

Cytotoxic Effects *In vitro* and *In vivo* of *Stephania glabra* (Roxb.) Miers Growing in Vietnam

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Abstract: From the tubers of *Stephania glabra* (Roxb.) Miers, we extracted three fractions: *n*-hexane (SM1), ethyl acetate (SM2) and aqueous (SM3) and isolated three compounds (oxostephanine, crebanine, dehydrocrebanine) to evaluate their cytotoxicity. The results of experiments showed that fraction SM2 had strong inhibitory effects against 6 cancer cell lines: N87, OVCAR-8, MDA-MB-231, HeLa, HepG2 and H358 with IC₅₀ values of 10.27; 12.21; 18.24; 22.84; 26.18; 30.09 µg/ml, respectively. This fraction also reduced Sarcoma180 tumor growth rate and its volume compared to the control group. Fraction SM1 showed weak effects against N87, HepG2 and HeLa cell line with IC₅₀ of 35.73; 87.2 and 94.6 µg/ml, respectively. Among three isolated compounds, oxostephanine had potent cytotoxic effects on 5 experimental cell lines: OVCAR-8, HeLa, HepG2, MDA-MB-231 and H358, with IC₅₀ of 0.34 ± 0.02; 0.66 ± 0.06; 0.7 ± 0.05; 1.02 ± 0.04 and 1.84 ± 0.02 (µg/ml), respectively. Dehydrocrebanine had cytotoxic efficacy on only two cancer cell lines: OVCAR-8 and MDA-MB-231 with IC₅₀ of 1.38 ± 0.05 and 5.00 ± 0.03 µg/ml, respectively. Fraction SM3 and crebanine did not show any effects against 6 experimental cell lines.

Key words: *Stephania glabra* • Cancer • Cytotoxicity • *In vitro* • *In vivo*

INTRODUCTION

Cancer is one of the top leading causes of death in the world today. The World Health Organization (WHO) has hypothesized that deaths from cancer will increase from 7.4 million in 2004 to 12 million in 2030 [1]. The rapid increase in the cancer incidence represents a real crisis for public health and health systems worldwide and cancer has always been an unsolved problem in the world. Most cancer treatment regimens consist of chemotherapy in conjunction with surgery or radiation. In spite of cancer chemotherapy treatment will cause side effects, it is still recognized as the first choice for the treatment of many cancers. However, recent studies show that plant have been a mainstay source of anti-cancer drugs and plants derived products have proved effective and safe in the management of cancers [2, 3]. *Stephania* genus have been reported to produce isoquinoline alkaloids, which reveal interesting pharmacological activity [4]. According to previous research, this genus contains various alkaloids

[5], such as cepharanthine, tetrandrine, showed remarkable cytotoxic activity on human cancer cells. Cepharanthine exerts potent cytotoxicity against colon and hepatoma cancer cell lines with IC₅₀ values between 2.4 and 5.3 µM [6]. Tetrandrine, extracted from the root of *S. tetrandra*, inhibits the proliferation and induces the apoptosis of several cancers, including breast cancer, lung cancer, neuroblastoma, Burkitt's lymphoma, hepatoma and leukemia [7]. Oxostephanine, isolated from the tubers and leaves of *S. dielsiana* growing in Vietnam, shows the significant activity against human epithelial type 2 (Hep-2) cells, rhabdomyosarcoma (RD) cells and lung carcinoma (LU) cell line (IC₅₀ of 0.566; 0.755 and 1.404 µg/ml, respectively) [8]. In addition, *Stephania glabra* (Roxb.) Miers, a precious medicinal plant growing in Vietnam, has long been used in folklore. It mainly contains over 30 alkaloids, which was reported to have anti-psychotic, anti-diabetic, antipyretic, analgesic, antimicrobial and anti-hypertensive activities [9]. However, there are few scientific work has been

conducted on cytotoxic activities mediated by *S. glabra* phytochemicals till now. The current report demonstrated novel anticancer activities of three substances (oxostephanine, crebanine and dehydrocrebanine) and three crude extracts of *S. glabra*.

