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EVALUATION OF THE CYTOTOXIC EFFECTS OF HELICIA NILAGIRICA BEDD IN VITRO

Zoology

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ABSTRACT

The cancer is a second largest killer disease and despite numerous advances made in the treatment strategies, the complete cure of cancer remains elusive. Therefore, the present study was undertaken to investigate the anticancer potential of aqueous extract of *Helicia nilagirica* (HNA) in vitro by MTT and clonogenic assays, where V79 and HeLa cells were treated with the different concentrations of aqueous extract of *Helicia nilagirica.* The treatment of V79 and HeLa cells with HNA resulted in a concentration dependent increase in cytotoxicity, which was maximum at the highest concentration of 400 µg/ml HNA in both the cell lines. The results of MTT assay were further confirmed by clonogenic assay, which also showed a concentration dependent decrease in the clonogenicity of HeLa cells.. To understand the mechanism of action the effect of HNA on glutathione (GSH) concentration, activities of glutathione-s-transferase (GST), catalase and superoxide dismutase (SOD) were studied at different post HNA treatment times. The exposure of HeLa cells to different concentrations of HNA at different post- treatment time alleviated the GSH content and also reduced the activities of antioxidant GST,CAT and SOD in a concentration and time dependent manner, except GST which was lowest and 6 h post-treatment and then marginally elevated at 12 h post-treatment.. The present study indicates that HNA exerted the cytotoxic effect on HeLa cells and recued the cell survival and this effect of HNA may be due to the alleviated level of the GSH, GST, catalase and SOD.

KEYWORDS

HeLa, MTT, clonogenic, glutathione, catalase

INTRODUCTION

Cancer a multistage disease has been the second leading cause of death worldwide. The number of cancer cases has been predicted to rise by almost 70% in 2020 (WHO, 2017) indicating the need to find new paradigms to treat or prevent the occurrence of cancer. The chemotherapy is an established mode of treatment of several neoplasia and it is the only treatment when a patient presents with metastasis (Harrington and Smith, 2009). Almost all the modern chemotherapeutic treatments available today are associated with several adverse side effects due to limitation in site specificity, causing strain to the patient/s (Ochwang'I *et al*., 2014). This indicates the need to focus on the use of alternative treatments and therapies against cancer, which are non-toxic or possess negligible side effects.

Plants have formed the major source of several modern chemotherapeutic drugs until their chemical synthesis began and they will continue to play a major role to treat cancer (Kinghorn *et al.*, 2016). Plant-derived drugs have gained interest for anticancer treatment as they are natural and readily available, readily administered orally as part of patient's dietary intake (Cornblatt *et al*., 2007; Amin *et al*., 2009). Since they are naturally derived compounds from plants they are generally more tolerated and non-toxic to normal human cells (Jagetia, 2007; Unnati *et al*., 2013; Jagetia and Baliga, 2016).

The National Cancer Institute collected about 35,000 plant samples from 20 different countries, and has screened around 114,000 extracts for anticancer activity. 60% of the commercially available anticancer drugs are derived from natural sources. The anticancer agents, vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apo-cynaceae), were the first agents to advance into clinical use for the treatment of cancer. Vinblastine and vincristine are used in combination with other cancer chemotherapy drugs, for the treatment of various kinds of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers (Moudi *et al*., 2013). The isolation of paclitaxel from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (*Taxaceae*) was clinically introduced to the US market in the early 1990s. Paclitaxel is active against a number of cancer types such as ovarian cancer, advanced breast cancer, small and non-small cell lung cancer, while *Taxus baccata* was reported to be used in India as a medicine for the treatment of cancer (Ahmed *et al*., 2013). The Camptothecin isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (*Nyssaceae*), derivatives of camptothecin, Topotecan and irinotecan, are used for the treatment of ovarian and small cell lung cancers, and colon cancers, respectively (Venditto and. Simanek, 2010). However, induction of various adverse side effects including myelosuppression, gastrointestinal, hair follicle damage, reproductive and nephrotoxicities by these drugs has been the major stumbling block which necessitates the need to identify effective newer biomolecules to kill cancerous cells and spare normal cells with very low or negligible toxicity.

