In vitro cytotoxicity of *Bryonia laciniosa* (Linn.) Naud. on human cancer cell lines

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Screening and isolation of active components from the herbs possessing anticancer potential appears to be a promising way of discovering novel chemotherapeutic compounds. A traditional medicinal herb *Bryonia laciniosa* (Linn.) Naud. is an important source of biologically active compounds used in Homoeopathy and has potential for further exploratory medicinal use, especially for its anticancer properties. In the present study water, methanol and chloroform extracts of *B. laciniosa* leaves were tested on human cancer and normal cell lines using three *in vitro* cytotoxicity assays, cell viability, SRB and clonogenic potential. The effect was compared with that of standard anticancer drugs doxorubicin and vincristine. Activation of caspase-8 and caspase-3 enzymes were assessed to evaluate the effect of extract on induction of apoptosis in cells. Of the different extracts, the aqueous extract demonstrated maximum cytotoxicity to cancer cells. The IC\textsubscript{50} value was estimated to be 18 µg/mL. Much higher concentration (85 µg/mL) of the extract was required to produce same effect on the normal cells. Nearly all cancer cells could be killed by the leaf extracts of *Bryonia in vitro*, whereas small fraction of cells from cancer cell lines showed resistance to doxorubicin even at concentration much higher than IC\textsubscript{50}. Results of caspase assay demonstrated activation of both caspase-8 and caspase-3 enzymes indicating induction of apoptosis in *Bryonia* leaf extract treated cells. The results thus show that aqueous extract of *B. laciniosa* leaves possess cytotoxicity to cancer cells and are able to kill all cancer cells without leaving residual population. The extract also shows a better ability to discriminate between cancer cells and normal cells as compared with standard drug using three *in vitro* assays.

**Keywords:** *Bryonia laciniosa*, Human cancer, Anticancer, Cytotoxicity, *In vitro.*

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**Introduction**
Recent investigations on medicinal evaluation of plants have shown greater interest in one of the traditionally used Indian medicinal plant, *Bryonia laciniosa* (Linn.) Naud. (Family-Cucurbitaceae). The name *Bryonia* meaning ‘to sprout’ in Greek, refers to vigorous growth of herbaceous stems that are produced annually from large perennial roots. The plant is valued as medicinal and ornamental plant. It is native to Europe, Mediterranean region and Central Asia\textsuperscript{1}. *Bryonia* genus belongs to a family of herbaceous vines climbing by means of tendrils Plate 1. The species of this genus are characterized by palmate 3-7 lobed membranous and glabrous leaves, creamy white flowers in axillary fascicles, spherical and smooth bright red colored berries and obovate, minutely scrobiculate creamy white colored seeds\textsuperscript{2}.

*Bryonia* was proved as a remedy by the German founder of Homoeopathy, Dr. Samuel Hahnemann in 1834\textsuperscript{3}. The evidences of use of *Bryonia* as a medicinal plant in India dates back even before that. It is an important constituent of formulation of anti-inflammatory drug in Homoeopathy. As a folk medicine, the plant is used in treatment of gastrointestinal, respiratory, rheumatic and metabolic disorders, as well as in liver and infectious diseases\textsuperscript{4,5}. *Bryonia* is known to contain cucurbitacins, which give it a strong toxic effect. The plant is traditionally known to show antitumor effect. *B. laciniosa* in particular is used for treatment of cancer among the tribal populations of South India\textsuperscript{6}.

Recent investigations on *B. laciniosa* have shown good activity of petroleum ether, chloroform and ethyl acetate extracts of the plant against growth and multiplication of brine shrimp\textsuperscript{7}. Antioxidant and antitumor role of methanol extracts of leaves have been demonstrated in animal models\textsuperscript{8}. An analgesic and antipyretic activity in animal models has also been exhibited by methanol extracts of its leaves\textsuperscript{8}. The goniothalamin isolated from whole plant has

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shown substantial antimicrobial and antifungal activity against a wide range of Gram negative bacteria and fungi. Significant anti-inflammatory activity of chloroform extract of plant has also been reported in experimental animal models of acute and chronic inflammation by Gupta et al. Phytochemical studies have resulted in isolation of compounds goniothalamin, bryonin, punicic acid and lipids from the plant.

The investigations and traditional uses cited above suggest that this plant could be a promising source of anticancer chemotherapeutic agent. A comprehensive study on plant appears important to explore it for more defined medicinal uses. In the present study we have investigated cytotoxic activity of leaves extracts using three in vitro assays, viz. cell viability, SRB and clonogenic employing human cancer and normal cell lines. The effect of Bryonia leaf extract on induction of apoptosis in cell lines has also been assessed in this study.

