinatorial chemistry as well as molecular modeling, medicinal plants remain an important source of new drugs, new drug leads and new chemical entities (Newman *et al*, 2000; Newman *et al*, 2003). It was reported that out of over 800 small molecule new chemical entities (NCEs) introduced between 1981 and 2002 nearly the half were natural products, semi-synthetic natural products, semi-synthetic natural products analogues or synthetic compounds based on natural products (Abu-Dahab and Afifi, 2007).

Cancer is a multi-step disease developed by environmental, physical, chemical, metabolic and genetic factors. It is one of the most prominent human diseases and currently there is considerable scientific and commercial interest in the discovery of new anticancer agents from natural plant sources (Kinghorn *et al*, 2003). The efficacy of natural products as anticancer agents was recognized in 1950s by the U.S. National Cancer Institute (NCI) and has since made a very large contribution in the discovery of new anticancer agents from natural product sources (Cragg and Newman, 2005). Mizoram is a small and hilly state located in the North-Eastern region of India. It lies between 21°.58′ and 24°.35′ N latitude and 92°.15′ and 93°.26′ E longitude (Lalramnghinglova, 1996).

It has rich deciduous type of forests with a variety of vegetation. Collection of information through literature search, personal interview with local herbal practitioners and elders from villages revealed that there are some plants used by the local people of Mizoram for the treatment of cancer-suspected diseases. *In vivo* antitumor potentials of some plants from this state against murine tumor model have been reported (Rosangkima and Prasad, 2004; Rosangkima *et al*, 2008; Rosangkima *et al*, 2010). However, in spite of the success of a natural product approach to anticancer drug discovery, the anticancer potentials of some traditional anticancer medicines from Mizoram against some human cancer cell lines are unexplored. Therefore, in the present study, the preliminary *in vitro* anticancer screening of some of the Mizoram traditional medicines was undertaken against Dalton's lymphoma, MCF-7 and HeLa cells.

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MATERIALS AND METHODS

Survey of anticancer medicinal plants

Survey of anticancer medicinal plants was carried out through literature search and consultation of local herbal practitioners and elders. Botanical identification of the plants was done by Dr. R. Lalfakzuala, Department of Botany, Mizoram University, Aizawl, Mizoram, and the voucher specimens were submitted to department of Zoology, Mizoram University, Aizawl. The following common questions were asked to the practitioners and elders during consultation: Local name of the plants traditional used as anticancer medicinal plant, type of cancer and other diseases for which it is used, part(s) of the plant used, methods of preparation and treatment doses.

Plant extract preparation

Different parts of selected traditional anticancer medicinal plants were collected and rinsed thoroughly with tap water, shade-dried and powdered with an electric-grinder. The powdered materials were subjected to sequential extractions with petroleum ether, chloroform and methanol using a Soxhlet apparatus until the solvents became clear. The remaining residues were processed for hot water extraction. Solvents from each extract were evaporated under reduced pressure at 45^{o}C to dryness. Petroleum ether extracts were discarded. Chloroform and methanol extracts were dissolved in dimethylsulfoxide (DMSO) and diluted with minimum essential medium (MEM) to get the required concentrations for the study. Aqueous extracts were dissolved in minimum essential medium (MEM).

Cell culture

Cell lines of different tissue origin such as MCF-7 (human breast tumor) 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^{o}C in a CO_2 incubator.

Cytotoxicity assay

Cytotoxicity of various plant extracts was determined by MTT assay (Mossman, 1983). MTT (3- (4, 5- dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide) 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. Briefly, 1x10^{4} cells were inoculated in 96 well plates in each well containing 200 µl of MEM and allowed to grow in CO_2 incubator for 24 h (37^{o}C, 5% CO_2). Thereafter, different concentrations of plant extracts (5, 10, 20, 50 and 100 µg/ml) were added and incubated for an additional 48 h followed by the addition of 20 µl MTT (3- (4, 5- dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide) stock solution (5 mg/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.