Helicia nilagirica Bedd. (Family: Proteaceae) locally known as Pasaltakaza is a tree, that grows up to a height of 12 meters and it grows in southern India, Indochina, Sri Lanka, , Burma (Myanmar), Japan, Taiwan, and Thailand. The *Helicia nilagirica grows*along streams some species are found on hilltops or ridges (Khamyong et al., 2004). Traditionally, *Helicia nilagirica* has been used as folk medicine in Mizoram, India by the Mizos since time immemorial . The decoction of leaves or bark of *Helicia nilagirica* is used to cure mouth ulcers, indigestion, stomach ailments, peptic ulcers, urinary tract infection gynaecological disorders and scabies and other skin diseases (Sawmliana 2003). In Sikkim the fruits of *H. nilagirica* are used to treat cough and cold (Chauhan 2001). A recent study has indicated that methnol extract of this plant possessed anti-inflammatory activity in rat cotton pellet granuloma model (Lalawmpuii *et al.* 2014). The systematic study on the anticancer properties of *Helicia nilagirica is lacking, which* indicates a need to evaluate its anticancer potential. Therefore, the present study was carried out to evaluate the anticancer activity of *Helicia nilagirica 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), reduced glutathione (GSH), ethylenediamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), crystal violet were obtained from Sigma Chemical Co. (Bangalore, India). Sodium bicarbonate (Na_2CO_3), potassium chloride (KCl) and hydrogen peroxide (H_2O_2) were procured from SD Fine Chemicals, Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium hydrogenphosphate (Na_2HPO_4), hydrochloric acid (HCl), n-butanol, Tris buffer (Tris (hydroxymethyl) aminomethane and ammonium oxalate were requisitioned from Merck India Limited, Mumbai, India. Trypsin EDTA 1X, 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 (DOX), was obtained from Getwell Pharmaceuticals, Gurgaon, India.

Preparation of the extract

The identification and authentication of *Helicia nilagirica* Bedd. (Family: Protaeceae) was done by Botanical Survey of India, Shillong. The non-infected stem bark of *Helicia nilagirica* was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season. The stem bark was peeled of the tree, cleaned chopped into small pieces, spread into the stainless steel trays and allowed to shade dry at room temperature in the dark, in the clean and hygienic conditions free from insects, animals, fungus, and other extraneous terrestrial materials. The dried tree stem bark was powdered in an electrical grinder at room temperature. A sample of 100 g of bark powder was sequentially extracted with petroleum ether, chloroform, ethanol and water using a Soxhlet apparatus. The extracts were evaporated to dryness under reduced pressure and stored at -80 until further use. Only the aqueous extract of *Helicia nilagirica* (HNA) was used for evaluation of the anticancer activity.

Dissolution of drug/s

The doxorubicin was freshly dissolved in MEM and the aqueous extract of *Helicia nilagirica* was dissolved in MEM, filtered and sterilized immediately before use.

Cell line and Culture

HeLa S3, 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, 1% L-glutamine and 50 µg/ml gentamicin sulfate at 37°C in an atmosphere of 5% CO_2 in humidified air in a CO_2 incubator (Eppendorf AG, Hamburg, Germany).

Experimental Design

Usually a fixed number of cells were inoculated into the desired culture vessels and they were divided into the different groups depending on the experimental protocol:

MEM group: The cells of this group served as negative control group.
HNA group: This group of cells was treated with different concentrations of HNA.

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

The cytotoxic effects of different concentrations of aqueous extract of *Helicia nilagirica* was studied by MTT assay in HeLa, and V79 cells as described by Mosmann (1983). Usually 10^3 cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37 ºC 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. The cells in microplates were exposed to different concentrations of HNA or doxorubicin and incubated in the CO_2 incubator for next 48 hours. Thereafter, 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 microplate reader (Spectramax M2). The cytotoxicity was calculated using the formula Control-Treatment/Control X 100. The IC50 was also determined.