Material and Methods

Plant material
The plant was collected in the months of July-Sept from western Ghat region of Maharashtra state in India. And it was taxonomically identified and confirmed by the Botanical Survey of India, western Circle, Pune and a voucher specimen no. ALMOD1 was retained in our laboratory for future reference.

Preparation of extracts
Five grams powder of surface sterilized and shed dried leaves was dissolved in 50 mL of deionized water, methanol and chloroform, respectively. The solutions were kept in shaking incubator at 25°C, 500 rpm for 48 h. The solutions were filtered and dried under reduced pressure. The dry extracts were weighed and dissolved in phosphate buffered saline (PBS) (water extract) and 10% dimethylsulfoxide (DMSO) (chloroform and methanol extracts) to prepare 10 mg/mL stock solution of each. Sterile conditions were maintained throughout the experiment.

Cell lines
Two cancer cell lines Mcf-7 (human breast adenocarcinoma), SiHa (human squamous cell carcinoma; cervix) and one non cancer cell line Vero (monkey kidney cell line) were obtained from National Animal Cell Repository at National Center for Cell Science, Pune. Vero cells were used as representative of normal cells in this study. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with Earle’s salts supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin and maintained at 37°C in humidified 5% CO₂ atmosphere.

Standard drugs
Two anticancer drugs, viz. doxorubicin hydrochloride and vincristine sulphate (VHB Medisciences Ltd., India) were used in the present study as positive control. The drugs were used within 15 days of purchase and were stored at prescribed temperature during the period of study. The dilutions of these drugs were made in PBS.

Cell viability assay for in vitro cytotoxicity assessment
Replicate cultures of the three cell lines, Mcf-7, SiHa and Vero were prepared by seeding 5 × 10⁴ cells in 3 cm size petri plates. Plates were incubated in
humidified 5% CO₂ atmosphere at 37°C. After 24 h, final concentrations ranging from 1 µg to 100 µg (1 µg, 10 µg, 25 µg, 50 µg, 100 µg/mL) of three leaf extracts were inoculated in duplicates. Equal amount of plain PBS was added to plates which served as control. As chloroform and methanol extracts were prepared in 10% DMSO, cytotoxic effect of 10% DMSO was tested by inoculating its dilutions on three cell lines. The two anticancer drugs doxorubicin hydrochloride and vincristine sulphate were used at the final concentrations 0.1 µg, 0.25 µg, 0.5 µg, 0.75 µg, 1 µg, 5 µg/mL, respectively. Plates were incubated in 5% humidified CO₂ atmosphere for next 48 h. At the end of exposure period, cells were trypsinized in 1 mL of trypsin-EDTA solution and counted for number of viable and nonviable cells by staining with trypan blue dye. IC₅₀ value of the drug was defined as the concentration of the drug that killed 50% of cells in comparison with the untreated cultures.

**Sulphorhodamine B (SRB) assay**

The SRB assay was performed as described by Wattanapiromsakul et al. Briefly, 0.2 mL of cell suspension containing 5 × 10⁵ cells were seeded per well of 96 well microtitration plate and incubated at 37°C for 24 h in 5% CO₂ atmosphere. The water, methanol and chloroform extracts of *B. laciniosa* leaves and the two reference drugs, vincristine sulphate and doxorubicin hydrochloride were then added to the microtest plates in triplicates at the same concentrations as that in cell viability assay. The plates were incubated for 48 h. After drug exposure, cells were fixed by adding 100 µl of ice cold 40% tri-chloro acetic acid (TCA) to each well. Cells were incubated at 4°C for 1 h and then washed with cold water. 50 µl of SRB stain (Sigma, 0.4%) was added to each well and left in contact with the cells for 30 min. The plates were rinsed with 1% acetic acid and 0.1 mL of 10 mM trizma base pH 10.5 (Sigma) was added to each well to solubalize the dye. Absorbance (OD) was read on plate reader (Biorad) at 405 nm wavelength on ELISA plate reader. The absorbance of the assay mixture was read at 405 nm wavelength on ELISA plate reader.

**Statistical analysis**

Regression analysis was used for determining IC₅₀ values. The paired t-test using SSPS 10.0 was used to determine the significance of difference between cancer and normal cells in all the cytotoxicity assays. P values less than 0.05 were considered to be consistent with statistical significance.