MATERIALS AND METHODS

Materials:

- Tubers of *Stephania glabra* (Roxb.) Miers were collected from Nam Dinh province, Vietnam during April, 2016.
- The tubers of *S. glabra* (2.3kg) were dried, powdered and then extracted with methanol at room temperature. The resulting extracts were concentrated under vacuum to yield crude extract (205g). The methanol extract (263g) was dissolved in water (1.5L) and subjected to liquid-liquid partitioning (3 times) using n-hexane, ethyl acetate (EtOAc), yielding 18g, 62g and 21g of n-hexane (SM1), ethyl acetate (SM2) and aqueous (SM3) residue, respectively. Three alkaloids, oxostephanine, crebanine and dehydrocrebanine were isolated from the SM2 extracts.
- Cancer cell lines: human cervical epithelioid carcinoma cells (HeLa ATCC), human non-small cell lung cancer cells (H358 ATCC), human ovarian carcinoma cells (OVCAR-8 ATCC), human gastric cancer cell lines (N87 ATCC), human breast cancer cells (MDA-MB-231 ATCC) and hepatocellular carcinoma (HepG2 ATCC).

Normal embryonic kidney cells (HEK293 ATCC).

Mouse sarcoma 180 cells (ATCC).

All cell lines were supplied from ATCC (ATCC- American type culture collection).

Taxol (Sigma-Aldrich) served as a positive control.

Swiss mice were purchased from Vietnam National Institute of Hygiene and Epidemiology, Hanoi and maintained at standard housing conditions.

Reagents and Instruments

Reagents: RPMI, fetal bovine serum and penicillin/streptomycin (Invitrogen): All cell lines were cultivated in RPMI or DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen).

Other Chemicals: CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega), Blue Trypan, PBS, Trypsin – EDTA (Invitrogen).

Instruments: Put Company Name and Country for Each

Instrument: Fluorescence microscope Axiovert40 CFL (Zeiss, Germany), centrifuge (BioSan, Germany), microplate reader (Bio-rad, Singapore), carbon dioxide (CO₂) incubator (ThermoFisher Scientific, USA), fume hoods (Terra Universal, USA), water bath (Mettler, Germany).

In vitro Cytotoxicity Assay

Principle: The sensitivity of cells to compounds or residues was determined *in vitro* by an MTS (3 - (4, 5-dimethylthiazol-2-yl) - 5 - (3-carboxymethoxyphenyl) - 2 - (4-sulfophenyl) - 2H - tetrazolium) based colorimetric assay (Promega). MTS, in the presence of phenazine methosulfate (PMS), produces a formazan product that has an absorbance maximum at 490 - 500nm. The quantity of formazan (presumably directly proportional to the number of viable cells) can be measured by the absorbance at 490 nm [10].

Procedure: Cell lines were seeded in a 96-well microplate, 180 µL/well at a density of 5000–7000 cells/well. The microplate was incubated for 24 h at 37°C, 5% CO₂. Samples at various concentrations were added to the microplate which was incubated for another 48 h.

Cell viability was determined by staining with MTS assay. MTS/PMS solution was added to all wells of each assay and plates were incubated at 37°C, 5% CO₂, for 2-4 h. The plates were read on a microplate reader at 490 nm. The IC₅₀ value (i.e. the concentration of compound that reduces the cell viability by 50%) was determined with the GraphPad Prism 5 Software by plotting the percent cell viability as a function of compound concentration.

In vivo Cytotoxicity Assay

Anti-Tumor Activity of Sample on S180 Tumor-Bearing

Mice: Male Swiss mice weighing 18 - 20 g, 5 weeks of age, obtained from National Institute of Hygiene and Epidemiology, were used. 0.2ml tumor cell suspension (10⁶ cell/ 0.2ml) were implanted subcutaneously into the right flank of the mice.

Experimental Design: The growth of tumors was measured in size, after seven day intervals.

At the beginning of the experiment, the mice were divided into two groups, as follows:

- The non-treated control group (group DCUT) was treated with the vehicle used to dilute the drug.

Table 1: Schedule of administration and dose for both groups of mice

Group	Number of mice	Administration (times)	Measure (times)	Dose	Time course (days)
DCUT	10	18	11	200µl distilled water/time	21
SM2	15	18	11	35 mg /kg/time	21

- Group DCUT consisted of animals treated by SM2 fraction

Dosage and Injection Method: The fraction SM2 was dissolved in distilled water and given to the mice by intraperitoneal injection to the stomach in timing of four times per week, on Monday, Tuesday, Thursday, Friday.

