RESEARCH PUBLICATIONS


BOOK CHAPTER


SEMINARS PRESENTED

1. Presented a research paper in the” International Conference on Radiation biology” held on Nov 11-13, 2014 at NASC Complex, Dev Prakash Shastri Marg, Pusa, New Delhi, India.

2. Presented a research paper in the “Mizoram Science Congress 2016” held on 13th-14th Oct at Mizoram University, Aizawl.

3. Oral presentation in the National Seminar on “Biodiversity, Conservation and Utilization of Natural Resources with reference to Northeast India” held on 30th-31st March 2017, organized by Department of Botany at Mizoram University, Aizawl.

SEMINARS PARTICIPATED

1. Participated in “Five Days Workshop on Instrumentation in Science” from 6th-10th Aug 2012 held at Mizoram University sponsored by UGC.

2. Attended “One Day Seminar on Animal Welfare and Ethics” on 6th June 2013 at Hotel Regency, Aizawl organized by Department of Pharmacy, RIPANS in association with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

3. Participated in the “International Conference cum Exhibition on Drugs Discovery and Development from Natural Resources” organized by the Department of Pharmacy, RIPANS under the sponsorship of the Ministry of Health and Family Welfare, Govt. of India on 5th-6th Feb. 2014 at Art and culture Auditorium, Berawtlang, Aizawl.

4. Participated in the National conference on “Advances in Cancer Genomics” held on 3th -31st May 2014 organized by Mizoram State Cancer Institute and Department of Biotechnology
Mizoram University sponsored by Department of Biotechnology, New Delhi coordinated by Indian Institute of Technology, Guwahati.

5. Participated in the workshop on “Exploring the Cancer genomics” held during 22\textsuperscript{nd}-27\textsuperscript{th} Feb. 2016 organized by Department of Biotechnology, Mizoram University sponsored by State Biotech- Hub facility, Department of Biotechnology (DBT), New Delhi.

6. Attended workshop on “Mechanisms of Adaptation in the Temporal Environment” on 23\textsuperscript{rd} May 2017 at Mizoram University organized by Department of Zoology, Mizoram University, Aizawl.
EVALUATION OF THE CYTOTOXIC EFFECTS OF HELICIA NILAGIRICA BEDD IN VITRO

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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-transferease (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 is 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 (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. (Apocynaceae), 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 Deccne (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 Pasaltaka 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 (Sawmlana 2003). In Sikkim the fruits of H. nilagirica are used to treat cough and cold (Chauhan 2001). A recent study has indicated that methanol 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 Heliciinilagirica in vitro.

MATERIALS AND METHODS

Chemicals
Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 5,5’-dithio-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), ethylendiamine tetra-acetic acid (EDTA), bovine serum albumin (BSA), crystal violet were obtained from Sigma Chemical Co. (Bangalore, India). Sodium bicarbonate (NaCO₃), potassium chloride (KCl) and hydrogen peroxide (H₂O₂) were procured from SD Fine Chemicals, Mumbai, India, whereas sodium hydroxide (NaOH), sodium chloride (NaCl), disodium hydrogenphosphate (NaH₂PO₄), 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: Protaceae) was done by Botanical Survey of India, Shillong. The non-infected stem bark of *Helicia nilagirica* was collected from Sialuk, 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°C until further use. Only the aqueous extract of *Helicia nilagirica* (HNA) was used for evaluation of the anticancer activity.

Dissolution of drugs

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 usually grown in 25 cm² 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 sulphate at 37°C in an atmosphere of 5% CO₂, 37°C in an atmosphere of 5% CO₂ in humidified air in a CO₂ 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 10⁵ cells were seeded into 96 well plates in 100 µl MEM. The cells were incubated at 37°C in a CO₂ incubator in an atmosphere of 5% CO₂ in 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 CO₂ 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 performed to study the effect of treatment time on the cytotoxicity of HNA on the 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 NaHPO₄ 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).

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:-

\[
\text{GST activity} = \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{1000/9.6 \times \text{Vol of sample}}
\]

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 20°C) was initiated by adding 1 ml of 30 mM H₂O₂ 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.

**STATISTICAL ANALYSES**

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. AP 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 determination of anticancer activity was calculated according to Puck and Marcus (1955).

\[
\text{PE} = \frac{(\text{Number of colonies counted} \times 100)}{(\text{Number of cells seeded})}
\]

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 (P CI Analytics Pvt. Ltd., Mumbai, India).

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).
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 potentiative control drug doxorubicin also showed a similar pattern (Figure 1). The IC50 was also calculated and found to be 306.71 µg/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 6 h 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 4 h 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 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 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 6 h, 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. emodi, Taxus brevifolia, Ochrosia elliptica and Campotheca acuminata (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 of secondary 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 less toxic effects and higher efficacy 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. The more intense color indicates more viable cells (Mossman, 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 Aphananthisis polyostachya, Tinospora cordifolia, Alstonia scholaris, Consolida orientalis, Ferula assafoetida, Coronil avaria and P. pellucida 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, Centarea, Cyamum, Tanacetum vulgar and Fragopogon 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 Aphananthisis polyostachya , 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-glutamlycysteiny1 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-glutamlycysteiny1 synthetase. Glycine is then added by glutathione synthetase to form GSH. The presence of sulphhydril (SH) group of the cysteiny1 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 sulphhydril groups lead to an alteration in the calcium homeostasis that eventually results in the loss of cell viability which is indispensable 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 isozymes 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.
The authors are thankful to the Department of Biotechnology, and University Grants Commission Government of India, New Delhi for providing financial assistance to carry out this study.

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.