**Results and Discussion**

Data on the viable cell counts obtained after exposure of cells to different concentrations of water, methanol and chloroform extracts of *B. laciniosa* leaves are given in Table 1. The effect of water extract on three types of cells Mcf-7, SiHa and Vero cell lines seeded in 25 cm² flasks were incubated for 24 h in humidified CO₂ incubator. Cells were then treated with IC₅₀ dose of *Bryonia* aqueous leaf extract (20 µg/mL for SiHa and 86 µg/mL for Vero cell lines). After 48 h incubation cells were trypsinized and 1 × 10⁶ cells of each culture were processed further as per the instructions of manufacturer. The absorbance of the assay mixture was read at 405 nm wavelength on ELISA plate reader.
chloroform extracts were observed. Much higher concentration (about 300 µg) of methanol extract was required to produce 100% cytotoxicity to cells. To achieve the IC_{50} values on Vero cells significantly higher concentration of all three extracts were needed (P<0.01). Thus of the three extracts tested, water extract showed highest cytotoxic activity to cancer cells and a weak activity to normal cells.

Subtle cytotoxic effects as induced by natural products such as *Bryonia* may be escaped in short-term dye exclusion assays. Hence, cytotoxicity was also assessed using an alternative assay SRB which reflects the total protein content in the cell and also using the most sensitive clonogenic assay which estimates the regenerative potential of the cells. In SRB assay, viable cells are estimated by staining total cellular protein with the dye SRB. It is shown to be more reliable and reproducible than MTT assay. The effect of *Bryonia* aqueous extract using SRB assay is presented in Figure 2. The overall effect of leaf extracts on cells was very similar to that demonstrated by cell viability assay. However, the IC_{50} values 28.0, 46.02, 98.08 µg/mL determined by aqueous extract, 62.4, 142.5, 201.2 µg/ml determined by methanol extract and 69.3, 155 and 213.7 µg/ml determined by chloroform extract on three cell lines using SRB assay appeared higher. (Data of methanol and chloroform extract SRB assay is not shown in Figure 2 as overall it is very similar to cell viability assay). Even using SRB assay, maximum cytotoxic effect was shown by the water extract.

The methanol and chloroform leaf extracts were prepared by dissolving the dried extracts in 10% DMSO. Therefore, the effect of different concentrations of 10% DMSO on three cell lines was 10% DMSO on three cell lines was

<table>
<thead>
<tr>
<th>Drug Conc µg/mL</th>
<th>WE</th>
<th>ME</th>
<th>CE</th>
<th>WE</th>
<th>ME</th>
<th>CE</th>
<th>WE</th>
<th>ME</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
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<td>5.47×10⁵</td>
<td>4.61×10⁵</td>
<td>6×10⁵</td>
<td>5.2×10⁵</td>
<td>1.02×10⁶</td>
<td>7.8×10⁵</td>
<td>7.4×10⁵</td>
<td>6.11×10⁵</td>
<td>1.96×10⁵</td>
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<td>4.95×10⁵</td>
<td>3.11×10⁵</td>
<td>5.6×10⁵</td>
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<td>7.25×10⁵</td>
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<td>5.51×10⁵</td>
<td>1.83×10⁵</td>
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<td>25</td>
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<td>2.41×10⁵</td>
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<td>3.14×10⁵</td>
<td>2.68×10⁵</td>
<td>2.91×10⁵</td>
<td>8.0×10⁴</td>
</tr>
<tr>
<td>IC_{50} Value (µg/mL)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tbody>
</table>

Viable cell count of Mcf-7, SiHa and Vero cells after 48 h exposure to different concentrations of water (WE), methanol (ME) and chloroform extracts (CE) of *Bryonia laciniosa* leaves. The IC_{50} values for each are denoted (P<0.02).

![Fig. 1](image1.png)

**Fig. 1**—Effect of *Bryonia laciniosa* aqueous leaf extract on three cell lines using cell viability assay. Cell Viability Assay: Effect of exposure of different concentrations of *Bryonia laciniosa* leaf aqueous extract for 48 h on Mcf-7, SiHa and Vero cells. The IC_{50} values were 18.20, 20.46 and 86.8 for Mcf-7, SiHa and Vero cells, respectively.