Determination of optimum exposure time for cytotoxicity

A separate experiment was conducted to study the effect of treatment time on the cytotoxicity of HNA on the cells, where grouping and other conditions were essentially similar to that described above except that the cells were exposed to HNA for different times and the cytotoxicity was determined by MTT assay as described above.

The Determination of anticancer activity

Another experiment was setup to evaluate the anticancer activity of HNA, where grouping and other conditions were similar to that described in the experimental design section. Usually 10^6 exponentially growing HeLa cells were seeded into several culture flasks and the cells were allowed to attach for 24 h. The cells were exposed to 5, 10 or 20 µg/ml DOX or 20, 300 or 400 µg/ml of HNA.
After 2 hours of drug/s treatment the media were removed and the flasks were washed twice with sterile PBS, subjected to trypsin EDTA treatment and the following studies were conducted.

Clonogenic Assay

Usually 200 HeLa cells were inoculated into several individual petridishes containing 5 ml MEM and allowed to grow for another 11 days. The resultant colonies of cells were stained with 1 % crystal violet in methanol and scored. The plating efficiency (PE) of the cells was determined and surviving fraction (SF) calculated according to Puck and Marcus (1955).

PE = (Number of colonies counted x 100)/(Number of cells seeded)
SF = (Number of colonies counted)/(Number of cells seeded) x (mean plating efficiency).

Biochemical assays

A separate experiment was performed to estimate the effect of HNA on the activities of various antioxidant enzymes in HeLa cells, where grouping and other conditions were essential 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. The cells were pelleted and weighed and 5% homogenate of the cells was prepared in PBS (pH 7.4) using sonicator (PCI Analytics Pvt. Ltd., Mumbai, India).

Total proteins

The proteins were estimated by standard procedure of Bradford (1976).

Glutathione estimation

Glutathione was estimated as described earlier (Moron *et al*., 1979). Briefly, 1.8 ml of 0.2 M Na_2HPO_4 was mixed with 40 µl 10 mM DTNB and 160 µl of cell homogenate. The mixture was incubated for 2 minutes at room temperature and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).
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Glutathione - S – transferase estimation

Glutathione-s-transferase activity was estimated by the method of Habig *et al*., (1974). Briefly, 0.5 ml of 0.1 M phosphate buffer pH 6.5, 0.1 ml of 20 mM 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 read at 340 nm at 1 min intervals for 6 minutes in UV-VIS Biospectrophotometer. The GST activity was estimated using the following formula:-
GST activity = Absorbance of sample – Absorbance of blank_× 1000/9.6 × Vol of sample

Catalase estimation

Catalase was assayed according to the technique of Aebi (1984). 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 initiated 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 by Fried (1975). Briefly, 100 µl of cell homogenate was mixed with 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, and 200 µl of 780 µM NADH and 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.

STATISTICALANALYSES

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 table 1-4 and figure 1-7 as mean ± standard error of the mean.

Determination of Cytotoxicity

The treatment of HeLa or V79 cells with different concentrations of HNA resulted in a concentration dependent rise in its cytotoxic effects (Figure 1)and a maximum cytotoxicity was recorded for the highest concentration of HNA. The positive control doxorubicin also showed a similar pattern (Figure 1). The IC50 was also calculated and found to be 306.71µg/ml for HeLa and 300.64 µg/ml HNA for V79 cells, respectively.

Determination of optimum exposure time for cytotoxicity

The optimum exposure time for cytotoxicity of HNA against the two cell lines was determined using MTT assay at 2, 4, and 6 hours. The highest percent of cytotoxicity was observed at 4 h for HeLa and 2 h post treatment time for V79 cells, respectively (Figure 2). The difference among various treatment times was statistically not significant. Therefore further experiments were setup using 2 h HNA treatment time.

Clonogenic Assay

The clonogenicity of HeLa cells declines in a concentration dependent manner after treatment with different concentrations of HNA and the cell survival reached a nadir at a concentration of 400 µg/ml (Figure 3). The IC50 of HNA was also calculated and found to be 208.69 µg/ml.