<table>
<thead>
<tr>
<th>Post Treatment Time (h)</th>
<th>Treatment (µg/ml)</th>
<th>Membrane</th>
<th>Helicia nilagrica (HNA)</th>
<th>Doxorubicin (DOX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.6±0.03</td>
<td>1.5±0.02</td>
<td>1.4±0.01*</td>
<td>1.4±0.01*</td>
</tr>
<tr>
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<td>1.6±0.02</td>
<td>1.5±0.01</td>
<td>1.4±0.01*</td>
<td>1.4±0.01*</td>
</tr>
<tr>
<td>12</td>
<td>1.6±0.01</td>
<td>1.5±0.00</td>
<td>1.4±0.00*</td>
<td>1.4±0.00*</td>
</tr>
</tbody>
</table>

*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 µmol/ mg protein and expressed as Mean ±SEM.

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<td>2</td>
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<td>1.5±0.001</td>
<td>1.4±0.001*</td>
<td>1.4±0.001*</td>
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<tr>
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<td>1.6±0.002</td>
<td>1.5±0.002</td>
<td>1.4±0.002*</td>
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<tr>
<td>12</td>
<td>1.6±0.007</td>
<td>1.5±0.007</td>
<td>1.4±0.007*</td>
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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. The results were determined as Unit/ mg protein and expressed as Mean ±SEM.

<table>
<thead>
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<th>Post Treatment Time (h)</th>
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<th>Helicia nilagrica (HNA)</th>
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<tbody>
<tr>
<td>2</td>
<td>1.6±0.001</td>
<td>1.5±0.001</td>
<td>1.4±0.001*</td>
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</tr>
<tr>
<td>6</td>
<td>1.6±0.002</td>
<td>1.5±0.002</td>
<td>1.4±0.002*</td>
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</tr>
<tr>
<td>12</td>
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<td>1.4±0.007*</td>
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*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 data 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 doxorubicin. N=3, *p<0.05. MEM- Minimum essential media (Negative Control), HNA- Aqueous extract of Helicia nilagirica (treatment), DOX- Doxorubicin (Positive Control).

Fig 3: Effect of different concentrations of the aqueous extract of Helicia nilagirica (HNA) and Doxorubincin (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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In vitro effect of tuibur (tobacco brew) on the viability of human blood lymphocytes

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The use of tobacco and its products are known to cause many illnesses including cancer. A smokeless tobacco locally manufactured called tuibur (tobacco brew) has been consumed by the Mizos from a very long time. In this experiment we aim to determine the cytotoxicity of tuibur by an in vitro study on tuibur-treated human peripheral blood lymphocytes. We have found that 24 h treatment of human lymphocytes with two grades of commercial tuibur and nicotine showed a concentration dependent decrease in cell viability. We, therefore, concluded that as the in vitro use of tuibur has an adverse effect on cell survival, its consumption might have potential side effects on the health of the users.

Key words: Cell viability, lymphocytes, tobacco, tuibur.

Introduction

Tobacco is linked with many diseases and has been known to contain more than eight thousand chemicals, out of which roughly 68 are probable carcinogens.1-3 Some of the common toxic chemicals include benzo[a]pyrene (B[a]P), N′-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N′-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosodimethylamine (NDMA), nitrite, cadmium, lead, arsenic, nickel, chromium, etc.4,5 The consumption of both smoking and smokeless tobacco is popular throughout the world and its detrimental effect could be observed from many medical records.

Besides its deleterious consequence upon the pulmonary system, it has been linked with many forms of cancer. In fact, many studies suggested that almost all known cancer could be linked to tobacco use.6,7 It would be safe to say that every nation throughout the globe has tobacco users in its population.8 The form of tobacco used may vary considerably. Some prefer smoking tobacco while others prefer smokeless tobacco, or both. But, it may be acceptable to say that more than half of the tobacco users used it in the form of smoking tobacco.9 The Mizo tribes living in the northeastern part of India use both smoke and smokeless tobacco.10 A form of smokeless tobacco locally called tuibur (tobacco brew) is used popularly and is commercially available in the
local market, generally in two grades, which largely depend on the amount of tobacco used in its production. The method of practice is the users of *tuibur* put the product in the mouth for roughly 5-10 minutes which is then spitted out. The duration is determined when the alkalinity of the *tuibur* is depleted.\(^{11}\)

In this experiment, we aimed to determine the effect of two grades of commercial *tuibur* on the viability of *tuibur*-treated human peripheral blood lymphocytes *in vitro*.

**Materials and Methods**

**Chemicals**

A small quantity of two grades of commercial *tuibur*, labelled as *tuibur*-A (special grade) and *tuibur*-B (ordinary grade), produced in a local industry were purchased from the market. Although there is no standard protocol, the manufacturers graded the *tuibur* depending on the quantity of tobacco used in its production. Pure nicotine (Cayman Chemical Company) and trypan blue (Sigma) were purchased from local supplier. RPMI-1640 media (HiMedia) was obtained from local supplier and prepared in the laboratory using standard protocol.

**Lymphocyte culture and treatment**

Lymphocyte culture were performed using the protocol described by Jagetia et al.\(^{12}\) Briefly, peripheral blood lymphocytes were collected by venipuncture in a heparinized vacutainer from a 27-year-old healthy male volunteer who has no known history of tobacco consumption. The collected blood was allowed to stand for roughly half an hour and the upper translucent layer containing lymphocytes was taken for culture. Approximately two million lymphocytes were cultured in different test tubes containing 2 ml RPMI-1640 culture media without the addition of any growth factor.

The tubes were separated into four groups (I, II, III & IV) and different volumes of *tuibur*-A and *tuibur*-B were added to group I & II (2.5, 5, 10, 20, 40, and 50 µl/ml) respectively. To group III, 2.5, 10, 20, 40, and 50 µg/ml of nicotine was added and this served as positive control. Group IV or blank acted as negative control and did not contain any chemical other than the cells and the media. All cultures were performed in triplicate. These tubes were incubated at 37°C for 24 h. After 24 h, the survival of the cells was checked by modified trypan blue exclusion assay.\(^{13}\) The number of living and dead cells were counted in a hemocytometer and the mean percentage of surviving cells was taken as viability.

