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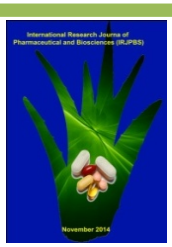

Evaluation of the anticancer activity of Chilauni, *Schima wallichii* (DC.) Korth. in vitro

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Abstract

The cytotoxicity of the ethanol extract of *Schima wallichii* (SWE) was assessed in HeLa and V79 cells by MTT assay. The HeLa, and V79 cells treated with different concentrations of SWE showed a concentrations dependent increase in its cytotoxic effect. Treatment of cells with SWE for different durations also increased its cytotoxic effects in a time dependent manner. The results of MTT assay were confirmed by clonogenic assay in HeLa cells, where the cells were treated with different concentrations of SWE. Treatment of HeLa cells with various concentrations of SWE reduced the clonogenicity of cells in a concentration dependent manner. The ability of SWE to induce apoptosis was studied by determining the caspase 8 and 3 activities at different post- treatment times. The SWE treatment marginally increased activity of both caspase 8 and 3 in a time dependent manner. The effect of SWE treatment was studied on the glutathione contents, glutathione-s-transferase, catalase and superoxide dismutase activities, where it was found to reduce the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase in a time dependent manner. Treatment of HeLa cells with ethanol extract of *Schima wallichii* increased the cytotoxic effect in a concentration dependent manner followed by a reduction in the clonogenicity of HeLa cells. The cell killing effect of SWE may be due to the caspase activation and reduction in the glutathione contents, and activities of glutathione-s-transferase, catalase and superoxide dismutase.

Keyword: *Schima wallichii*, MTT, Clonogenic, Caspase.

INTRODUCTION

The natural products have formed main source to cure different diseases including cancer since time immemorial [1-3]. The plants synthesize several secondary metabolites for different purposes and these are boon to human healthcare. These molecules are highly

complex in structure and it is difficult to undertake their chemical synthesis. This has rekindled the interest of researchers and of pharmacological industries on the isolation of these secondary metabolites to develop them in to modern drug entities in recent years [1]. The cancer has emerged has a major disease that has been putting higher financial burden on the healthcare of families and also of Governments who had to allocate higher financial resources to cancer centers. The fact remains that despite higher allocation and availability of most modern therapy for cancer patients the mortality rates remains higher in cancer patients [4].

The chemotherapy has been the major treatment modality, especially in advanced stages of cancer. Several chemotherapeutic drugs isolated from plant/natural products are in the market [2, 3]. The cytotoxic chemotherapeutic drugs are used alone or as an adjuvant therapy in patient to improve the survival [5]. Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer, and while the actual compounds isolated from the plant frequently may not serve as the drugs, they provide leads for the development of potential novel agents. The vinca alkaloids isolated from periwinkle plant *Catharanthus roseus* initially used to treat hematologic malignancies find their wide use to treat different types of solid neoplasia [6, 7]. Similarly, epipodophyllotoxins are used to treat several malignant neoplasia [8]. The taxols isolated from Pacific yew are also useful in the treatment of wide range of tumors [9]. The management of malignancies frequently requires the use of treatment modalities that are associated with significant toxic effects. The acceptability of specific therapy can be assessed by comparing its benefits with its potential cost in terms of toxicity [10]. This indicates the need to continue the screening of natural products for treatment of cancer.

Schima wallichii (DC.) Korth. (Chilauni) belong to the tea family, Theaceae. It is an evergreen tree inhabiting warm temperate to subtropical climates. The tree is found across southern and South East Asia, and stretch from the eastern Himalaya of Nepal to eastern India across Indochina, Southern China, Taiwan and the Ryukyu Islands.

It is commonly known as needle wood tree and grows up to 35 m high. However, in some places it may be seen only 40 ft high [11]. Locally, it is called “khiang” in Mizo language. *Schima wallichii* is known to possess several medicinal properties. The leaves and the stem bark are normally used traditionally for its medicinal properties. The bark is used as an antiseptic for cuts and wounds. It acts as vermicide, mechanical irritant and as a cure against gonorrhea [12]. Decoction of bark reduces fever and is said to be effective against head lice [13]. The bark juice is given to disinfest the animal from liver flukes ([14]. The sap from the stem is used for curing ear infection [15]. Fruit decoction is used by the people of Western Mizoram, India against snakebite [16, 14]. The young plants, leaves and roots are also used medicinally against fever and the bark is anthelmintic and rubefacient [17]. The leaves of *Schima wallichii* are known to have antitumor and antimutagenic properties [18, 19]. The astringent corollas are used to treat uterine disorders and hysteria [20]. The anticancer activity of *Schima wallichii* ethanol extract has not been studied in vitro therefore, the present study was undertaken to investigate its cytotoxic effect in vitro.

MATERIALS AND METHODS

Chemicals

Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 5,5'dithio 2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitronbezene (CDNB), DMSO (Dimethyl sulphoxide). 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), sodium pyruvate, thiobarbituric acid (TBA) and crystal violet, were obtained from Sigma Chemical Co. (Bangalore, India). Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), potassium chloride (KCl), hydrogen peroxide (H_2O_2), were procured from SD Fine Chemicals, Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sulphuric acid (H_2SO_4), hydrochloric acid (HCl), n-butanol were purchased from Merck India Limited, Mumbai, India. Trypsin EDTA MTT (3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), minimum essential medium (MEM), fetal bovine serum (FBS), and reduced nicotinamide adenine dinucleotide (NADH) were purchased from HiMedia, Mumbai, India. Doxorubicin was supplied by Biochem Pharmaceutical Industries, Mumbai, India. The caspase kits were purchased from LabGills, Kolkata, India.

Preparation of the extract

The non-infected stem bark of *Schima wallichii, Chilauni* (DC.) Korth. was collected from Bazar veng, Lunglei, Mizoram during the months of April and May. The authentication and identification of *Schima wallichii* was done by the Botanical Survey of India, Shillong. The bark was cleaned and shade dried at room temperature in clean and hygienic conditions. The dried bark was powdered using an electrical grinder. The dried powder of *Schima wallichii* was extracted sequentially with petroleum ether, chloroform, ethanol and distilled water according to increasing polarity using a Soxhlet apparatus. The liquid extracts were filtered and concentrated by evaporating them to dryness under reduced pressure. The concentrated extracts were stored at -80 until use. The ethanol extract was used for the study and it will be called as SWE henceforth.