Glutathione

The glutathione content of HeLa cells treated with different concentrations of HNA declined in a concentration dependent manner at all the post treatment times (Figure 4) and this decline was statistically significant when compared with the untreated control group (Table 1). A maximum of 2.7 fold reduction in the glutathione content was observed at 6h post treatment at a concentration of 400 µg/ml (Table 1). The doxorubicin treatment also showed a pattern similar to HNA treatment (Figure 4).

Glutathione-s-transferase

Treatment of HeLa cells with different concentration of HNA showed a concentration dependent reduction in the GST activity at all the post treatment times (Figure 5). The maximum reduction (4.09 fold) was found at a concentration of 400 µg/ml after 4h treatment time (Table 2). The DOX treated group also reduced the enzyme activity in a concentration dependent manner. The reduction at all concentrations was found to be statistically significant ($p<0.05$).

Catalase

The activity of catalase in the HeLa cells treated with different concentrations of HNA showed a concentration dependent decrease at all post treatment times (Figure 6). A maximum of 2.87 fold decrease in catalase activity was observed at 400 µg/ml, at 6 h post treatment when compared with the non-drug treated control group. The catalase activity declined significantly in the HeLa cells treated with different concentrations of HNA or doxorubicin (Table 3).

Superoxide dismutase

The treatment of HeLa cells with different concentrations of HNA or DOX caused a significant but concentration dependent attrition in the SOD activity at all post treatment times (Figure 7 and Table 4). A maximum of 7.11 fold and 7.59 fold decrease in the SOD activity was observed for 400 µg/ml and 20 µg/ml of HNA and DOX, respectively, at 6h, post treatment (Table 4).

DISCUSSION

Chemotherapy has been a major treatment modality to treat various malignant cancers, either alone or in combination with radiation or surgery. It has also been used as a palliative treatment where the complete cure of cancer has not been affected (Morgan *et al*., 2004; Roeland). The active principles in chemotherapy have been derived from plants such as *Catharanthus roseus, Podophyllum peltatum, P. emodii, Taxus brevifolia, Ochrosia elliptica* and *Campototheca acuminate* (Kinghorn and Balandrin 1993). However, most of the modern chemotherapeutic agents have limitations in terms of toxicity, lack of tumor selection, ineffective against drug resistant cancers, expensive and teratogenic (Mellor and Callaghan 2008; Valko and McLeod 2009). Moreover, the patients who survive chemotherapy have shown the development second malignancies associated with chemotherapeutic treatment (Morton *et al*., 2014). Therefore, screening for non-toxic, cheaper, higher efficacy and better selectivity cancer drug/s, which are devoid of all the side effects of modern molecules is needed. The use of plants for treating various ailments have been practiced by humans since time immemorial and there is an unending quest in finding new and improved chemotherapeutic drugs till today. Since herbal products have been traditionally accepted and known to have lesser or no adverse effects, it is imperative to search new molecules from the plants. This has been the impetus to determine the anticancer activity of the stem bark of *Helicia nilagirica in vitro.*

Cell culture has provided a fast, efficient and economical way of cytotoxicity screening, elucidation of mode of action of drugs in a controlled and systematic manner with high resolution in a short period of time. The present study was also carried out using HeLa, a cervical cancer cells and non-cancerous V79, a Chinese hamster lung cells to estimate the cytotoxic effects of HNA by employing MTT assay. This assay is a rapid and a standard technique to test the cytotoxicity of drugs where metabolically active cells increase the formation of formazan crystals by mitochondrial succinate dehydrogenase and the level of enzyme activity is a measure of the viability of the cells and more intense color indicates more viable cells (Mossmann, 1983). The treatment of HeLa and V79 cells with HNA reduced the cell survival indicated by a concentration dependent rise in the cytotoxic effect. There seems to be no reports on the cytotoxicity of HNA and this is probably the first report where HNA has been found to be cytotoxic. However, other plants such as *Aphanamixis polystachya* ,*Tinospora cordifolia*, *Alstonia scholaris, Consolida orientalis*, *Ferula assafoetida*, *Coronill avaria* and *P. pellucidum* extract have been reported to induce cytotoxicity in HeLa cells in vitro (Jagetia *et al.,* 1994; Jagetia and Rao., 2006;Jagetia and Baliga, 2005; Widowati *et al.,* 2013; Jagetia and Venkatesha, 2016). Similarly, *Arctium lappa, Artemisia absinthium, Calendula officinalis, Centaurea, Cyanus, Tanacetum vulgare* and *Tragopogon pratensis* have been reported to be cytotoxic to J-45.01 human acute T leukemia cells (Wegiera *et al.,* 2012). The cytotoxicity of HNA was further confirmed by performing clonogenic assay on the HeLa cell line. The IC50 was calculated to be 208.69 µg/ml.