**Statistical analysis**

All statistical analysis were performed using Microsoft Excel 2013 and OriginPro-8. Correla-

---

**Table 1** | Mean percentage of viable human peripheral blood lymphocytes for blank and treatment with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

<table>
<thead>
<tr>
<th>Concentration (µl/ml or µg/ml)</th>
<th>Tuibur-A (%)</th>
<th>Tuibur-B (%)</th>
<th>Nicotine (%)</th>
<th>Blank (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td>2.5</td>
<td>100.00±0.00</td>
<td>100.00±0.00</td>
<td>98.25±0.06</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>96.39±0.58</td>
<td>95.06±0.40</td>
<td>96.55±0.25</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>90.34±1.86</td>
<td>90.35±0.97</td>
<td>92.32±0.59</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>86.41±0.62</td>
<td>87.03±0.29</td>
<td>91.1±0.23</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>79.43±2.22</td>
<td>85.65±1.20</td>
<td>87.9±0.50</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>71.07±1.97</td>
<td>79.15±0.58</td>
<td>85.57±0.14</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>65.57±0.62</td>
<td>76.14±1.11</td>
<td>82.71±2.31</td>
<td>-</td>
</tr>
</tbody>
</table>
A correlation coefficient was performed to determine relationship between different treatment concentrations and viability within a group. Student’s t-test was employed to determine significant difference between the treatment groups.

**Results**

The pH of *tuibur*-A and *tuibur*-B were found to be 9.81 and 10.09 respectively. Table 1 and Figure 1 showed the mean percentage of viable cells for the different treatment groups. The negative control showed 100% viability while *tuibur*-A, *tuibur*-B and nicotine showed a concentration dependent viability. Lymphocytes treated with a maximum concentration of 50 µl/ml of *tuibur*-A and *tuibur*-B showed 65.57% and 76.14% viability respectively while a minimum concentration of 2.5 µl/ml of both the two *tuibur* grades resulted in 100% viability in both the groups. A maximum concentration of 50 µg/ml and a minimum concentration of 2.5 µg/ml of nicotine showed 82.71% and 98.25% viability respectively. A strong negative correlation was observed between cell viability and concentration of *tuibur*-A (-0.994), *tuibur*-B (-0.969) and nicotine.

### Table 2 | Student’s t-test between different treatment groups at 95% confidence interval.

<table>
<thead>
<tr>
<th>Student’s t-test between</th>
<th>p-value at 95% CI</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tuibur</em>-A &amp; <em>Tuibur</em>-B</td>
<td>0.60</td>
<td>No significant difference</td>
</tr>
<tr>
<td><em>Tuibur</em>-A &amp; Nicotine</td>
<td>0.27</td>
<td>No significant difference</td>
</tr>
<tr>
<td><em>Tuibur</em>-B &amp; Nicotine</td>
<td>0.45</td>
<td>No significant difference</td>
</tr>
<tr>
<td>Control &amp; <em>Tuibur</em>-A</td>
<td>≤0.00</td>
<td>Significant difference</td>
</tr>
<tr>
<td>Control &amp; <em>Tuibur</em>-B</td>
<td>≤0.00</td>
<td>Significant difference</td>
</tr>
<tr>
<td>Control &amp; Nicotine</td>
<td>≤0.00</td>
<td>Significant difference</td>
</tr>
</tbody>
</table>

Figure 1 | Graph showing mean percentage of viable human peripheral blood lymphocytes treated with different concentration of *tuibur*-A, *tuibur*-B and nicotine.
nicotine (0.979). This means higher the concentration of the chemicals, lower the viability and vice versa.

Statistical analysis by t-test at 95% CI (Table 2) between mean percentage of viable cells for blank and tuibur-A, blank and tuibur-B, blank and nicotine showed a significant difference (p-value=0.00). However, comparison of tuibur-A and tuibur-B (p-value=0.60), tuibur-A and nicotine (p-value=0.27), tuibur-B and nicotine (p-value=0.45) showed that there is no significant difference in mean percentage of viable cells between these groups.

**Discussion**

Tobacco is known to contain enormous amount of different chemicals, many of which have been reported to have carcinogenic and cytotoxic properties.\(^1\,\,14\) Most studies, if not all, reported the use of tobacco in any form only have negative impact on the physiological well-being of the users. There have been only a handful of literatures on the scientific investigation of tuibur. A preliminary report on the chemical composition of tuibur showed the presence of polycyclic aromatic hydrocarbons and carbonyl compounds in the tar phase.\(^11\)

An epidemiological study among the Mizos showed that tuibur users have a higher risk of developing gastric cancer and the combine use and frequency of smoking, betel, tuibur and sahdah were reported to have a significant influence on the risk of gastric cancer.\(^10\) Phukan et al.\(^15\) have also reported tuibur use as a risk factor for gastric cancer. Besides gastric cancer patients in Mizoram, tuibur consumers were found to have a variety of mtDNA D-loop region mutations and polymorphisms.\(^16\) Individuals with Arg/Pro genotype, GSTM1 null genotype and GSTT1 non-null genotype were also suggested to have a higher risk of gastric cancer if they have habits of using tuibur and smoking tobacco.\(^17\,\,18\)

The damaging effect of tobacco may be attributed to its vast array of chemical compositions. Heavy metals like cadmium and lead present in tobacco have also been found to cause glomerular dysfunction. Many of these effects may be because of nicotine’s ability to affect certain antioxidant enzymes like lipid peroxidase, superoxide dismutase, catalase, glutathione-s-transferase, glutathione reductase, etc.\(^3\) Cytological studies have reported nicotine to inhibited cell proliferation and decreased protein synthesis in a dose dependent manner in cultured periodontal ligament fibroblast,\(^19\) while it was also reported to stimulate endothelial cell DNA synthesis and proliferation at concentrations lower than \(<10^{-8}\) M. The cytotoxicity of nicotine was reported to be at a higher concentration, i.e. \(>10^{-6}\).\(^20\)