Clonogenic assay

The cytotoxic effect of potent plant extracts was also further confirmed by clonogenic assay (Puck and Marcus, 1956), 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 was counted. The plating efficiency (PE) and surviving fraction (SF) were calculated as mean ± S.D. 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.

Phytochemical analysis

Phytochemical analyses of potent plant extracts were performed using standard procedures (Sofowora, 1988; Trease and Evans, 1989; Harborne, 1998). The tests for phytomchemical screening include: tests for alkaloids, flavonoids, terpenoids, phenols, saponins, tannins, carbohydrates and cardiac glycosides.

Test for alkaloids: 0.5 g of extract was diluted to 10 ml with acidified alcohol, boiled and filtered. To 5 ml of the filtrate 2 ml of dilute ammonia and 5 ml of chloroform were added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent (potassium mercuric iodide) was added to one portion and Draggendorff's reagent (solution of potassium bismuth iodide) to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) indicated the presence of alkaloids.

Test for flavonoids: In *Alkaline Reagent Test*, extract was treated with a few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicated the presence of flavonoids. In *Lead acetate Test*, extract was treated with a few drops of lead acetate solution. Formation of yellow precipitate indicated the presence of flavonoids.

***Test for terpenoids* (Salkowski test):** To 0.5 g of the extract 2 ml of chloroform was added. Concentrated H_2S0_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for phenols: Extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

Test for saponins: In *Froth Test*, extract was diluted with distilled water and then shaken in a graduated cylinder for 15 minutes. Formation of layer of foam indicated the presence of saponins. In *Foam Test*, 0.5 gm of extract was shaken with 2 ml of water. The persistence of foam for ten minutes indicated the presence of saponins.

Test for tannins: 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added. The appearance of brownish green or a blue-black colour indicated the presence of tannins.

***Test for carbohydrates* (Molisch's Test):** Extract was dissolved in distilled water and then treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicated the presence of Carbohydrates.

***Test for cardiac glycosides* (Keller-Killiani test):** 0.5 g of extract was dissolved in 5 ml water and to it 2 ml of glacial acetic acid containing one drop of ferric chloride solution was added followed by an underlaying with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides.

Statistical analysis

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

RESULTS

A total of eight test parts from eight different plants such as leaves of *Lonicera macrantha*, *Senecio scandens*, *Croton caudatus*, *Mussaenda macrophylla*, *Blumea lanceolaria*, stem bark of *Dillenia pentagyna*, fruit of *Solanum khasianum* and root of *Ageratum conyzoides* were selected for the present study. Ethnobotanical information of the selected anticancer medicinal plants is presented in Table 1. Three different extracts were prepared for each test part using solvents with different polarities. The percentage yield of extracts from different plants ranges from 0.71% to 4.71%. The lowest yield (0.71%) was observed with the chloroform extract of *S. khasianum*, while the highest yield (4.7%) was found with the chloroform extract of *L. macrantha* (Table 2).

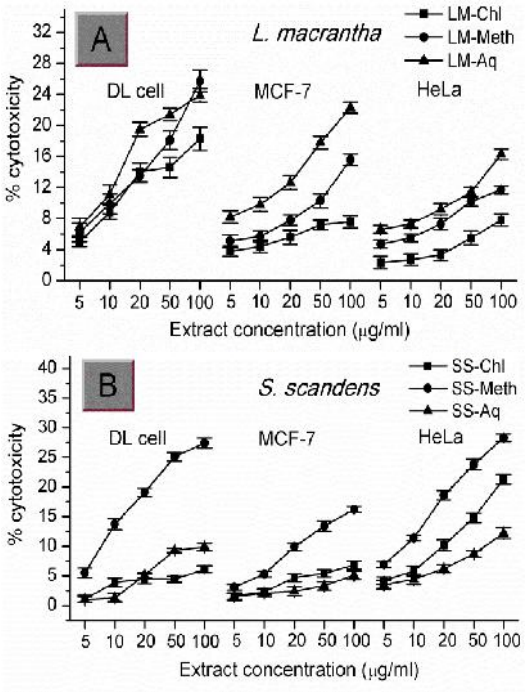
In vitro anticancer screening of chloroform, methanol and aqueous extracts of each plant was conducted using Dalton's lymphoma, MCF-7 and HeLa cells. The results of present study shows that out of 24 different extracts studied, only three extracts from two different plant species such as methanol and aqueous extracts of *S. khasianum* fruit (SKF-Meth and SKF-Aq) and methanol extract of *D. pentagyna* stem bark (DP-Meth) exhibited potent anticancer activity against DL, MCF-7 and HeLa cells in a concentration-dependent manner while aqueous extract of *C. caudatus* leaf (CC-Aq) exhibited concentration-dependent anticancer activity only on DL cells (Graph 1A – 1H).