The clonogenic assay is the gold standard to test the reproductive integrity of cells and HNA treatment has been found to reduce the Clonogenic potential of HeLa cells in a concentration dependent fashion. Likewise *Aphanamixis polystachya , Tinospora cordifolia*, and *Alstonia scholaris* have been reported to retard the clonogenicity of HeLa cells (Jagetia *et al.,* 1994; Jagetia and Rao., 2006; Jagetia and Venkatesha, 2016). Similarly, a natural product berberine has been found to reduce the clonogenic potential of HeLa cells in a concentration dependent manner (Jagetia and Rao, 2017).

Glutathione (γ -glutamylcysteinyl glycine) is a tripeptide synthesized in most cells and it is formed by the ATP dependent condensation of glutamic acid and cysteine, catalyzed by γ-glutamylcysteinyl synthetase. Glycine is then added by glutathione synthetase to form GSH. The presence of sulfhydryl (SH) group of the cysteinyl moiety is a powerful reducing agent and a strong nucleophile that is able to react with cellular toxicants directly or via the catalysis of the glutathione S-transferase family of enzymes. It is also a co-factor for several metabolic enzymes and is involved in intracellular transport, functions as an antioxidant and radioprotectant and facilitates protein folding and degradation (Halliwell and Gutteridge 1999; Gamcsik *et al*., 2012; Lu, 2013). In cancer cells the rise in GSH beyond normal level is an indication of chemotherapy resistance whereas low level of GSH has been reported to enhance oxidative stress, and subsequently cause cell death and apoptosis of the tumor cells. The loss of essential sulfhydryl groups lead to an alteration in the calcium homeostasis that eventually results in the loss of cell viability which is indispensible for chemotherapy to be effective (Mayer *et al*., 1987; Neal *et al*., 2003; Ramsay and Dilda, 2014). Treatment of the HeLa cells with different concentration of HNA showed a concentration dependent reduction of GSH content which showed the effectiveness of HNA against neoplastic cells.

Glutathione-S-transferase isoenzymes are ubiquitous which catalyze the conjugation of reduced glutathione (GSH) with compounds that contain an electrophilic centre through the formation of a thioether bond between the sulphur atom of GSH and the substrate (Chasseud 1979; Mannervik 1985; Laborde, 2010). A number of GST isoenzymes also exhibit GSH-dependent catalytic activities such as reduction of organic hydroperoxides, isomerisation of various unsaturated compounds and also several non-catalytic functions such as sequestering of carcinogens, modulation of signal transduction

pathways etc. (Ketterer *et al*. 1990; Jakoby and Habig 1980; Cho *et al*. 2001). The over expression of GST in cancer cells are common and can induce chemoresistance and resistance to apoptosis which makes it a promising target for research on the GST inhibitors to sensitize tumor cells (McIlwain *et al*., 2006; Zeng *et al*., 2014). The decline in GST activity after treatment with the HNA could be an indication that this extract act as an inhibitor compound for GST and thereby killing the cancer cells.

Catalases catalyze the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) in the presence of iron or manganese as a cofactor (Kodydková *et al*., 2014). It is localized in peroxisomes in eukaryotic cells. Suppression of catalase has been reported to induce the increase production of H_2O_2 which block TNF-induced NF-κB activation and sensitizes cells to apoptosis (Yang *et al*., 2011). The HNA also reduces the activity of catalase which may have contributed to its anticarcinogenic activity.