Onion bulbs treated with tuibur showed a reduced root growth, reduced mitotic index, formation of micronuclei, lagging chromosomes, and c-mitosis.\(^21\) A study on seven smokeless tobacco aqueous extracts showed a concentration-dependent effects on the growth and viability of oral bacteria cultured under anaerobic conditions.\(^22\) These effects may be a result of increase superoxide anion production, lipid peroxidation, DNA fragmentation and DNA ladders caused by the use of chewing tobaccos.\(^23\)

Our result showed concentration dependent cell viability for the tuibur and nicotine treatment groups while the untreated negative control group showed 100% viability. We are uncertain as to what chemical(s) in the tobacco brew would cause the cells to die. But from the nicotine treatment group, we may be able to say, although carefully, that the nicotine might contributed significantly in this result. However, one study suggested other biologically active compounds like NNN, NNK, etc., other than nicotine present in tobacco leave extract to be the source of cytotoxicity.\(^24\)

Another probable factor for the decrease in viability of the tuibur treatment groups would be the change in pH of the culture media. As we have shown in our result, the pH of both the two grades of tuibur are alkaline in nature, a slight rise in pH of the culture media was observed after the addition of both the tuibur (data not shown). This change in pH may be a factor that leads to decrease cell viability. In conclusion, our
result showed that 24 h treatment of human lymphocytes with *tuibur* and nicotine may have an adverse effect on their survival and hence these chemicals might have cytotoxic properties. Therefore, the consumption of *tuibur* might have potential side effects on the health of the users.

**Acknowledgement**

The authors would like to thank Mr. B. Sanga Ralte for his valuable suggestions in the statistical analysis.

**References**


The Phytochemical and Thin Layer Chromatography Profile of Ethnomedcinal Plant *Helicia Nilagirica* (Bedd)

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Abstract

The plants have provided valuable medicines in the form of secondary metabolites synthesized by them for various purposes. The present study deals with the phytochemical profiling of *Helicia nilagirica* using various phytochemical procedures and thin layer chromatography. The mature non-infected stem bark of *Helicia nilagirica* was collected, dried, powdered and subjected to sequential extraction with increasing polarity using petroleum ether, chloroform, ethanol and distilled water. The different extracts were cooled and evaporated to dryness with rotary evaporator. The phytochemical analyses were carried out on chloroform, ethanol and aqueous extracts. The chloroform extract revealed the presence of flavonoids, tannins, terpenoids, cardiac glycosides, whereas alkaloids, saponins and carbohydrates were completely absent. Similarly, the ethanol extract contained flavonoids, tannins, phenols and cardiac glycosides. The aqueous extract showed the presence of saponins, tannins, cardiac glycosides and carbohydrates. The TLC profile also showed the presence of different phytochemicals in the different extracts as indicated by different Rf values using various solvent systems.

Keywords: *Helicia nilagirica*; Phytochemical; Flavonoid; Thin layer chromatography

Introduction

Medicinal plants have been used as the main traditional herbal medicinal system amongst rural dwellers worldwide since antiquity and they are still in use for healthcare. The earliest written evidence of use of plants as medicine has been found around 5000 years before on a Sumerian clay slab from Nagpur which comprised of 12 recipes for drug preparation consisting over 125 plants including poppy, henbane and mandrake. The Rig Veda, the ancient knowledge book of Hindus dating between 3500 B.C. to1800 B.C. mentions several plant-based drugs for human healthcare and is the earliest systematic record in this regard. In 2500 BC the Chinese emperor has written a book for 365 drugs some of which have been used even today include *Rhei rhisoma*, camphor, *Theae folium*, *Podophyllum*, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra [1,2]. Over the years plants have been used as the main source of medicine especially in the developing countries and more than 80% of the world's population relies on traditional medicine for their primary healthcare needs [3].

Phytochemicals are natural bioactive chemical compounds produced by various plants to protect themselves from environmental hazards such as pollution,
stress, drought, UV exposure and pathogenic attack [4].

These compounds are known as secondary plant metabolites and include organic substances like alkaloids, carotenoids, glycosides, terpenoids, steroids, tannins, flavonoids, saponins, vitamins, mucilages, minerals, organic acids etc. [5-8]. These secondary metabolites are of great health benefits to humans. Some of the beneficial roles of phytochemicals include low toxicity, low cost, easy availability with an extensive range of therapeutic activities such as antioxidant, antimicrobial, hypoglycemic, antidiabetic, antimalarial, anticholinergic, antileprotic and antineoplastic. These phytochemicals also help in the modulation of detoxification of enzymes, stimulation of the immune system, decrease in platelet aggregation and modulation of hormone metabolism [9,10]. Even with a remarkable progress made in synthetic drugs in modern medicine, therapies using medicinal plants still make a major contribution to the pharmaceutical industry because of their safety, easy availability, and cost effectiveness. They also have synergistic effect with other biologically active ingredients due to the presence of beneficial minerals [11,12].