- Group SM2: SM2 doses of 35 mg/kg body weight /0.2 ml were administered each times.
- Control group (group DCUT): The control group received the same amount of sterile distilled water (Table 1).

Determination of Tumor Size: Tumor size was determined every other day. Tumor volumes were determined according to the formula:

$$V = 0.4 \times a \times b^2$$

where

a: is the largest dimension of the tumor

b: is the smallest dimension.

V: is the volume of tumor

Statistical analyses were performed using GraphPad Prism 5.

RESULTS

In vitro Cytotoxic Effects of Crude Extracts: Different concentrations of crude extracts used in *In vitro* cytotoxicity assay, are shown in Table 2.

The IC_{50} values of crude extracts were shown in Table 3.

Among all extracts, fraction SM2 displayed strongest inhibitory effects against 6 cancer cell lines: H358, OVCAR-8, N87, MDA-MB-231, HepG2 and HeLa. Both fraction SM2 and Taxol showed lower cytotoxicity in the control HEK293.

The cytotoxicity curves of fraction SM2 were very similar to that of Taxol, revealed that SM2 has typical effects of an anticancer agent.

In vitro Cytotoxic Effects of Substances: Different concentrations of 3 compounds used in *In vitro* cytotoxic assay, are shown in Table 4.

Concentration-response curves of isolated compounds (BVE 7.3, BVE 10.3 and III5) for the growth of six tumor cell lines were shown in Fig. 2 and 3.

The IC_{50} values of isolated compounds were shown in Table 5.

Among three compounds isolated from fraction SM2, compound III.5 (oxostephanine) displayed potential inhibitory effects against 5 cancer cell lines: HeLa, HepG2, OVCAR-8, MDA-MB-231 and H358. The IC_{50} values were quite low on all of these cell lines, especially on HeLa, HepG2 and OVCAR-8 ($IC_{50} < 1 \mu\text{g/ml}$). Although fraction SM2 showed highest cytotoxic effects against N87 cell line in previous experiments, there was no cytotoxicity of III.5 on its ($IC_{50} = 5 \mu\text{g/ml}$), indicating unknown constituents of SM2 that have potent antitumor effect. Thus, more studies need to be conducted to search for new bioactive compounds in *S. glabra*.

Compound BVE 7.3 (dehydrocrebanine) also demonstrated bioactivity against OVCAR-8 and MDA-MB-231 cell line with IC_{50} values of 1.38 ± 0.05 and $5.00 \pm 0.03 \mu\text{g/ml}$, respectively.

Compound BVE 10.3 (crebanine) showed no cytotoxicity against any of 6 cell lines.

Specially, this compound at low concentrations can enhance the proliferation of human cervical epithelioid carcinoma cells (HeLa).

Results of *In vivo* antitumor experiment were shown in Table 6.

In Group DCUT, tumor volume increased significantly and consecutively. In contrast, the tumor size of mice in Group SM2 was considerably smaller than that of animals in Groups DCUT from day 3/8 to 20/8. In addition, the slope of increasing tumor volume during the course was significantly faster in the control group (DCUT). At baseline, tumor volumes were not significantly different between the controls and treated mice, although control mice had significantly higher volumes at one week and continued to be higher at three weeks.

Table 2: Various concentrations of crude extracts and Taxol

Sample (µg/ml)	C1	C2	C3	C4	C5	C6	C7	C8	C9
Fraction (SM1, SM2, SM3)	1000	500	250	100	50	25	10	5	2.5
Taxol	30	3	0.3	0.03	0.003				

Table 3: IC₅₀ value of SM1, SM2 & SM3 fractions and Taxol on cell lines (µg/ml)

No.	Sample	HeLa	HepG2	OVCAR-8	N87	H358	MDA-MB-231	HEK 293
1	SM1	94.6	87.2	174.1	35.73	ND	ND	ND
2	SM2	22.84	26.18	12.21	10.27	30.09	18.24	48.12
3	SM3	ND	ND	ND	ND	ND	ND	ND
4	Taxol	0.011	0.032	0.03959	0.45	0.7	0.5	13.6

ND: not determined (results were observed at more than 5000 µg/ml; or the data did not follow the model of linearity).