Superoxide dismutases (SOD) are the enzymes that catalyze the dismutation of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen. They consist of three isoforms in mammals: the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3), all of which require catalytic metal (Cu or Mn) for their activation. The mechanism of dismutation of O_2^- to H_2O_2 by SOD involves alternate reduction and reoxidation of a redox active transition metal including copper (Cu) and manganese (Mn) at the active site of the enzyme (Abreu, 2010). The role of SOD level on tumor invasiveness is controversial as far as different studies are concerned on different tumors. In one study MnSOD overexpression protected HeLa cervical carcinoma cells from growth suppression under the condition of serum deprivation, which was suggested to be related to changes in the intracellular oxidative processes of these cells (Palazzotti 1999). When human prostate carcinoma cells were transfected with the cDNA for MnSOD, the clones overexpressing MnSOD grew more slowly under basal cell culture conditions in vitro than control cells (Li 1998). In one model recombinant CuZnSOD increased colon carcinoma liver metastasis in mouse models (Nonaka 1993), whereas in another mouse model recombinant CuZnSOD reduced fibrosarcoma pulmonary metastasis (Yoshizaki 1994). Therefore, modulation of the oxidant/antioxidant balance toward a more reduced state is likely to have a controlling influence limiting the survival and invasion of most cancer cells. The HNA also alleviated the activity of SOD which caused change in the cellular oxidant/antioxidant balance thereby increasing tumor cell kill in the present study.

The exact mechanism of action of cell killing by HNA is not clearly understood however, the present study clearly indicates that HNA has been able to reduce the activities of GST, catalase and SOD which are involved in the failure of chemotherapy. This reduced activity may have played a major role in the cell killing by HNA in the present study. The GSH is another molecule which in indicated in the development of chemo resistance and it reduction by HNA would have made cells for sensitive to its cytotoxic effect and its decline may have also stimulated cell death by apoptosis. Although the effect of HNA at molecular level has not been studied. It is plausible that HNA may have blocked the transcription of NF-κB, COX-II, Nrf2 and some cell cycle proteins that help cancer cell division and ensure higher survival (Sobolewski *et al*., 2010; Lu and Stark, 2015; Choi and Kwak, 2016) and their inhibition may have led to the effective cell killing by HNA. The suppression of these proteins have been found to enhance cancer cell killing (Xu *et al*., 2014; Pozdeyev *et al*., 2015; Menegon *et al*., 2016). The HNA may have also stimulated apoptotic pathway by upregulating p53 and Bax proteins bring effective cell killing.

CONCLUSIONS

The exact mechanism underlying the antineoplastic activity of the HNA is unknown, however cytotoxic effect against HeLa cells was observed with MTT and clonogenic assays. The alleviation in the level of glutathione, GST, catalase and SOD might have increased the level of oxidative stress, leading to DNA damage and cell death. The HNA may also have suppressed the transcription of Nrf2, NF-κB and COX-II genes at molecular level and up regulated p53 and Bax leading to effective cell kill in the present study.