*Helicia nilagirica* Bedd. (Family: Proteaceae) locally known as Pasaltakaza, is a medium-sized tree, which grows up to a height of 12 meters. It is widely distributed in Sri Lanka, southern India, Burma (Myanmar), Indochina, Japan, Taiwan, and Thailand. It is also found scattered in lowland to montane rain forests, up to an altitude of 500 - 3,350 meters. Some species are found in habitats along the streams whereas other species are found on hilltops or ridges [13]. It has been used as folk medicine since time immemorial in Mizoram, India by the Mizo people. The decoction prepared by boiling the leaves or bark is used to treat various stomach ailments including peptic ulcers, indigestion, mouth ulcer, urinary tract infection and gynecological disorders. The stem bark juice is applied to reduce muscular swelling and treat cuts and wounds. The stem bark is also used in scabies and other skin diseases [14]. *H. nilagirica* has been shown to possess anti-inflammatory and antioxidant properties recently [15,16]. *H. nilagirica* has been found to be cytotoxic in cultured HeLa cells indicating its anticancer potential in treating neoplastic disorders [17]. The fruits of *H. nilagirica* have been used as a medicine to cure cough and cold in Sikkim [18]. *Helicia nilagirica* has been used in traditional medicine by the people of Mizoram and other states for healthcare and the information has been collected by meeting the elders who practice ethnomedicine. There are only very few reports about phytochemical analysis of *Helicia nilagirica* and these studies were done on methanol extract and not all parameters were evaluated [14,19]. The ethnomedicinal use and scanty information on the phytochemical analysis of *Helicia nilagirica* stimulated us to investigate its phytochemical composition.

**Materials and Methods**

**Chemicals and reagents**

Potassium iodide, bismuth nitrate, sulphuric acid ferric chloride, hydrochloric acid, aluminium chloride, ammonium hydroxide, glacial acetic acid, chloroform, ethanol, methanol, n-butanol, ethyl acetate, sodium chloride, sulphuric acid, olive oil, and Whatman filter paper were procured from Sd fine Chemical Ltd., Mumbai, India. The TLC plates were commercially procured from Merck India, Mumbai.

**Collection and extraction**

The mature and non-infected stem bark of *Helicia nilagirica* Bedd. (Family: Proteaceae) locally known as Pasaltakaza was collected from Sialsuk, Aizawl District of Mizoram, India during the dry season. Identity of *Helicia nilagirica* Bedd was authenticated by the Botanical Survey of India, Shillong. The cleaned and non-infected bark was spread into stainless steel trays and allowed to dry in the shade at room temperature in dark in clean and hygienic conditions to avoid entry of insects, animals, fungus, and extraneous terrestrial materials. The exhaust and free air circulation was allowed. The dried bark was powdered in a grinder at room temperature. A sample of 100 g of stem bark powder was extracted sequentially in chloroform, ethanol and water in a Soxhlet apparatus. Each extract was concentrated to dryness under reduced pressure and stored at -80°C until further use.

**Phytochemical Screening**

The different extracts of *Helicia nilagirica* were analyzed for the presence of various phytochemicals using standard procedures as described below.

**Alkaloids**

The presence of alkaloids was determined by mixing 0.1g of the extract with 0.5 ml of Mayer’s reagent and Dragendorff’s reagent. The formation of a creamy (Mayer’s reagent) or reddish-brown precipitate (Dragendorff’s reagent) indicated the presence of alkaloids [20,21].

**Tannins**

About 0.5 g of dried powdered samples was boiled in 20 ml of water in a test tube and filtered. A few drops of 0.1% ferric chloride was added to the filtrate. The formation of...
brownish green or a blue-black colour indicated the presence of tannins [20,21].

**Phlobatannins**

The aqueous extract of *Helicia nilagirica* was boiled with 1% aqueous hydrochloric acid and deposition of a red precipitate indicated the presence of phlobatannins [20,21].

**Saponins**

About 2 g of the powdered sample was boiled with 20 ml of distilled water in a water bath for 10 minutes and filtered while hot and cooled before conducting the following tests:

**Frothing:** 3 ml of filtrate was diluted up to 10 ml with distilled water and shaken vigorously for 2 minutes. The formation of a fairly stable froth indicated the presence of saponins in the filtrate.

**Emulsification:** 3 drops of olive oil was added to the solution obtained by diluting 3 ml filtrate to 10 ml distilled water and shaken vigorously for a few minutes. The formation of a fairly stable emulsion indicated the presence of saponins [20,21].

**Flavonoids**

Three different methods were used to test the presence of flavonoids in all the extracts [20,21,22]. 5 ml of dilute ammonia solution was added to a portion of the aqueous filtrate of each sample followed by the addition of concentrated $\text{H}_2\text{SO}_4$. Appearance of a yellow colour (disappeared on standing) in each extract indicated the presence of flavonoids.  

a) A few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow colour indicated the presence of flavonoids.  

b) A portion of the sample was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colour indicated the presence of flavonoids.

**Terpenoids**

The presence of terpenoids was detected as follows:

**Salkowski test:** Five ml of each extract was mixed with 2 ml of chloroform, with a careful overlaying of 3 ml concentrated sulphuric acid. The formation of a reddish-brown precipitate at the interface indicated the presence of terpenoids [23].

**Cardiac glycosides (Keller-Killani test)**

The cardiac glycosides were determined by adding 5 ml of each extract in 2 ml of glacial acetic acid containing one drop of ferric chloride solution that was underlayed with 1ml of concentrated sulphuric acid. The appearance of brown ring at the interface indicated the presence of deoxysugar, which is a characteristic of cardenolides [20,21].

**Carbohydrates**

The presence of carbohydrates in each extract of *Helicia nilagirica* was detected by the Benedict’s test, where the filtrates of the extracts were treated with Benedict's reagent and heated gently. The appearance of orange red precipitate indicated the presence of reducing sugars.

**Quantitative Determination of The Phytochemicals**

The quantitative determination of saponins and flavonoids was also carried out as described below.

**Determination of Saponins**

20 g of *Helicia nilagirica* powder was weighed in a conical flask and 100 ml of 20 % aqueous ethanol was added. The sample was heated over a hot water bath for 4 h with continuous stirring at 55 °C. The mixture was filtered and the residue was re-extracted with another 200 ml of 20 % ethanol. The combined extracts were reduced to 40 ml in a water bath at 90 °C. The concentrate was transferred into a 250 ml separating funnel with the addition of 20 ml of diethyl ether and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight and saponin contents were calculated as percentage [24].