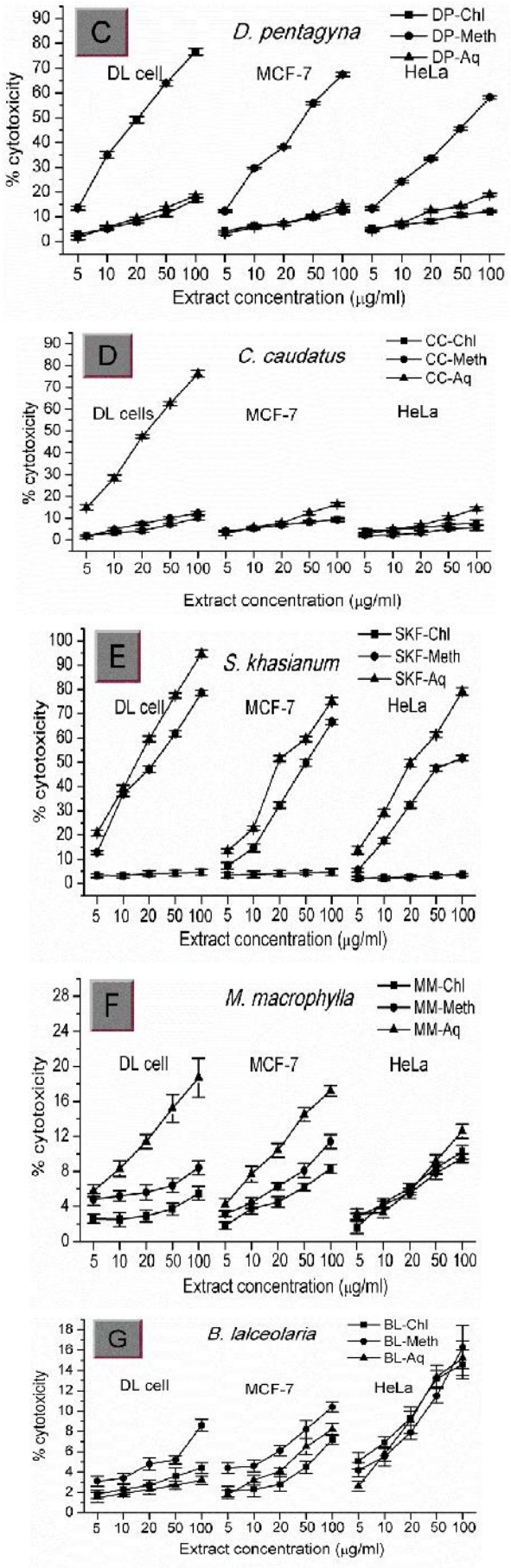
Table 1 Traditional anticancer medicinal plants of Mizoram and their ethno-medicinal values.