Preparation of drug/s

The doxorubicin was freshly dissolved in distilled water, whereas the *Schima wallichii* ethanol extract was dissolved in 1% ethanol in water and diluted with MEM. The dissolved extract was filter sterilized before use.

Cell line and Culture

HeLa and V79 cells were procured from the National Centre for Cell Science, Pune, India. The cells were routinely grown in 25 cm^2 culture flasks (HiMedia, Mumbai, India) containing Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, and 1% L-glutamine at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

Experimental Design

A known amount of cells were inoculated into several microplate wells and the cells were divided into the different groups depending on the experimental protocol:

MEM group: The cells of this group were used as negative control group.

SWE group: This group of cells was treated with different concentrations of SWE.

DOX group: The cell were treated with 5, 10 or 20µg/ml of doxorubicin (DOX) and served as positive control.

MTT assay

The cytotoxic effects of 12.5, 25, 50, 100, 200, 300 and 400 µg/ml of SWE was studied by MTT assay in HeLa, and V79 cells as described [21]. Usually 10^4 cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37°C in a CO_2 incubator in an atmosphere of 5% CO_2 in 95 % humidified air. The cells were allowed to attach for 24 hours. Thereafter, different concentrations of SWE or doxorubicin were added into each well of the microplate and incubated in the CO_2 incubator. After 48 hours of drug/s treatment, 20 µl of MTT was added into each well and the microplates were incubated for another 2 hours. The drug containing media were removed and the insoluble purple formazan formed was dissolved with lysis buffer and incubated once again for 4 hours after which the absorbance was measured at 560 nm using a micorplate reader (Biorad, USA). The cytotoxicity was calculated by the formula: Control-Treatment/Control X 100.

An another experiment was setup to study the effect of treatment duration where all the conditions were similar to that described above except that the cells were treated with 50, 100 or 200 µg/ml of SWE for 2, 4 and 6 h and processed for MTT assay as described above.

The Determination of clonogenic potential

The anticancer activity of SWE was determined by inoculating 10^6 exponentially growing HeLa cells into several culture flasks. The cells were allowed to attach for 24 h and were divided into the following groups:

MEM group: The cells of this group served as negative control group.

SWE group: This group of cells was treated with 50, 100 or 200µg/ml SWE for 6h.

DOX group: The cell cultures of this were treated with 5, 10 or 20 µg/ml DOX, and served as a positive control.

After 6 hours of drug treatment the medium from each flask decanted and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and processed for clonogenic assay [22].

Usually 200 HeLa cells were seeded into several individual petridishes containing 5 ml MEM, left undisturbed and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. The clone containing a minimum number of 50 cells was considered as a colony. The plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated according to the following formulae:-

PE = (Number of colonies counted x 100) / (Number of cells seeded)

SF = (Number of colonies counted) / (Number of cells seeded) x (mean plating efficiency).

Apoptosis assay

For the study of apoptosis, the cells were terminated at 6, 12 and 24 hours post drug treatment. The activity of caspase 8 and 3 was determined according to the manufacturer's protocol after treatment of HeLa cells with 100 µg/ml ethanol extract of *Schima wallichii.*

Biochemical assays

A separate experiment was performed to estimate the effect of SWE on the activities of various antioxidant enzymes in HeLa cells, where grouping and other conditions were essentially similar to that described for anticancer activity except that the cell cultures were terminated at 2, 6 and 12 hours post drug treatment. The drug containing media were removed; the cells were washed with sterile PBS and dislodged using trypsin EDTA treatment. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using ultra sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

Glutathione estimation

Glutathione was estimated as described earlier [23]. The concentration of glutathione was measured by its reaction with DTNB (Ellman's reagent) to give a compound that absorbs light at 412 nm. Briefly, 1.8 ml of 0.2M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The mixture was allowed to stand for 2 minutes and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).

Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of [24]. Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1ml of 20mM CDNB, and 8.8 ml distilled water were mixed and incubated at 37°C for 10 min. After incubation, 0.5 ml of 20 mM GSH and 0.1 ml of cell homogenate were added. The absorbance was recorded at 340 nm at 1 min intervals for 6 minutes using UV-VIS Biospectrophotometer.

Catalase estimation

Catalase was assayed according to technique described [25]. Briefly, in a 3 ml cuvette, 20 µl of sample was diluted with 1.98 ml of 50 mM phosphate buffer (pH 7.0). The reaction (maintained at 20ºC) was started by adding 1 ml of 30 mM H_2O_2 and the decrease in absorbance was monitored at 240 nm for 60 seconds in a UV-VIS Biospectrophotometer.

Superoxide dismutase estimation

SOD activity was estimated as described [26]. Briefly, 100 µl of cell homogenate, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitrobluetetrazolium, 200 µl of 780 µM NADH were incubated for 90 seconds at 30°C. The reaction was terminated by adding 1000 µl of acetic acid and 4 ml n-butanol. The absorbance was recorded at 560 nm using a UV-VIS Biospectrophotometer. The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme and the test with SOD enzyme samples using the formula (Blank-Sample)/Blank X 100.

Statistical Analysis

The statistical analyses were performed using Origin Pro 8. All the results are expressed as mean ± standard error mean (S.E.M). Experimental data were analyzed by one way ANOVA

followed by Tukey's test for multiple comparisons for different parameters between the groups. A p value of < 0.05 was considered as significant.

RESULTS

The results are expressed in tables 1-8 and figures 1-11 as mean±standard error of the mean.

Determination of Cytotoxicity

Treatment of HeLa, and V79 cells with different concentrations of SWE caused a concentration dependent increase in the cytotoxicity and the maximum cytotoxic effect was observed at the highest concentrations of SWE used. Treatment of HeLa cells with different concentrations of SWE induced highest toxic effect at a concentration of 400µg/ml which was 62.21% (Table 1). Similarly, SWE induced maximum cytotoxicity of 73.62% at 400 µg/ml in V79 cells. However, 50% cytotoxicity was found at 100 µg/ml for the extract for both of the cells used. The standard drug DOX was used as the positive control (Table 1, Figure 1 and 2).