Table 4: Various concentrations of the three isolated compounds and Taxol

Sample (µg/ml)	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
3 compounds	32	16	8	4	2	1	0.5	0.25	0.125	0.0625
Taxol	30	3	0.3	0.03	0.003					

Table 5: The IC₅₀ values of isolated compounds: III5 (oxostephanine), BVE10.3 (crebanine), BVE 7.3 (dehydrocrebanine) and positive control (Taxol) against six cancer cell lines (µg/ml)

No.	Compounds	HeLa	HepG2	OVCAR-8	N87	H358	MDA-MB-231
1	BVE 7.3	30.50 ± 0.07	8.90 ± 0.09	1.38 ± 0.05	ND	37.91 ± 0.1	5.00 ± 0.03
2	III5	0.66 ± 0.06	0.70 ± 0.05	0.34 ± 0.02	28.35 ± 0.07	1.84 ± 0.02	1.02 ± 0.04
3	BVE 10.3	ND	ND	ND	ND	ND	ND
4	Taxol	0.011 ± 0.004	0.032 ± 0.002	0.040 ± 0.005	0.450 ± 0.003	0.700 ± 0.002	0.500 ± 0.007

* ND: not determined (results were observed at more than 5000 µg/ml; or the data did not follow the model of linearity)

Table 6: The median and interquartile range of tumor volume (cm³) during the course of treatment

Time of measurement						
Group	30/7	1/8	3/8	5/8	7/8	
DCUT	0.1944 (0.1176-0.2696)	0.3600 (0.1658 – 0.3872)	0.32 (0.2496-0.4574)	0.4032 (0.2896-0.7468)	0.6912 (0.4992-1.0496)	
SM2	0.2024 (0.1792-0.2592)	0.2754 (0.2022-0.3879)	0.3258 (0.2775-0.4356)	0.32 (0.2835-0.5577)	0.5012 (0.2887-0.8943)	
Time of measurement						
Group	9/8	12/8	14/8	16/8	18/8	20/8
DCUT	0.8112 (0.5862-1.4786)	0.6912 (0.5838-1.6178)	0.7436 (0.6499-1.7614)	0.8788 (0.6624-1.7361)	1.0192 (0.7785-2.3362)	1.2600 (1.0982-2.892)
SM2	0.4482 (0.2916-0.7098)*	0.4420 (0.2511-0.7098)*	0.5324 (0.2328-0.8112)*	0.4420 (0.2512-0.7774)*	0.6336 (0.0500-1.0575)*	0.7294 (0.0500-1.0573)*

*p<0.05

Table 7: The assessment of the mice, before and after treatment

Group	Total number of mice at the start (n)	Total number of mice at the end (n)	Mortality rate		Regression rate	
			n	Percentage (%)	n	Percentage (%)
DCUT	10	9	1	10.00	0	0
SM2	15	14	1	6.67	5	35.70

On the final day of experiment, percentage of tumor regression in Group SM2 was 35.7% (Table 7). These

results indicated that treatment with 35 mg/kg/day of SM2 suppressed the growth of sarcoma 180 tumors.

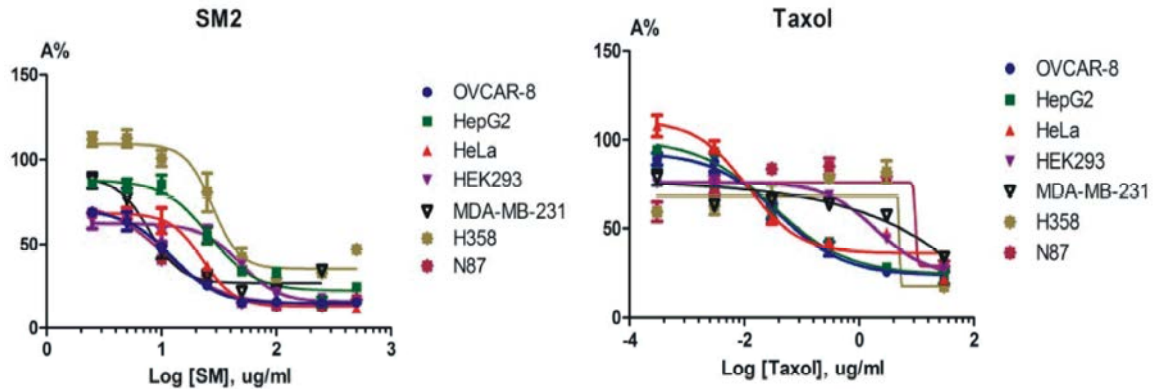


Fig. 1: Concentration-response curves of the fraction SM2 and Taxol for the growth inhibition of six tumor cell lines (GraphPad Prism 5)