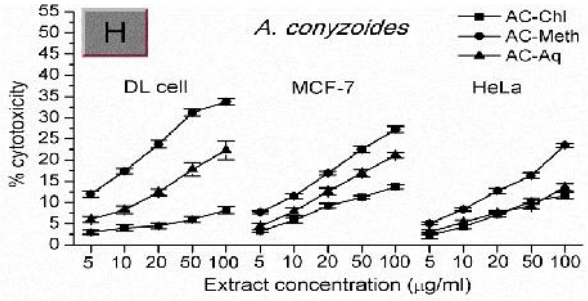
Plant species, (Local name)	Family	Part used	Reported local therapeutic uses
Lonicera macrantha (D. Don) spreng., (Leihruisen)	Caprifoliaceae	Leaf	Diarrhoea, dysentery, stomachache, cancer
Senecio scandens Buch.-Ham., (Sai-ek-hlo)	Asteraceae	Leaf	Stomach and other cancers
Dillenia pentagyna Roxb., (Kaihzawl)	Dillenaceae	Stem bark	Stomachache and stomach cancer
Croton caudatus Geiseler, (Chepa-khak)	Euphorbiaceae	Leaf	Stomachache, cancers, indigestion, diarrhea,
Solanum khasianum C.B. Clarke, (Rulpuk/At-hlo)	Solanaceae	Fruit	Toothache, cancers
Mussaenda macrophylla Wall., (Vakep)	Rubiaceae	Leaf	Diarrhoea, dysentery, Indigestion, cancers
Blumea lalceolaria Roxb., (Buarze)	Asteraceae	Leaf	Stomach cancer, stomachache
Ageratum conyzoides L. (Vailen-hlo)	Asteraceae	Root	Stomach cancer, dysentery

Table 2 Percentage yield of different plant extracts (% yield)

Name of plants	Chloroform extract	Methanol extract	Aqueous extract
Lonicera macrantha (Leaf)	4.71	2.74	4.65
Senecio scandens (Leaf)	1.91	1.07	2.74
Dillenia pentagyna (Stem bark)	3.22	2.79	3.12
Croton caudatus (Leaf)	2.46	2.23	2.86
Solanum khasianum (Fruit)	0.71	0.83	1.15
Mussaenda macrophylla (Leaf)	1.62	2.04	1.78
Blumea lalceolaria (Leaf)	2.37	2.06	2.14
Ageratum conyzoides (Root)	1.85	2.78	2.54







Graph 1 *In vitro* anticancer activity different extracts (-Chl, -Meth and –Aq) of *L. macrantha* (**A**), *S scandens* (**B**), *D. pentagyna* (**C**), *C. caudatus* (**D**), *S. khasianum* (**E**), *M. macrophylla* (**F**), *B. lalceolaria* (**G**) and *A. conyzoides* (**H**) expressed as % cytotoxicity against DL, MCF-7 and HeLa cell. Results are expressed as mean ± S.D. N = 6.

The most potent anticancer activity was observed with the aqueous extract of *S. khasianum* fruit (SKF-Aq) with an IC_{50} value of 18.24, 23.65 and 21.26 µg/ml on DL, MCF-7 and HeLa cells respectively which is followed by methanol extract of stem bark of *D. pentagyna* (DP-Meth) with an IC_{50} value of 25.84, 41.58 and 76.82 µg/ml on DL, MCF-7 and HeLa cells respectively (Table 3). Aqueous extract of *C. caudatus* leaf (CC-Aq) showed anticancer activity on DL cells with an IC_{50} value of 29.72 µg/ml.

Table 3 The IC_{50} values (µg/ml) of some plant extracts on different cancer cells at 48 hours.

Plants species	Extracts	IC50 values (µg/ml)		
		DL cell	MCF-7	HeLa
S. khasianum	SKF-Meth	**27.44**	**71.23***	**62.47***
S. khasianum	SKF-Aq	**18.24**	**23.65**	**21.26**
D. pentagyna	DP-Meth	**25.84**	**41.58***	**76.82***
C. caudatus	CC-Aq	**29.72**	-	-

* IC_{50} values more than 30 µg/ml are considered inactive. SKF-Meth = methanol extract of *S. khasianum*, DP-Meth = methanol extract of *D. pentagyna*, CC-Aq = aqueous extract of *C. caudatus*.

Table 4 Survival analysis of cancer cells treated with extracts of *S. khasianum* (fruit), *D. pentagyna* (stem bark), *C. caudatus* (leaf) and *S. scandens* (leaf).