**Determination of Flavonoids**

Ten g of the bark powder of *Helicia nilagirica* was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through Whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated to dryness over a water bath and weighed [25].
Determination of Moisture Content

Determination of the amount of volatile matter (i.e., water drying off from the drug) in the *Helicia nilagirica* is a measure of loss after drying of substances appearing to contain water as the only volatile constituent. The powdered bark of *Helicia nilagirica* was accurately weighed, placed (without preliminary drying) in a tared evaporating dish, dried at 105°C for 5 hours, and weighed again. The percentage moisture content was calculated with reference to the initial weight. The moisture content was calculated using the following formula:-

\[
\text{Moisture content} = \frac{\text{Pw} - \text{Fw}}{\text{W}} \times 100
\]

Where Pw = Preweighed sample
Fw = Final weight of the dried sample
W = Total weight of the sample

Ash values

The ash values including total and acid insoluble ash were determined to estimate the total amount of the inorganic salts present in the drug. The ash contents remained after ignition of plant material was determined by two different methods to measure total and acid insoluble ash contents.

Total Ash Contents

The method measures the total amount of material remaining after ignition including both ‘physiological ash’, derived from the plant tissue itself, and ‘non-physiological ash’ which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

**Procedure:** Two grams of ground air-dried material of *Helicia nilagirica* was accurately weighed in a previously ignited and tared crucible. The material was spread as an even layer and ignited by gradually increasing the temperature up to 500-600°C until it became white, indicating the absence of carbon. The crucible was cooled and weighed. The percentage of total ash content was calculated according to the following formula. Total ash content = \( \frac{\text{Pw} - \text{Fw}}{\text{W}} \times 100 \)

Where Pw = Preweighed crucible
Fw = Final weight of the crucible containing ash
W = Total weight of powdered plant material

Extractive Values

These are used to determine the amount of the matter which is soluble in the solvents used including alcohol and water. The percentage of alcohol and water-soluble extractives were calculated and used as standards.

**Determination of Alcohol-Soluble Extractive**

**Procedure:** Five grams of air dried coarsely powdered material was macerated in 100 ml of alcohol in a closed conical flask for twenty-four hours, with frequent shaking during first six hours and allowed to stand for next eighteen hours thereafter it was filtered rapidly with caution to avoid loss of solvent. The 25 ml of the filtrate was evaporated to dryness at 105°C in a tared flat-bottomed shallow dish and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air-dried material.

**Determination of Water-Soluble Extractive**

**Procedure:** Five grams of coarsely powdered air-dried material was macerated in 100 ml of chloroform-water (0.1%) in a closed flask for 24 h, shaken frequently until six hours and allowed to stand for another eighteen hours. Thereafter it was filtered rapidly, with precautions to avoid loss of solvent by evaporation. The 25 ml of the filtrate was evaporated to dryness at 105°C in a tared flat-bottomed shallow dish and weighed. The percentage of water soluble extractive was calculated with reference to the air-dried material. All the tests were done in triplicate.

**TLC Analysis**

TLC is a simple and rapid technique that is able to determine the number of components present in solution and helps in finding a suitable solvent for separating the components by column chromatography as well as for monitoring reactions’ progress. The chloroform ethanol and aqueous extracts of *H. nilagirica* were spotted on to a number of TLC plates (Merck India, Mumbai) in 1 mm diameter above the bottom of the plates and placed into different mobile phases. The extracts were allowed to move on the adsorbent (Stationary) phase according to the solvent system used. Several combinations of solvents of increasing polarity were evaluated as mobile phase for TLC run to determine the number of compounds present in different extracts of *Helicia nilagirica*. The different solvent systems were used as mobile phase for TLC, which consisted of chloroform: methanol (9:1, 8:2), pure chloroform, chloroform: ethyl acetate (1:1) and methanol: hydrochloric acid (9:1) solvent combinations. The resultant spots were observed under visible and ultra-violet light at 254 nm and 365 nm. The measure of the distance of a compound travelled is considered as the...
retention factor ($F_r$) value which was calculated using the following formula:

$$F_r = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

**Results**

The results of phytochemical analyses and TLC profiling of *Helicia nilagirica* are presented in Table 1-6 and Figures 1-3.

Figure 1: TLC profile of different extracts of *H. nilagirica* in different solvent systems observed under normal light to detect various phytochemicals present in the extracts. (aqueous, chloroform and ethanol). Lanes in each TLC plate: Left- aqueous; middle- chloroform and right-ethanol.

Figure 2: TLC profile of *H. nilagirica* with different solvent systems observed under UV 365 nm to detect phytochemicals present in the different extracts. Lanes in each TLC plate: Left- aqueous; middle- chloroform and right-ethanol.
Phytochemical analysis

The phytochemical screenings of chloroform extract of *Helicia nilagirica* showed the presence of flavonoids, tannins, terpenoids, diterpenes, cardiac glycosides, whereas the alkaloids, saponins and carbohydrates were conspicuous by their absence (Table 1). The phytochemical analysis of ethanol stem bark extract of *Helicia nilagirica* revealed that it contained only flavonoids, phenols, tannins and cardiac glycosides. The other phytochemicals like, alkaloids, saponins, terpenoids and carbohydrates, flavonoids, phenol and terpenoids were not detected (Table 1). In aqueous extract tannins, saponins, cardiac glycosides and carbohydrates were detected, whereas all other phytochemicals could not be detected and the phlobatannins were absent in all extracts (Table 1).

<table>
<thead>
<tr>
<th>Tests</th>
<th>Chloroform extract</th>
<th>Ethanol extract</th>
<th>Water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diterpenes</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Saponins</td>
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<td>-</td>
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<td>Cardiac glycosides</td>
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<tr>
<td>Carbohydrates</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1: Results of the Phytochemical analysis of *Helicia nilagirica*.