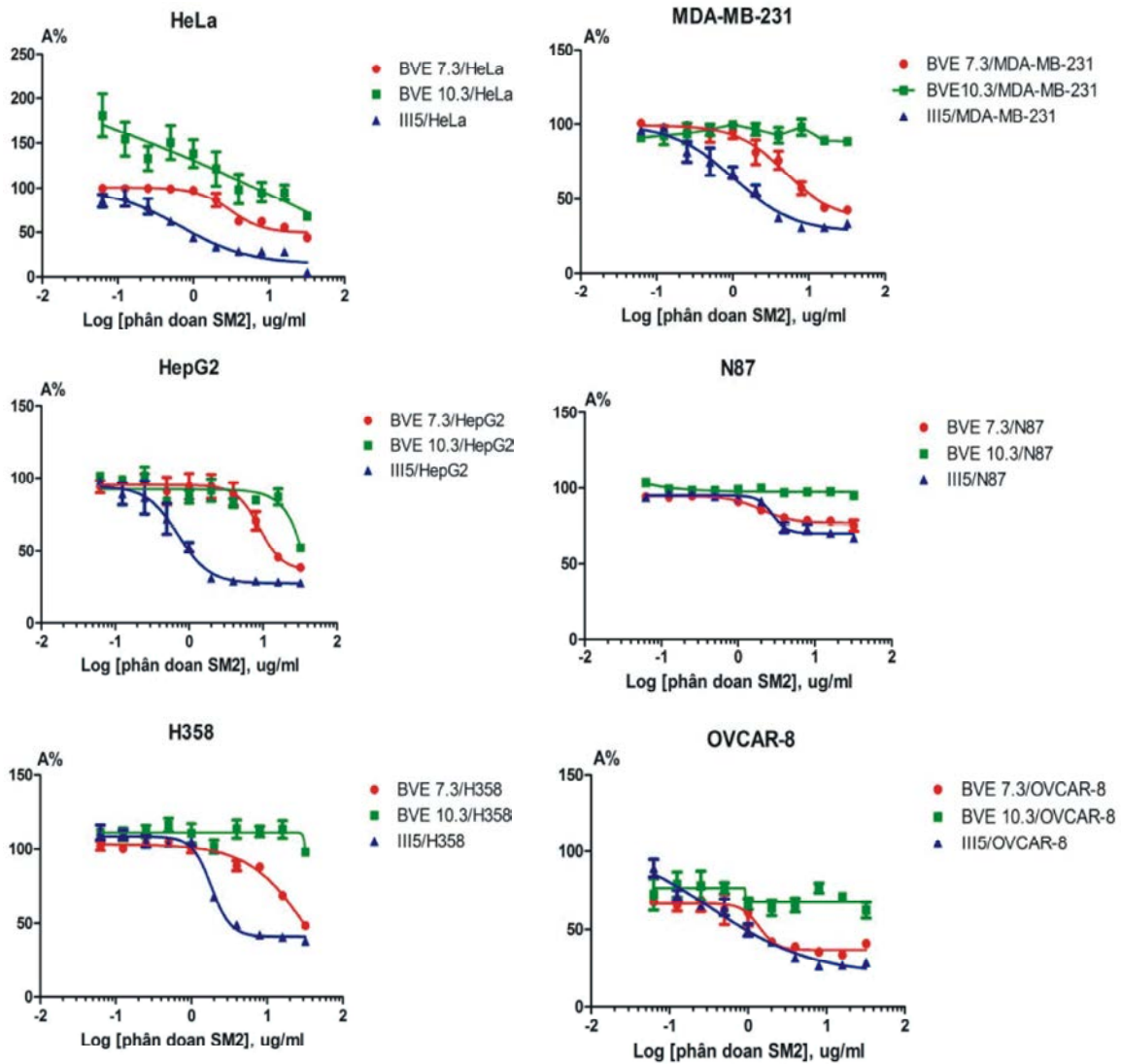


Fig. 2: Concentration-response curves of isolated compounds (BVE 7.3, BVE 10.3 and III5) for the growth of each cell line