Extracts	Dose (µg/ml)	SF ± S.D.		
		DL cells	MCF-7	HeLa
Control	0	0.971±0.12	0.985±0.14	0.973±0.11
	5	0.863±0.09	0.903±0.12	0.889±0.13
	10	0.622±0.08	0.744±0.09	0.721±0.11
SKF-Meth	20	0.571±0.09	0.604±0.09	0.607±0.09
	50	0.493±0.07	0.511±0.08	0.542±0.08
	100	0.476±0.08	0.483±0.09	0.509±0.08
	5	0.714±0.05	0.943±0.10	0.784±0.12
	10	0.371±0.05	0.735±0.06	0.429±0.08
SKF-Aq	20	0.228±0.09	0.478±0.07	0.378±0.07
	50	0.135±0.10	0.218±0.05	0.228±0.06
	100	0.078±0.05	0.164±0.06	0.137±0.07
	5	0.954±0.13	0.961±0.08	0.958±0.13
	10	0.862±0.08	0.853±0.09	0.871±0.08
DP-Meth	20	0.746±0.08	0.762±0.08	0.760±0.08
	50	0.602±0.09	0.632±0.07	0.677±0.07
	100	0.447±0.07	0.452±0.09	0.597±0.09
	5	0.893±0.12	0.955±0.11	0.961±0.09
	10	0.742±0.09	0.937±0.12	0.932±0.08
CC-Aq	20	0.681±0.07	0.914±0.07	0.906±0.08
	50	0.372±0.08	0.886±0.09	0.864±0.07
	100	0.286±0.06	0.874±0.07	0.857±0.08

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

Treatment with different concentrations of SKF-Meth, SKF-Aq, DP-Meth and CC-Aq resulted in a concentration-dependent significant decline in the clonogenicity of DL, MCF-7 and HeLa cells (Table 4). Maximum decline in reproductive capacity of all cell types was observed at 100 µg/ml of each extract which is the highest concentration used in the present study. Analysis of survival fraction also suggest that plant extracts showed more significant decline in the number of colonies of DL cells when compared to MCF-7 and HeLa cells.

Three most potent anticancer extracts such as SKF-Meth, SKF-Aq and DP-Meth were processed for phytochemical analysis. This phytochemical analysis revealed the presence of important medicinal phytochemicals such as alkaloids and flavonoids in all the three extracts (Table 5). The phytochemical screening also shows moderate concentration of alkaloids and flavonoids, traces of phenols, saponins, tannins and carbohydrates and absence of terpenoids in SKF-Meth. Phytochemical analysis of aqueous extract of *Solanum khasianum* fruit (SKF-Aq) also revealed that alkaloids and saponins are present in larger amount, whereas flavonoids are present in small quantities. DP-Meth shows moderate concentration of alkaloids, saponins and tannins, absence of terpenoids, carbohydrates and cardiac glycosides. All the extracts tested negative for the presence of terpenoids and cardiac glycosides.

Table 5 Preliminary phytochemical analysis of extract of *S. khasianum* and *D. pentagyna*.

Phytochemicals	Tests	SKF-Meth	SKF-Aq	DP-Meth
Alkaloids	- Draggendorff's test	++	+++	++
	- Mayer's test	++	+++	++
Flavonoids	-Alkaline reagent test	+	+	+
	-Lead acetate test	+	-	-
Terpenoids	- Salkowski test	-	-	-
Phenols	- Ferric chloride test	+	-	+
Saponins	-Froth test	+	+++	++
	-Foam test	+	++	++
Tannins	-Ferric chloride test	+	-	+++
Carbohydrates	- Molisch's Test	+	-	-
Cardiac glycosides	- Keller-Killiani test	-	-	-

- = shows absence of phytochemicals.
+ = shows presence of phytochemicals.
++ = shows moderate concentration of phytochemicals.
+++ = shows high concentration of phytochemicals.