Quantitative determination of phytochemicals

The quantitative determination of the chemical constituents showed that *Helicia nilagirica* contained 9.26% and 0.26% flavonoids and saponins, respectively (Table 2).
Table 2: Quantitative determination of the chemical constituent of *Helicia nilagirica*.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Flavonoid</th>
<th>Saponin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helicia nilagirica</em></td>
<td>Quantity</td>
<td>Output</td>
</tr>
<tr>
<td>10g</td>
<td>0.9256g</td>
<td>9.256</td>
</tr>
</tbody>
</table>

**Determination of moisture content**

The drying of 500 g of *Helicia nilagirica* bark yielded 296 g of dried bark, and this reduction in weight was due to 40.6% loss in its water contents. The analysis of dried bark of *Helicia nilagirica* showed presence of 25.63% moisture (Table 3).

<table>
<thead>
<tr>
<th>Weight before drying (kg)</th>
<th>Weight after drying (kg)</th>
<th>Loss after drying (%)</th>
<th>Moisture content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.296</td>
<td>40.6</td>
<td>25.63</td>
</tr>
</tbody>
</table>

Table 3: Weight loss on drying fresh bark of *Helicia nilagirica*.

**Determination of total ash content**

The ash content of the crude bark powder was found to be 3.24% (Table 4).

<table>
<thead>
<tr>
<th>Total ash (%)</th>
<th>Ethanol-soluble extractive (%)</th>
<th>Water-soluble extractive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.24</td>
<td>3.4</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 4: Physicochemical parameters of dried bark powder of *Helicia nilagirica*.

**Determination of extractive values**

The *Helicia nilagirica* bark was found to contain 3.4% ethanol-soluble and 8% water-soluble extractives (Table 4).

**Extract yield:** The extraction of *Helicia nilagirica* stem bark yielded 2%, 4% and 6% chloroform, ethanol and water extracts (Table 5).

<table>
<thead>
<tr>
<th>Dried powder</th>
<th>Chloroform extract (%)</th>
<th>Ethanol extract (%)</th>
<th>Water extract (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100g</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 5: Yield of various extracts of *Helicia nilagirica*.

**TLC Analysis**

The evaluation of chloroform and ethanol extracts of *Helicia nilagirica* showed the presence of different components as indicated by a varying number of spots and colours on a TLC plates using UV visualization method (Table 6).

<table>
<thead>
<tr>
<th>Extract</th>
<th>Solvent</th>
<th>Day light</th>
<th>R&lt;sub&gt;v&lt;/sub&gt; value</th>
<th>UV 254 nm</th>
<th>R&lt;sub&gt;v&lt;/sub&gt; value</th>
<th>UV 365 nm</th>
<th>R&lt;sub&gt;v&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>CHCl₂</td>
<td>Streak</td>
<td>-</td>
<td>Two spots</td>
<td>0.92, 0.53</td>
<td>5 spots</td>
<td>0.92, 0.86, 0.57, 0.5 &amp; 0.42</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH₂OH·H₂O</td>
<td>Streak</td>
<td>-</td>
<td>3 spots</td>
<td>0.57, 0.28 &amp; 0.09</td>
<td>Not clear</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous</td>
<td>CH₂OH·H₂O</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>CHCl₂</td>
<td>One spot</td>
<td>0.94</td>
<td>1 spot</td>
<td>0.94</td>
<td>3 spots</td>
<td>0.94, 0.88 &amp; 0.84</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH₂OH·H₂O</td>
<td>Streak</td>
<td>-</td>
<td>3 spots</td>
<td>0.90, 0.82 &amp; 0.5</td>
<td>1 spot</td>
<td>(bluish) 0.38</td>
</tr>
<tr>
<td>Aqueous</td>
<td>CH₂OH·H₂O</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>CH₂OH</td>
<td>Not visible</td>
<td>-</td>
<td>1 spot</td>
<td>0.94</td>
<td>3 spots</td>
<td>(1 bluish, 2 red)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH₂OH</td>
<td>Not visible</td>
<td>-</td>
<td>Not clear</td>
<td>-</td>
<td>1 spot</td>
<td>(bluish) 0.11</td>
</tr>
<tr>
<td>Aqueous</td>
<td>CH₂OH</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>CH₂CO₂H</td>
<td>One spot</td>
<td>0.9</td>
<td>3 spots</td>
<td>0.90, 0.69 &amp; 0.48</td>
<td>4 spots</td>
<td>0.90, 0.86 &amp; 0.84</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH₂CO₂H</td>
<td>Streak</td>
<td>-</td>
<td>3 spots</td>
<td>0.69, 0.42 &amp; 0.23</td>
<td>Not visible</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous</td>
<td>CH₂CO₂H</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
<td>Not visible</td>
<td>-</td>
</tr>
</tbody>
</table>
Discussion

Plants synthesize several phytochemicals, which have played an important role in the development of new therapeutic agents. The preliminary qualitative phytochemical analysis of the bark of *Helicia nilagirica* revealed the presence of phenol, flavonoid, tannins, saponins, cardiac glycosides and carbohydrates. These phytochemicals synthesized by plants are essential for the growth, pathogen attack, pollination, defence and other activities of plants [26,27]. However, at the same time these phytochemicals are of great use for humans as a source of drugs and other healthcare agents [28-30].

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure which are ubiquitously present in plants. Approximately, more than 10000 flavonoids have been identified [31,32]. They can be divided into a variety of classes such as flavones (e.g., apigenin, and luteolin), flavanols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others [33,34]. They are the hydroxylated phenolic substances synthesized by plants in response to microbial infection [35]. Flavanols are the most abundant flavonoids in foods and they are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [36]. Flavonoids have been consumed by humans since the advent of human life on earth. They have extensive biological properties that promote human health and help to reduce the risk of diseases and they are known to possess antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anticancer and antiviral properties [37-41]. The *Helicia nilagirica* have shown the presence of flavonoids like other plants. These results are in conformation with earlier studies, where the presence of flavonoids has been reported in the methanol extract of *H. nilagirica* [15]. The flavonoids have also been detected in *H. nilagirica* recently [19]. Earlier, flavonoids were detected in *Croton caudatus* and *Oxylium indicum* and several other plants from our laboratory [28,29].