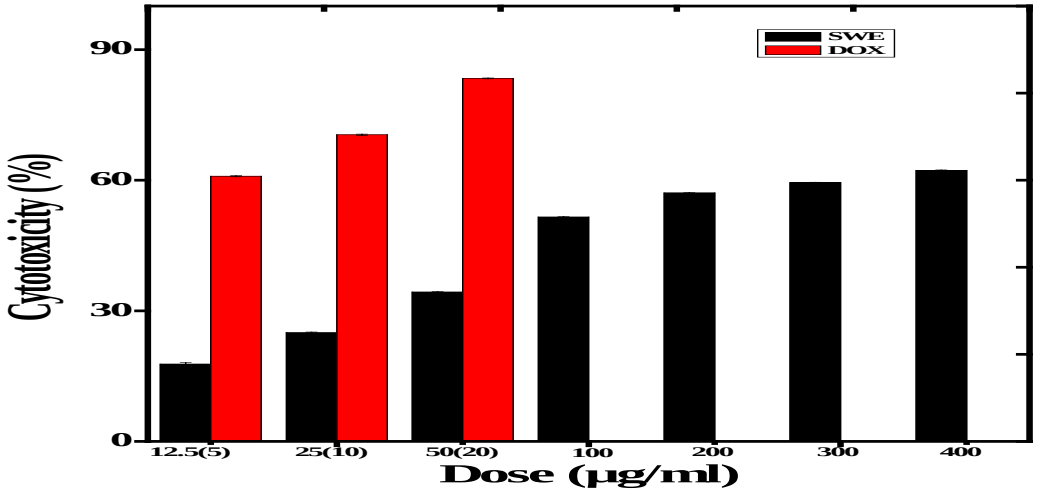

Figure 1: Cytotoxic effects of ethanol extract of *Schima wallichii* on HeLa cells evaluated by MTT assay. N=8;p<0.05

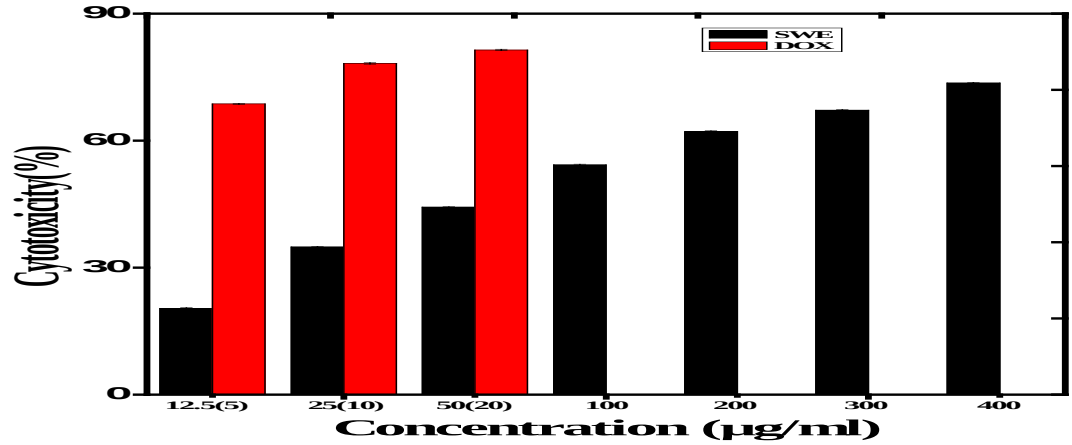

Figure 2: Cytotoxic effects of ethanol extract of *Schima wallichii* on V79 cells evaluated by MTT assay. N=8; p<0.05

Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxic effect of SWE was also evaluated by MTT assay. The SWE treatment resulted in a time dependent increase in the cytotoxicity in HeLa, and V79 cells and maximum cytotoxic effect was observed in the cells treated with SWE for 6 h (Table 2). The HeLa and V79 cells showed 50% cytotoxicity at the maximum exposure time which was selected for further experimentation (Figure 3 and 4).

Table 1 :The cytotoxic effect of different concentrations of ethanol extract of *Schima wallichii* in HeLa and V79 cell lines by MTT assay. p<0.05

Cell line	Treatment	Dose	Cytotoxicity (%)±SEM
Hela	DOX	5	60.92 ± 0.14
		10	70.44 ±0.16
		20	83.38 ±0.11
	SWE	12.5	17.76 ±0.38
		25	25.00 ± 0.12
		50	34.31 ± 0.12
		100	51.52 ± 0.17
		200	57.07 ±0.12
		300	59.44 ± 0.08
		400	62.21 ± 0.15
V79	DOX	5	68.67 ± 0.11
		10	78.26 ± 0.19
		20	81.42 ± 0.17
	SWE	12.5	20.41 ± 0.18
		25	34.88 ± 0.15
		50	44.30 ± 0.08
		100	54.27 ± 0.17
		200	62.13 ± 0.18
		300	67.20 ± 0.16
		400	73.62 ± 0.16

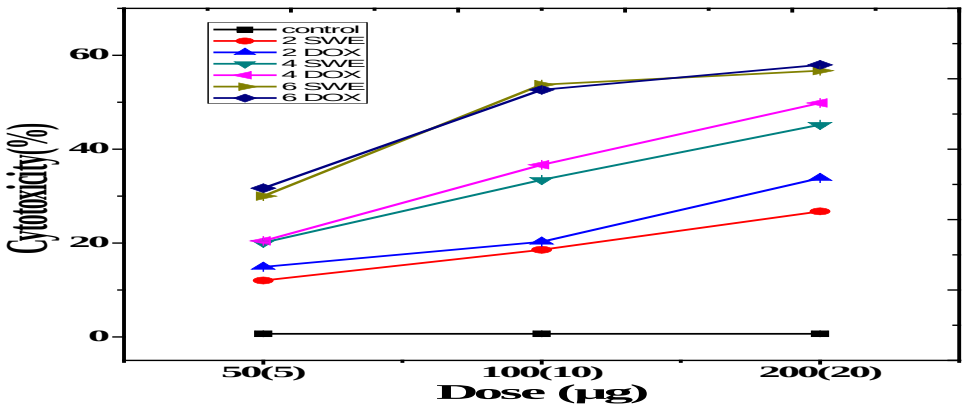

Figure 3: Cytotoxic effects of ethanol extract of *Schima wallichii* or DOX at different exposure times on HeLa cells by MTT assay. N=8; p<0.05.