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 experimental cancer models (Rosangkima & Prasad, 2004; Damle *et al*, 2013; Patel & Patel, 2011). In the present study, *in vitro* anticancer activity/cytotoxicity of different plant extracts were determined by MTT assay. The principle of MTT assay is based on the reduction of a soluble tetrazolium salt, by mitochondrial dehydrogenase activity of viable cancer cells, into a soluble colored formazan product that can be measured spectrophotometrically after dissolution (Edrini *et al*, 2002). The IC_{50} value was used as a parameter for cytotoxicity. The criterion for cytotoxicity for the plant crude extracts, as established by the US National Cancer Institute (NCI), is an IC_{50} value lower than 30 µg/ml (Suffness & Pezzuto,1990). Thus, based on this NCI criterion, the results of preliminary *in vitro* anticancer screening indicated that out of 24 different extracts tested from eight plants, only four extracts, such as methanol extracts of *S. khasianum* fruit, *D. pentagyna* stem bark, aqueous extracts of *S. khasianum* fruit and *Croton caudatus* leaf showed potent cytotoxic activity on Dalton's lymphoma. However, aqueous extract of *S. khasianum* fruit exhibited potent cytotoxic activity on MCF-7 and HeLa cells also. Comparison of the cytotoxic potentials of these plant extracts depicted that cytotoxic activity was highest with the aqueous extract of *S. khasianum* fruit.

Clonogenic cell survival is a basic tool that was described in the 1950s for the study of radiation effects. Loss of reproductive integrity and the inability to proliferate indefinitely are the most common features of cell death. Therefore, a cell that retains its ability to synthesize proteins and DNA and go through one or two mitoses, but is unable to divide and produce a large number of progeny is considered dead. This is very commonly referred to as loss of reproductive integrity or reproductive death and is the end point measured with cells in culture. On the other hand, a cell that is not reproductively dead and has retained the capacity to divide and proliferate indefinitely can produce a large clone or a large colony of cells and is then referred to as "clonogenic." The ability of a single cell to grow into a large colony that can be visualized with the naked eye is proof that it has retained its capacity to reproduce. The loss of this ability as a function of dose of chemotherapeutic agents is described by the dose-survival curve (Elkind & Whitmore, 1967; Hall, 2000; Elkind & Sutton, 1960). Therefore, cytotoxic potential of these four potent plant extracts was also investigated through clonal culture of cancer cell lines. It was observed that all the four plant extracts produced cytotoxicity on the cell lines in a concentration-dependent fashion supporting the *in vitro* cytotoxic potentials of the extracts on cancer cells.

Plants have many phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer properties. Some studies have reported that extracts from natural products such as fruits, vegetables and medicinal herbs have positive effects against cancer compared with chemotherapy or hormonal treatments (Wu *et al*, 2002).

The antidiuretic, anti-inflammatory, anticancer, antiviral, antimalaria and antibacterial activities of the medicinal plants are due to the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, phlobatannins and reducing sugars (Balakrishnan & Sharma, 2013). Medicinal plants are used for discovering and screening of the phytochemical constituents for the manufacturing of new drugs. The phytochemical analyses of the medicinal plants have commercial interest in research institutions and pharmaceutical companies for manufacturing new drugs for the treatment of various diseases. The result of present studies shows the presence of alkaloids and flavonoids in the methanol and aqueous extracts of *S. khasianum* fruit and methanol extract of *D. pentagyna* stem bark in large quantities. The alkaloids and flavonoids present in these plant extracts could be an important contributory factors involved in their anticancer potentials.

CONCLUSION

Based on the results of present studies, it may be concluded that out of 24 different extracts from eight plants of Mizoram studied, three extracts such as methanol extracts of *S. khasianum* and *D. pentagyna* as well as aqueous extracts of *S. khasianum* and *C. caudatus* exhibited potent anticancer activity on Dalton's lymphoma, MCF-7 and HeLa cell lines *in vitro* in a concentration-dependent manner. The most potent anticancer activity was observed with the aqueous extract of *S. khasianum* fruit (SKF-Aq) with an IC_{50} value of 18.24 µg/ml followed by methanol extract of stem bark of *D. pentagyna* (DP-Meth) with an IC_{50} value of 25.84 µg/ml. It may be suggested that the important phytochemicals present in the plant extracts may play an important role in the anticancer activity of the extracts. However, further studies are required to establish their anticancer efficacy using more human cancer cell lines and also to observe any adverse effects in normal cells.

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