The presence of tannins in the *Helicia nilagirica* is in conformation with other studies where various plants have been reported to contain tannins [28,29]. Tannins are polyphenols which occur widely in vascular plants particularly associated with woody tissues. They are water soluble and have molecular weights ranging between 500 and 3000 Daltons. Based on the chemical structures, tannins are divided into two groups: hydrolysable, and condensed. The hydrolysable tannins consist of gallic acid esters, and elagic acid glycosides [41]. They possess an amazing astringent property, which is mainly related to their drug applications. They are known to be antimicrobial, antifungal, anthelmintic, antiviral, antiulcer. They are known to hasten the healing of wounds and alleviate inflammation in mucous membranes [42-44]. They exert internal anti-diarrheal and antiseptic effects by waterproofing the outer layers of more exposed mucous membranes. Tannins are also haemostatic, and can serve as an antidote in poisoning cases [45]. In the process of healing wounds, burns and inflammations, tannins help by forming a protective layer (tannin-protein/tannin-polysaccharide complex), over injured epithelial tissues permitting the healing process below to occur naturally. Studies show that many tannins act as radical scavengers, intercepting active free radicals [41]. Various degenerative diseases such cancer, multiple sclerosis, atherosclerosis and aging process itself are associated with high concentrations of intercellular free radicals and tannins are useful in these conditions.

Terpenoids are synthesized from five carbon isoprene units mainly isopentenyl pyrophosphate and its isomerdimethylallyl pyrophosphate by the enzyme terpene synthases. They are classified according to whether they contain two (C₁₀), three (C₁₃), four (C₂₀), six (C₃₀) or eight (C₄₀) isoprene units. They range from the essential oil components, the volatile mono-and sesquiterpenes (C₁₀ and C₁₃) through the less volatile diterpenes (C₂₀) to the involatile triterpenoids and steroids (C₃₀) and carotenoid pigments (C₄₀). Each of these various classes play a significant role in plant growth, metabolism or ecology [20]. Approximately 40,000 terpenes have been identified and the possible functions of majority of these molecules are not known [46]. Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer. They possess antimicrobial, antifungal, antiparasitic, antiviral, anti-allergic, antispasmodic, anti-malarial, antihyperglycemic, anti-

<table>
<thead>
<tr>
<th>Extract</th>
<th>Tannins</th>
<th>Flavonoids</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>0.86</td>
<td>0.84</td>
<td>2 spots (1 reddish, 1 bluish)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.82</td>
<td>0.84</td>
<td>Not clear</td>
</tr>
<tr>
<td>Aqueous</td>
<td>Not visible</td>
<td>Not visible</td>
<td>Not clear</td>
</tr>
</tbody>
</table>

Table 6: TLC profile of the different extracts of *Helicia nilagirica* on pre-coated aluminium TLC plates.
inflammatory and immunomodulatory properties [47-51]. Terpenoids can also be used as protective substances in storing agriculture products as they are known to have insecticidal properties as well [52].

Saponins are naturally occurring structurally and functionally diverse phytochemicals that are widely distributed among seventy families of plants. They are glycosides of both triterpenes and sterols [53]. Due to the presence of both the hydrophobic aglycone backbone and hydrophilic sugar molecules the saponins are highly amphipathic and possess foaming and emulsifying properties. They play an important role in plant ecology and they are also exploited for a wide range of commercial applications in the food, cosmetic and pharmaceutical sectors [54,55]. These molecules are potent membrane permeabilizing agents with immunostimulatory, hypocholesterolemic, anti-carcinogenic, anti-inflammatory, anti-microbial, anti-protozoan, molluscidal and have anti-oxidant properties [56,57]. Saponins also act as antitumor agents by inhibiting tumor cell growth and inducing apoptosis [42]. Terpenoids and saponins were detected in Helicia nilagirica like other plants. An earlier study has also reported the presence of saponins in Helicia nilagirica [19]. Likewise, several other plants have been found to synthesize terpenoids and saponins [28,29].

Cardiac glycosides composed of two structural features: The sugar (glycoside) and the non-sugar (aglycone-steroid) moieties and they act on the contractile action of the cardiac muscle. These compounds have long been used for the treatment of cardiac arrhythmias and congestive heart failure due to their capability to increase the contractile force [58]. Digitalis is the most commonly used cardiac glycoside, which directly inhibits the proliferation of androgen dependent and androgen independent prostate cancer cell lines by initiating apoptosis and increasing intracellular Ca$$^{2+}$$ [59]. Cardiac glycosides have been reported to inhibit the four genes that are over expressed in prostate cancer cells including the inhibitors of apoptosis and transcription factors [59]. Cardiac glycosides have been reported to act as active anticancer agents [60,61]. They are also reported to have antiviral properties against human cytomegalovirus [62]. The Helicia nilagirica has been found to contain cardiac glycosides. Similarly, the glycosides have been detected in the methanol extract of stem bark of H. nilagirica [15]. The other plants have been reported to contain the cardiac glycosides [28,29]. The presence of these phytochemicals may have been responsible for its anticancer activity in our earlier study [17].

Conclusions

The phytochemical analyses have revealed the presence of different phytochemicals including, phenols, flavonoids, tannins, terpenoids, saponins, cardiac glycosides in the different extracts. However, alkaloids and phlobatannins were not present in this plant. The presence of various phytochemicals affirms its ethnomedicinal use in Mizoram.

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Conflict of interest statement

The authors do not have any Conflict of interest statement to declare.

References