Table 2: Effect of treatment duration on the cytotoxic effects of ethanol extract of *Schima wallichii* (SWE) and DOX in HeLa and V79 cell lines by MTT assay.

Cell line	Treatment	Dose	Cytotoxicity (%)±SEM		
			Post-treatment time (h)		
			2	4	6
Hela	DOX	5	14.92 ± 0.18	20.45 ± 0.16	31.68 ± 0.16
		10	20.27 ± 0.17	36.67 ± 0.11	52.64 ± 0.13
		20	33.84 ± 0.15	49.83 ± 0.15	57.95 ± 0.13
	SWE	50	12.02 ± 0.1852	20.11 ± 0.33	30.00 ± 0.14
		100	18.57 ± 0.14	33.47 ± 0.22	53.74 ± 0.15
		200	26.74 ± 0.15	45.22 ± 0.34	56.73 ± 0.15
V79	DOX	5	19.82 ± 0.29	22.89 ± 0.12	26.03 ± 0.15
		10	27.38 ± 0.28	31.82 ± 0.18	38.93 ± 0.14
		20	39.71 ± 0.22	45.76 ± 0.20	52.06 ± 0.12
	SWE	50	17.00 ± 0.22	19.35 ± 0.14	22.73 ± 0.15
		100	24.76 ± 0.20	30.11 ± 0.16	43.56 ± 0.11
		200	37.36 ± 0.27	40.76 ± 0.14	51.87 ± 0.09

N=8, p<0.05.

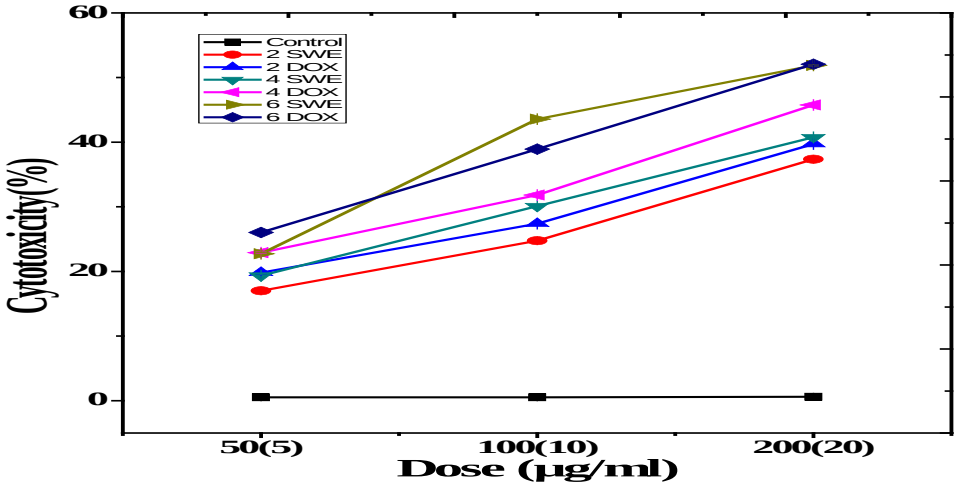

Figure 4: Cytotoxic effects of ethanol extract of *Schima wallichii* or DOX onV79 cells at different exposure times by MTT assay. N=8; p<0.05.

Clonogenic Assay

Treatment of HeLa cells with different concentrations of SWE caused a concentration dependent decline in the clonogenicity of cells. A maximum decline in the clonogenicity was observed for 200 µg/ml SWE, where extreme reduction in the cell survival (0.15) was observed. (Figure 5). The reduction in clonogenic potential by SWE was comparable to positive control DOX except the fact that the doses required by SWE were ten times greater than the DOX (Figure 5).

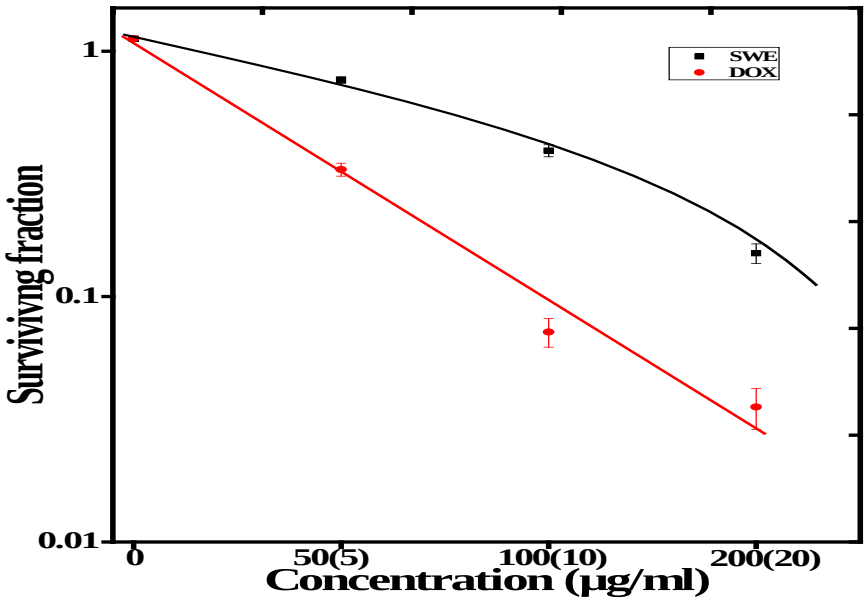

Figure 5: Effect of different concentrations of the ethanol extract of *Schima wallichii* or doxorubucin (DOX) treatment on the survival of HeLa cells. N=3.

Apoptosis

Caspase 8

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent increase in the activity of caspase 8 at different post treatment time. The maximum activity was observed at 48h post drug treatment. (Table 3, Figure 6).

Table 3: Effect of 100 µg/ml ethanol extract on the caspase activity in HeLa cells at different post treatment times.