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Table 1: Alterations in the Glutathione contents of HeLa cells induced by different concentrations of *Helicia nilagirica* (HNA) and doxorubicin. The results were determined as µmol/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	2.64±0.20	1.56±0.02*	1.44±0.01*	1.41±0.03*	1.10±0.003*	0.93±0.09*	0.87±0.03*
6	2.53±0.24	1.69±0.04*	1.41±0.009*	0.99±0.01*	1.01±0.003*	0.93±0.003*	0.78±-0.02*
12	2.33±0.06	1.01±0.01*	0.94±0.04*	0.88±0.04*	0.73±0.006*	0.59±0.01*	0.57±0.008*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), **n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 2: Alterations in the GST activity of HeLa cells treated with different concentrations of *Helicia nilagirica* (HNA) and doxorubicin. The results were determined as unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	0.10±0.003	0.08±0.001*	0.05±0.002*	0.04±0.001*	0.08±0.001*	0.05±0.001*	0.02±0.001*
6	0.10±0.001	0.08±0.002*	0.04±0.003*	0.02±0.003*	0.07±0.001*	0.03±0.001*	0.02±0.003*
12	0.10±0.007	0.09±0.003	0.06±0.002*	0.04±0.007*	0.09±0.002	0.05±0.006*	0.04±0.003*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), , **n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 3: Alterations in the catalase activity of HeLa cells treated with different concentrations of *Helicia nilagirica* (HNA) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20
2	7.64±0.19	6.11±0.50*	5.16±0.33*	4.58±0.33*	6.19±0.23	5.35±0.19*	3.68±0.16*
6	7.26±0.50	5.35±0.50*	3.82±0.38*	2.86±0.31*	4.37±0.18*	4.01±0.33*	2.58±0.16*
12	6.59±0.16	5.02±0.43*	2.77±0.34*	2.29±0.33*	4.09±0.11*	2.69±0.20*	1.91±0.38*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), **, n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

Table 4: Alterations in the SOD activity of HeLa cells treated with different concentrations of *Helicia nilagirica* extract (HNA) and doxorubicin (DOX). The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

Post Treatment Time (h)	MEM	Treatment (µg/ml)					
		Helicia nilagirica (HNA)			Doxorubicin (DOX)		
		200	300	400	5	10	20

2	3.03±0.12	2.87±0.13	2.39±0.06*	1.54±0.07*	2.63±0.13	2.11±0.10*	1.47±0.10*
6	2.84±0.12	1.82±0.09*	1.34±0.06*	1.04±0.18*	1.45±0.06*	1.16±0.10*	0.79±0.06*
12	2.52±0.07	0.61±0.02*	0.36±0.02*	0.35±0.003*	0.54±0.006*	0.40±0.002*	0.33±0.006*

*p<0.05 when treatment groups are compared with concurrent control (MEM) group.
Standard error of the mean (SEM), , **n=5.**
No symbol= no significant difference.
Aqueous extract of Helicia nilagirica (HRA), Doxorubicin (DOX).

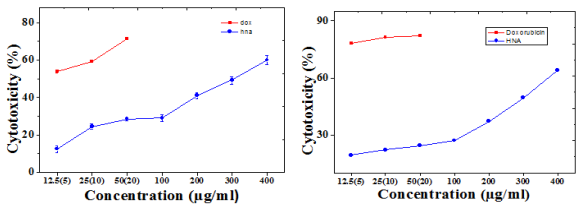

Fig. 1: Cytotoxic effect of different concentrations of aqueous extract of *Helicia nilagirica* in HeLa and V79 cell lines by conventional MTT assay. The results were determined as percentage (%) cytotoxicity and expressed as Mean ± SEM, n=5, p<0.05.

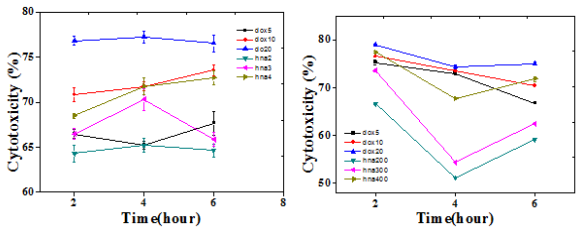

Fig 2: Cytotoxic effect at different exposure time of the aqueous extract of *Helicia nilagirica* and DOX in HeLa and V79 cell lines by MTT assay. The data are expressed as Mean ± SEM, n=5, p<0.05.

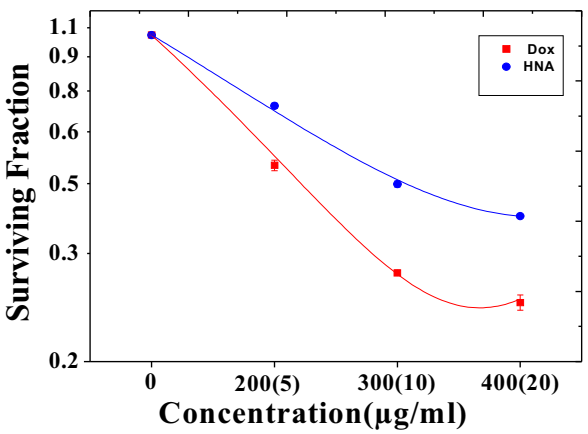

Fig 3: Effect of different concentrations of the aqueous extract of *Helicia nilagirica* (HNA) and Doxorubucin (DOX) treatment on the survival of HeLa cells.