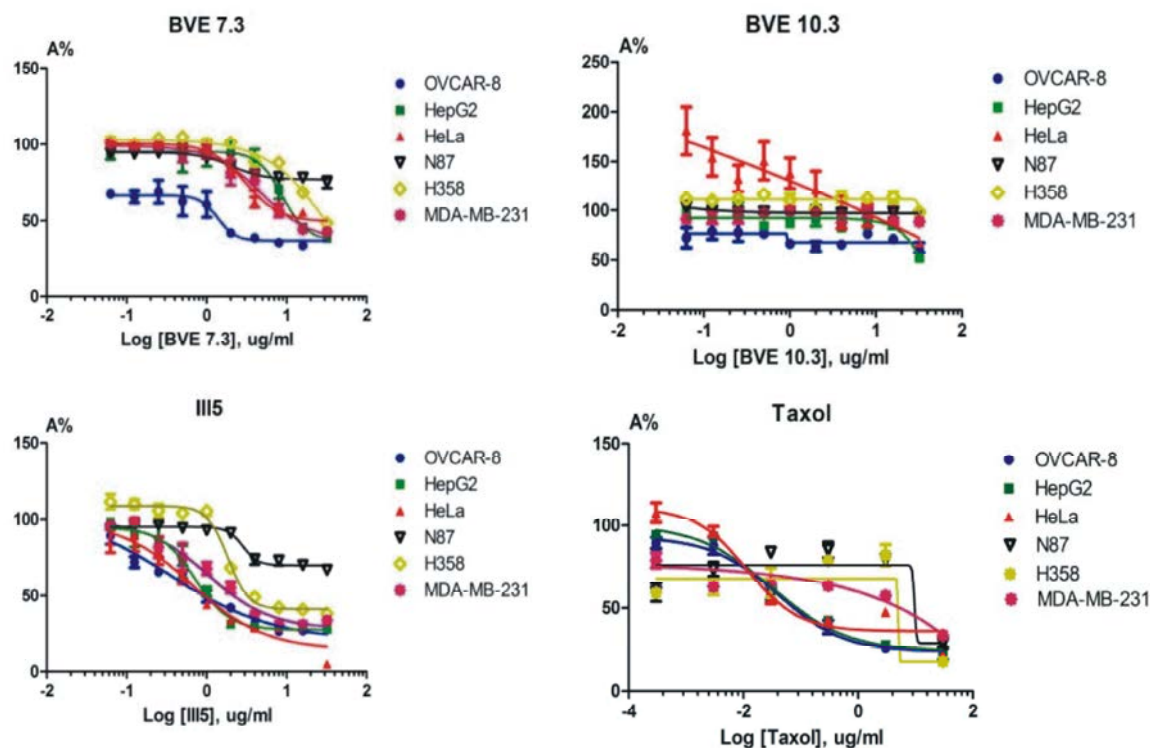


Fig. 3: Concentration-response curves of each isolated compound for the growth of six tumor cell lines

DISCUSSION

Effects of crude extracts from *S. glabra*: Fraction SM2 displayed promising anticancer activity against all of six cell lines: H358, OVCAR-8, N87, MDA-MB-231, HepG2 and HeLa. However, this fraction had less effect on normal embryonic kidney cells (HEK293). The cytotoxicity curves of fraction SM2 were similar to that of Taxol, so this fraction has considerable anticancer activity on these types of cancer.

In vivo testing showed that fraction SM2 also had a significant antitumor effect, with greater than 35% of tumor-bearing mice having reduction in tumor volume to pre-treatment size. Tumor growth of the remaining mice were distinctly slowed down, compared with control group.