	Post treatment time (h)	Treatment (µg/ml)		
		Control	SWE	DOX
Caspase 8	12	0.807 ±0.016	0.824 ±0.018	1.004 ±0.012*
	24	0.815 ±0.002	0.831 ±0.036*	1.035 ±0.114*
	48	0.838 ±0.004	0.856 ±0.011*	0.939 ±0.011*
Caspase 3	12	0.217 ±0.001	0.230 ±0.008*	0.246 ±0.006*
	24	0.236 ±0.005	0.252 ±0.008*	0.260 ±0.004*
	48	0.244 ±0.007	0.259 ±0.006*	0.278 ±0.016*

N=3

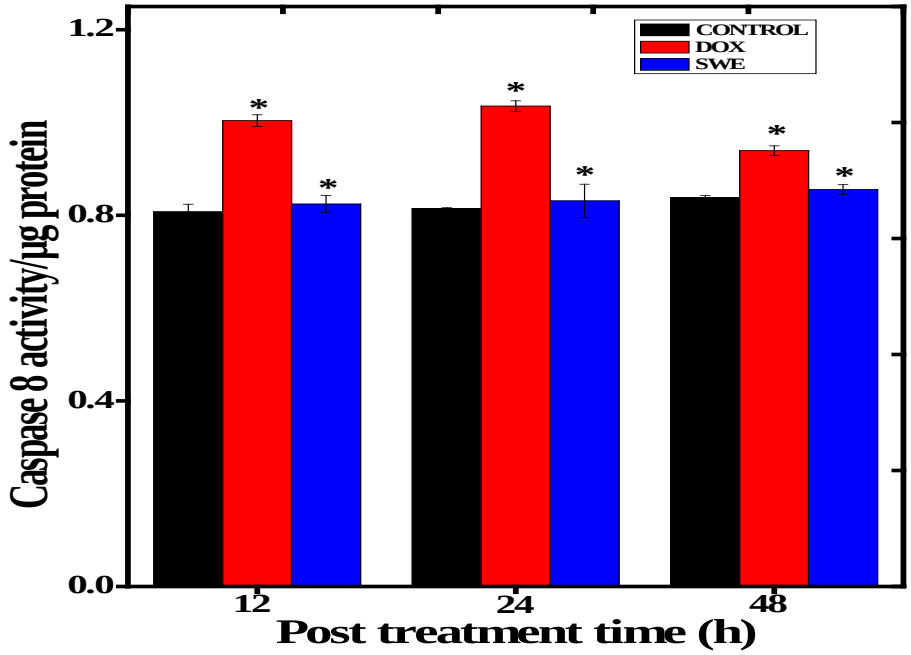

Figure 6: The caspase 8 activity in HeLa cell treated with *Schima wallichii* (SWE)or doxorubicin (DOX) at different post treatment times. N=3. p<0.05

Caspase 3

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent increase in the activity of caspase 3 at different post treatment time. The maximum activity was observed at 48h post drug treatment. (Table 3, Figure 7).

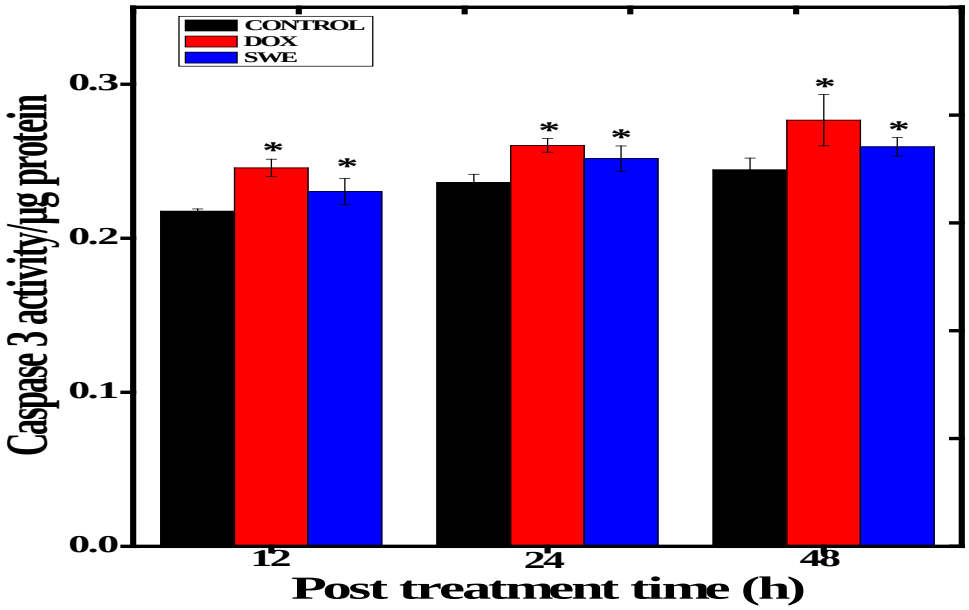

Figure 7: The caspase 3 activity in HeLa cell treated with *Schima wallichii* (SWE) or doxorubicin (DOX) at different post treatment times. N=3. p<0.05

Glutathione

Treatment of HeLa cells with different concentrations of SWE caused a statistically significant and concentration dependent reduction in glutathione contents at all the post-treatment times (Figure 8). The concentration of glutathione also declined in a similar fashion in DOX treated group (Table4). The glutathione concentration also showed a time dependent reduction and maximum decline was observed for 200 µg/ml SWE at 12 h post treatment.

Glutathione-s-transferase

The GST activity declined in a concentration dependent manner in HeLa cell exposed to 50-200 µg/ml SWE (Figure 9). The GST activity also reduced with assay time and a nadir was observed at 12 h post-drug treatment (Table 5). The decline in GST activity in SWE group was comparable to DOX treatment.

Catalase

The assay of catalase activity showed a concentration dependent alleviation in HeLa cells with increasing concentration of SWE. The catalase activity also declined with post-treatment assay time and the lowest catalase activity was recorded at 12 h post SWE treatment

Table 4: Effect of 100 µg/ml ethanol extract of *Schima wallichii* on the glutathione contents in HeLa cells at different post-treatment assay times.