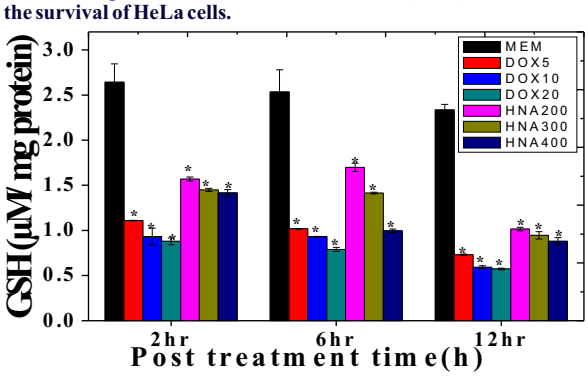

Fig 4: Alteration in the GSH content of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

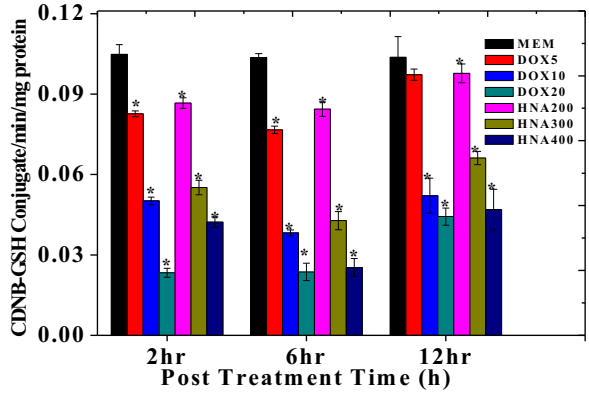

Fig 5: Alteration in the GST activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

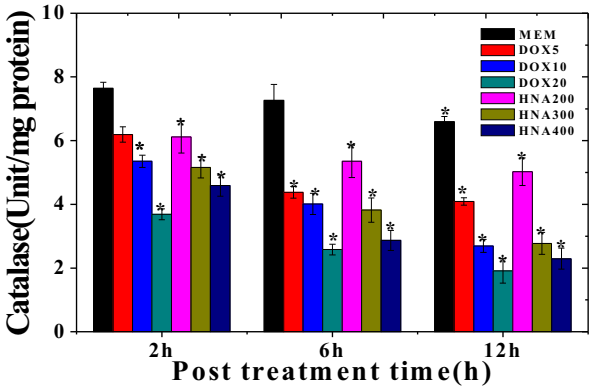

Fig 6: Alteration in the catalase activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

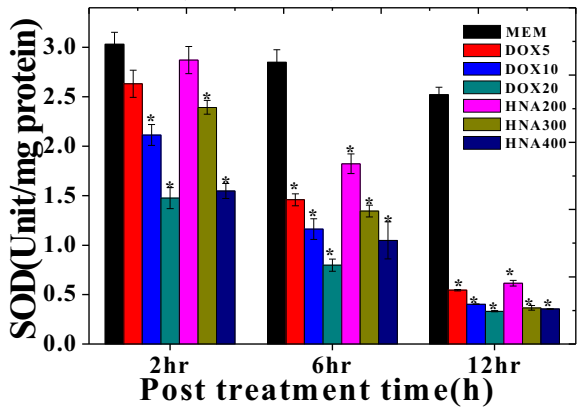

Fig 7: Alteration in the catalase activity of HeLa cells induced by different concentrations of *Helicia nilagirica* and doxorubicin. N=3. *P<0.05. MEM- Minimum essential media (Negative Control), HNA-Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

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