In folklore, the decoction of *S. glabra* is used in the treatment of diabetes, fever, gastric problem, amoebic dysentery, as an anthelmintic in rheumatic bodyache, blood dysentery, in leprosy and even anticancer drug [11]. Number of researchers have given different reports on the therapeutic activities of this medicinal plant, including antimicrobial, hypotensive, analgesic and antipyretic, antihyperglycemic, antihistaminic and anthelmintic effects [11].

This study determined the promising anticancer potential of the plant which could be used as a source of novel drug to fight cancer. Much interest is given these days towards naturally-derived phyto-components as they are considered to have less side effects compared to current treatments such as chemotherapy. Also, there is a growing demand for alternative treatments with naturally-derived anticancer agents with plants being the desired source. Medicinal plants like *S. glabra* can be of significant use in this context. However, further investigations are needed to be conducted to isolate and characterise the specific bioactive compounds responsible for such activities.

***In vitro* Cytotoxic Effects of Isolated Compounds from *S. glabra*:** From fraction SM2, we have isolated four compounds, including oxostephanine, crebanine, dehydrocrebanine and dehydrocorydalmine. Because of small amount of dehydrocorydalmine, only three remaining compounds were tested for cytotoxic effects. Among these compounds, oxostephanine showed substantial inhibitory effects against 5 cancer cell lines: HeLa, HepG2, OVCAR-8, MDA-MB-231 and H358. The IC_{50} values were quite low on all of these cell lines,

specially on HeLa, HepG2 and OVCAR-8 ($IC_{50} < 1 \mu\text{g/ml}$). However, oxostephanine did not show effect against N87 cell line ($IC_{50} = 5 \mu\text{g/ml}$).

Oxostephanine showed highest cytotoxicity on human ovarian carcinoma cells (OVCAR-8), with IC_{50} value of $0.34 \pm 0.02 \mu\text{g/ml}$. Results of exposure of the cells lines to the compounds for 24 hours at a concentration of $1 \mu\text{g/ml}$ enhanced induction of apoptosis. Oxostephanine caused about 5.5-fold increase in apoptosis (35.6% compared with 5.6%). Apoptosis induction is an important indicator of the ability of chemotherapeutic drugs to induce death of tumor cells. This result suggested that one of the causes of cell death upon exposure to these compounds was apoptosis. This result also is a good agreement with a previous studies by Wirasathien and co-worker [12], Makarasen and co-worker [13] which revealed that oxostephanine has strong inhibitory activities against various cancer cell lines.

CONCLUSIONS

In vitro Cytotoxic Activities: Fraction SM2 displayed potent inhibitory effects against 6 cancer cell lines: N87, OVCAR-8, MDA-MB-231, HeLa, HepG2, H358 with IC_{50} values of 10.27; 12.21; 18.24; 22.84; 26.18; 30.09 $\mu\text{g/ml}$, respectively.

Fraction SM1 showed weak effects against N87, HepG2, HeLa cell line with IC_{50} of 35.73; 87.2; 94.6 $\mu\text{g/ml}$, respectively.

Fraction SM3 did not show effects against any of 6 experimental cell lines.

Oxostephanine demonstrated its cytotoxicity on 5 experimental cell lines: human ovarian carcinoma (OVCAR-8), human cervical epithelioid carcinoma (HeLa), hepatocellular carcinoma (HepG2), human breast cancer (MDA-MB-231), human non-small cell lung cancer (H358), with IC_{50} of 0.34 ± 0.02 ; 0.66 ± 0.06 ; 0.7 ± 0.05 ; 1.02 ± 0.04 ; 1.84 ± 0.02 ($\mu\text{g/ml}$), respectively.

Dehydrocrebanine displayed cytotoxic effects against human ovarian carcinoma (OVCAR-8) cells with IC_{50} of 1.38 ± 0.05 and human breast cancer cells (MDA-MB-231) with IC_{50} of $5.00 \pm 0.03 \mu\text{g/ml}$.

Crebanine did not show any effects against 6 experimental cell lines.

In vivo Antitumor Activities: Fraction SM2 has reduced Sarcoma180 tumor growth rate and tumor volume compared to the control group. At the end of experiment, the mean tumor volume in Group SM2 was 0.68 cm^3 (32.6% of control).

Fraction SM2 did not significantly affect the survival of experimental mice. Mortality rate of treated group was 6.7%, whereas regression rate was 35.7%.

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