Post treatment time (h)	µmol/mg protein±SEM						
	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	2.86 ±0.005	2.22 ±0.002*	2.01 ±0.001*	1.77 ±0.006*	1.65 ±0.002*	1.28 ±0.002*	0.88 ±0.008*
6	2.38 ±0.029	2.08 ±0.003*	1.62 ±0.008*	1.36 ±0.004*	1.25 ±0.002*	0.96 ±0.003*	0.68±0.003*
12	2.18± 0.002	1.85± 0.003*	1.31± 0.007*	1.18 ±0.002*	0.87 ±0.005*	0.655 ±0.001*	0.55 ±0.003*

N=5. p<0.05

Table 5: Alteration in the glutathione-s-transferase activity of HeLa cells treated with different concentrations of *Schima wallichii* or doxorubicin.

	nmol/mg protein ±SEM						
Post treatment time (h)	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	0.214 ±0.001	0.148 ±0.005*	0.118 ±0.004*	0.08 ±0.004*	0.09 ±0.006*	0.072 ±0.006*	0.034 ±0.004*
6	0.189 ±0.002	0.122 ±0.004*	0.092 ±0.005*	0.066 ±0.0058*	0.072 ±0.004*	0.046 ±0.002*	0.030 ±0.003*
12	0.169 ±0.003	0.088 ±0.002*	0.068 ±0.004*	0.046 ±0.002*	0.05 ±0.003*	0.032 ±0.004*	0.016 ±0.004*

N=5, p<0.05

(Table 5). There was an abrupt decline in the catalase activity at 6 h post treatment when compared to 2 h after SWE treatment. The DOX treatment also resulted in an identical decline in catalase activity at all post treatment times (Figure 10).

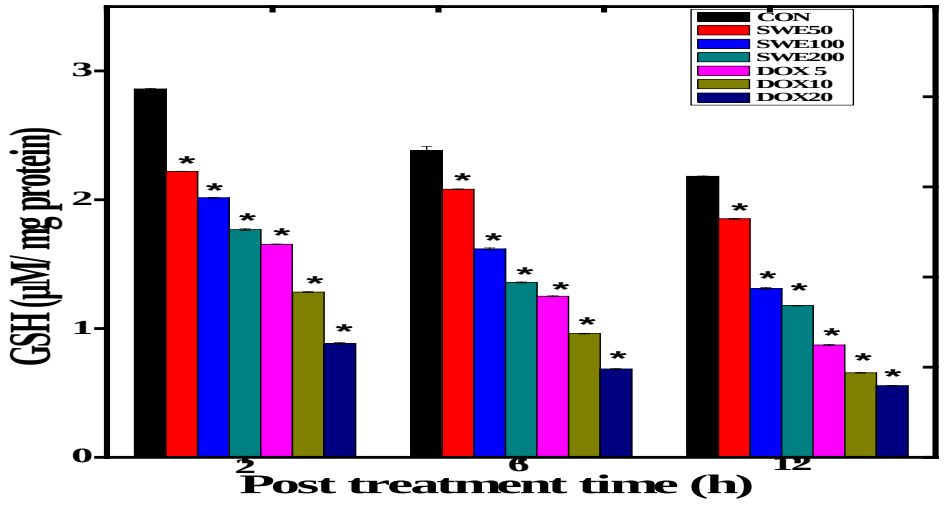

Figure 8: Alteration in the glutathione contents in HeLa cells treated with various concentrations of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;p<0.05 when treatment groups compared with concurrent control (SPS) group.***

Table 6: Alterations in the catalase activity of HeLa cells treated with different concentrations of *Schima wallichii* ethanol extract (SWE) or doxorubicin (DOX).

Post treatment time (h)	Unit/ mg protein±SEM						
	Control (MEM)	Treatment (µg/ml)					
		Schima wallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	8.36 ±0.03	7.19 ±0.02*	5.28 ±0.01*	2.87 ±0.005*	5.91 ±0.03*	3.85 ±0.02*	3.09 ±0.009*
6	8.71 ±0.03	4.25 ±0.02*	3.47 ±0.81*	1.19 ±0.003*	4.25 ±0.02*	2.19 ±0.02*	1.73 ±0.003*
12	7.46 ±0.02	2.55 ±0.02*	1.20 ±0.003*	0.77 ±0.006*	2.56 ±0.03*	1.15 ±0.02*	0.095 ±0.01*

N=5, p<0.05

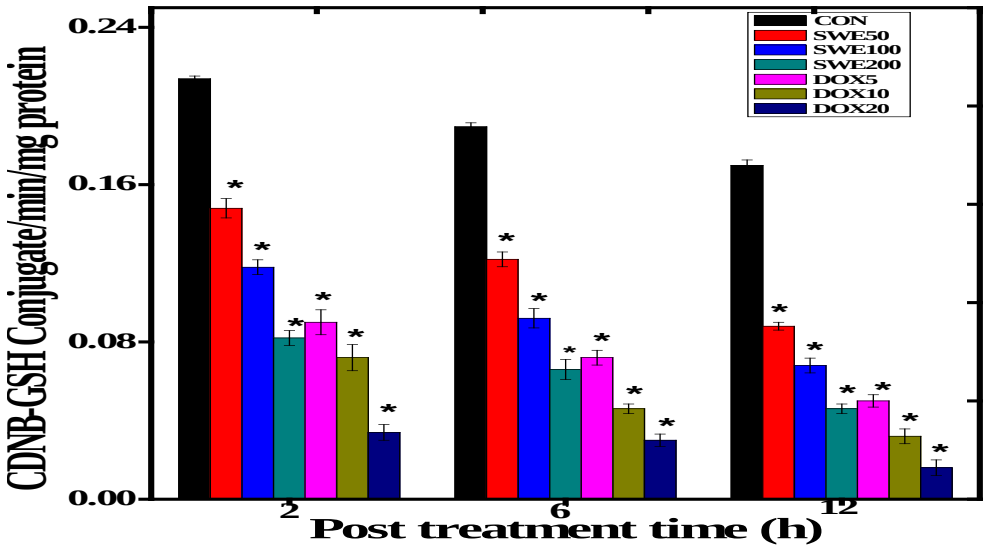

Figure 9 :Alteration in the glutathione-s-transferase (GST) activity in HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;p<0.05 when treatment groups compared with concurrent control (SPS) group.***

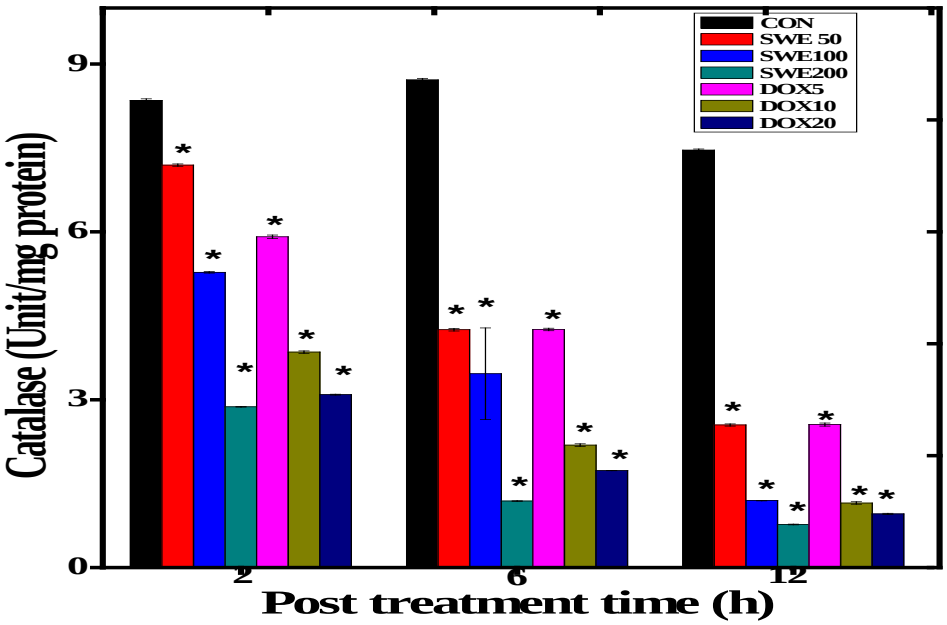

Figure 10 : Alteration in the catalase activity on HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;p<0.05 when treatment groups compared with concurrent control (SPS) group.***

Superoxide dismutase

The exposure of HeLa cells to different concentrations of SWE caused a concentration dependent but gradual reduction in the SOD activity (Figure 11). The SOD activity also showed a time dependent decrease with a maximum reduction in the SOD activity at 12 h post – treatment (Table 7). The positive control DOX also showed a pattern similar to that of SWE treatment

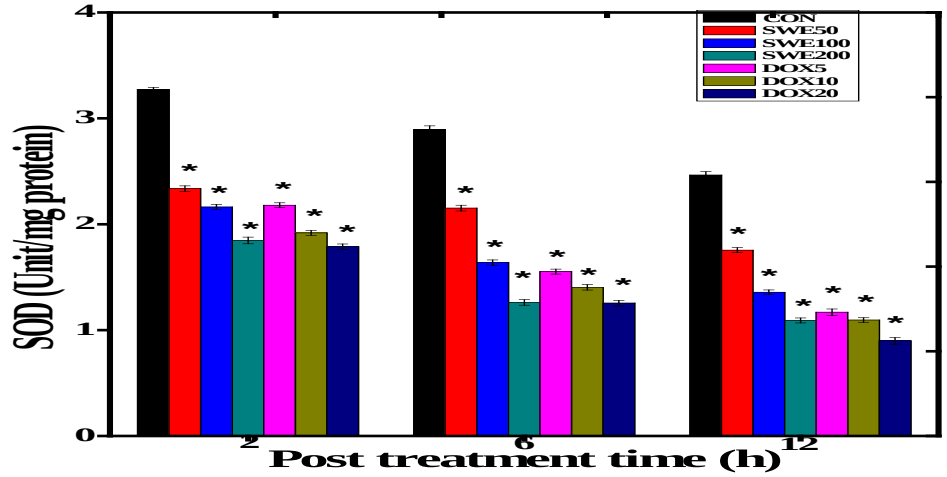

Figure 11 : Alteration in the Catalase activity on HeLa cells treated with various doses of *Schima wallchii* ethanol extract (SWE) or doxorubicin (DOX) at different post treatment times. N=5;.p<0.05 when treatment are compared with concurrent control (SPS) group.***

Table7: Alteration in the SOD activity HeLa cells treated with different concentrations of *Schima wallichii* ethanol extract (SWE) or doxorubicin (DOX).

	Unit/ mg protein±SEM.						
Post treatment time (h)	Control (MEM)	Treatment (µg/ml)					
		Schimawallichii (SWE)			Doxorubicin (DOX)		
		50	100	200	5	10	20
2	3.27 ±0.02	2.34 ±0.03*	2.16 ±0.02*	1.85 ±0.03*	2.18 ±0.02*	1.92 ±0.02*	1.79 ±0.03*
6	2.90 ±0.03	2.15 ±0.03*	1.64 ±0.03*	1.26 ±0.03*	1.55 ±0.02*	1.41 ±0.03*	1.25 ±0.03*
12	2.46 ±0.03	1.75 ±0.02*	1.36 ±0.02*	1.09 ±0.02*	1.17 ±0.03*	1.09 ±0.02*	0.9 ±0.03*

N=5. p<0.05

DISCUSSION

Several studies have focused on natural anticarcinogenic agents. Many natural products have been identified to treat malignant neoplasia [2, 3]. The importance of natural products in medicine cannot be underestimated. The Federal Drug Administration, USA has approved approximately 547 products from natural resources or their derivatives for clinical use [27]. This indicate that plants still forms the major source for drug development and the screening of plants provides a major avenue for new drug discovery. Therefore the present study was designed to evaluate the antineoplastic action of *Schima wallichii* in vitro.

The MTT assay is a rapid and standard technique to determine the cytotoxicity of any drug in various cultured cells. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. Hence, the weaker the color formed, the more are the dead cells. The treatment of HeLa and V79 cells with different concentrations of SWE caused a concentration dependent rise in the cytotoxicity of ethanol extract of *Schima wallichii*. MTT assay has been used to test cytotoxicity in various cell lines earlier [21]. The cytotoxicity of ethanol extract of *Consolida orientalis* L, *Ferula assa-foetida* L, *Coronilla varia* L, *Orobanche orientalis* G. Beck on HeLa cells increased in a concentration dependent manner [28]. The other plants like *Alstonia scholaris, Consolida orientalis*, *P. pellucidum, Tinospora cordifolia*, *Ferula assafoetida* and *Coronilla varia* extract have been shown to exert cytotoxicity in cultured HeLa cells earlier [29-33].

The clonogenic assay is the most confirmatory test, which indicates the reproductive integrity and the extent of cell survival and it is also a long-term assay which takes cares of the delayed effects induced by drug treatments [34,35]. The cytotoxic effect of SWE was further confirmed by clonogenic assay where SWE treatment caused a concentration dependent decline in the clonogenicity of HeLa cells. There are no reports where SWE has been used to evaluate the reproductive potential of any cell line earlier. However, other plants including

Alstonia scholaris, Aphanamixis polystachya and *Tinospora cordifolia* have been reported to reduce the clonogenic potentials of HeLa cells earlier [29, 31,33].

The cancer cells show elevated oxidative stress however, excess oxidative stress also kills cancer cells by eliciting various mechanisms of cell death and agents than can induce greater amount of oxidative stress may be useful in killing the cancer cells [36-38]. Therefore, effect of SWE on oxidative stress was also studied in HeLa cells.

Glutathione (γ -glutamylcysteinyl glycine) is the most abundant non-protein thiol in the cell. It plays various physiological roles including counter balancing the excess free radicals produced in the cells during numerous physiological processes [39,40]. Glutathione occurs in two forms namely the reduced form (GSH) and the oxidized form (GSSG). In normal conditions, GSH protects the cells against the damaging effects of free radicals, xenobiotics, ionizing radiations and some cytokines. It also regulates DNA synthesis, cell proliferation and the carcinogenic mechanisms. In cancer cells, its higher amount is indicated in tumor microenvironment-related aggression, apoptosis evasion, colonizing ability, and multidrug and radiation resistance [41-43]. Increased concentration of GSH in the tumor cells have been known to make the tumor refractory to treatment, while depletion of glutathione has been reported to enhance the cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of the calcium homeostasis and eventually loss of cell viability [44, 45]. Therefore, glutathione attrition in cancer cells may enhance the cytotoxic effects of chemotherapeutic agents [46]. A similar connection is true in the present study, where SWE reduced the glutathione concentration in a concentration and time dependent manner. Earlier, *Alstonia scholaris* has been reported to reduce the glutathione contents in tumor cells [47]. The glutathione-S-transferases are a multi-gene family of enzymes, which carry out detoxification and activation of certain chemicals [48]. The GSTs are overexpressed in a wide variety of tumors and their negative modulation has emerged as a promising therapeutic target as they have been implicated in the resistance to cancer therapy. The augmented activity of GST in tumor cells is associated with suppression of tumor cell kill by apoptosis [49,50]. The SWE treatment has decreased the GST activity significantly and that may be one of the reasons of increased cell killing effect in the present study. Several GST inhibitors have been shown to reduce drug resistance by sensitizing tumor cells to anticancer drugs and bring effective cell killing [51,52]

The catalase is involved in the detoxification of H_2O_2 into water and molecular oxygen. However, it also plays a crucial role in various other processes. High levels of catalase have been found in patients with lung cancer, whereas the patients suffering from breast cancer, head and neck cancer, gynecological cancer, lymphoma, prostate cancer and urological cancer showed decreased levels of catalase [53]. The higher activity of catalase has been linked to suppression of apoptosis in tumor cells undergoing chemotherapy [54]. The untreated HeLa cells have shown higher catalase activity, whereas SWE treatment reduced the activity of catalase indicating that SWE action may be mediated by reducing catalase activity in HeLa cells. Superoxide dismutase (SOD) present in all oxygen metabolizing cells and it catalyzes the dismutation of the superoxide radical (O_2) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [55]. Four different forms of SOD are known until now [56] of which the Cu-Zn SOD is the most abundant and comprises approximately 90% of total SOD activity in a eukaryotic cell [57]. All the SODs are very efficient scavengers of the superoxide radical and cell damage occurs when there is not enough SOD to handle the flux of O_2 [58, 59]. Malignant

cells have been reported to show elevated Cu-Zn SOD activity [60]. The role of SOD in cancer is controversial as it is found to be overexpressed in some cancers, whereas other cancer show reduced expression [61]. The SOD are overexpressed in late stage of cancer, especially metastatic tumors [61] indicating that high levels of SOD in tumors may make them refractory to therapy. The SWE treatment reduced SOD activity in HeLa cells, which may account for its higher cell killing activity.

The exact mechanism of action of SWE is not known. It may have utilized different pathways to kill the cells. The present observations indicate that SWE treatment reduced the levels of GSH, GST, catalase and SOD, which may have increased the oxidative stress and stimulated the mechanisms that may have brought apoptotic and non-apoptotic form of cell death. The analysis of caspase 8 and 3 in HeLa cells indicate that part of the cell death seems to be mediated by apoptosis and increased oxidative stress indicate SWE also induced non-apoptotic form of cell death. The increased oxidative stress by SWE may have triggered events that may have damaged the cellular genome thereby bringing cell death in the present study. It is plausible that SWE may have increased the activation of P53 and related proteins to stimulate apoptotic form of cell death. The SWE may have inhibited the transcription of NF-κB, COX-II and Nrf2 elements which are responsible for tumor cell proliferation and therapy resistance [62-64]. In fact the down modulation of these proteins have been reported to enhance cell killing [65-67]. The SWE may have also used some other unknown mechanisms to kill the HeLa cells in the present study.

It is concluded that SWE administration caused effective killing of HeLa cells and the anticancer activity of SWE may due to reduction in the GSH and other antioxidant enzymes including GST, CAT and SOD. SWE may have also retarded the transcriptional activation of Nrf2, NF-κB and COX-II which may have contributed in killing the HeLa cells. The stimulation of caspase 8 and 3 in the present study indicates that SWE induced apoptotic mode of cell death in some of the cells if not all. Our results demonstrates that SWE act as an anticancer agent. However, further studies are required to ascertain its potential as an anticancer agent in different tumor models and understanding its mechanism of action.

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