![Fig. 2](image2.png)

**Fig. 2**—Effect of *Bryonia laciniosa* leaf aqueous extract on three cell lines using SRB assay. Plot of OD readings obtained at 490 nm for SRB assay of *Bryonia laciniosa* aqueous leaf extract on Mcf-7, SiHa and Vero cells against concentration of aqueous extract. The IC_{50} values calculated by this assay were 28.03, 46.02 and 92.98 µg, respectively.
evaluated by both cell viability and SRB assays to serve as control. The O.D. readings of SRB assay after 48 h. exposure of 1.25, 2.5, 5 and 10% DMSO on Mcf-7, SiHa and Vero cell lines are shown in Table 2. Very similar results were observed in cell viability assay (Data not shown). No measurable cytotoxic effect of 10% DMSO was thus displayed by the tested DMSO concentrations on three cell types.

Two anticancer drugs vincristine sulphate and doxorubicin hydrochloride were used as positive controls in this study. The viable count of three types of cells after treatment of these drugs for 48 h is given in Table 3. Certain numbers of cells were still obtained even at the highest concentration tested. The IC\text{50} values of the two drugs 1.12 and 0.33 µg on Mcf-7 and 3.8 and 0.73 µg for Vero cells, respectively were found to be much lower than the plant extracts, but the difference between cancer cells and normal cells were marginal (P = 0.721 and P = 0.33 for difference between effect on Mcf-7 and Vero cells by the two drugs, respectively using SRB assay).

Comparison of graphs depicting effect of vincristine on three cell lines (Figure 3) with that of aqueous extract (Figure 1) shows that 100% cell death of Mcf-7 cells could be obtained with aqueous leaf extract, while even at higher concentration vincristine was unable to kill 100% Mcf-7 cells. Similar results were observed in SRB assay where the surviving fraction was much higher with Doxorubicin (Figure 4).

The cytotoxic effect of aqueous B. laciniosa leaf extract was further tested using clonogenic assay which estimates the regenerative potential of the drug treated cells. Any subtle metabolic effect rendered by drug may get reflected by reduced ability of the cells to proliferate. It can be detected by number and size of the colonies formed by cells after exposure to drug. The number of colonies formed on two cancer cell lines.

<table>
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<th>DMSO Conc µg/mL</th>
<th>O. D. 490 nm</th>
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<tr>
<td></td>
<td>Mcf-7</td>
</tr>
<tr>
<td>Control</td>
<td>1.17</td>
</tr>
<tr>
<td>1.25%</td>
<td>1.12</td>
</tr>
<tr>
<td>2.5%</td>
<td>1.16</td>
</tr>
<tr>
<td>7.5%</td>
<td>1.16</td>
</tr>
<tr>
<td>10%</td>
<td>1.19</td>
</tr>
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O. D. readings at 490 nm of SRB assay for Mcf-7, SiHa and Vero cells after 48h exposure to different concentrations of 10% DMSO.

<table>
<thead>
<tr>
<th>Drug Conc µg/ml</th>
<th>Drug: Vincristine</th>
<th>Drug: Doxorubicin</th>
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<tbody>
<tr>
<td></td>
<td>Mcf-7</td>
<td>SiHa</td>
</tr>
<tr>
<td>Control</td>
<td>2.4×10^3</td>
<td>1.3×10^3</td>
</tr>
<tr>
<td>0.125</td>
<td>1.9×10^3</td>
<td>1.25×10^3</td>
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<tr>
<td>0.25</td>
<td>1.32×10^3</td>
<td>1.2×10^3</td>
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<tr>
<td>0.50</td>
<td>0.5×10^3</td>
<td>1.03×10^3</td>
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<tr>
<td>0.75</td>
<td>0.45×10^3</td>
<td>7.6×10^3</td>
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<td>1.0</td>
<td>0.36×10^3</td>
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<td>0.2×10^3</td>
<td>0.56×10^3</td>
</tr>
<tr>
<td>5.0</td>
<td>0.1×10^3</td>
<td>0.16×10^3</td>
</tr>
<tr>
<td>IC\text{50} Value (µg/ml)</td>
<td>1.12</td>
<td>2.95</td>
</tr>
</tbody>
</table>

Viable cell count of Mcf-7, SiHa and Vero cells after exposure to different concentrations of standard anticancer drug Vincristine sulphate and Doxorubicin hydrochloride for 48hrs and the their IC\text{50} values. The right half of table shows O.D. reading at 490 nm and IC\text{50} values of SRB assay for drug doxorubicin hydrochloride (P<0.02).
lines on exposure to different concentrations of water extract are shown in Plate 2 & Figure 5.

Very few colonies appeared on Mcf-7 and SiHa cells on treatment with more than 25 µg of drug. The approximate values of IC$_{50}$ concentration of the drug appeared to be 9.7 and 14.6 µg, respectively for two cancer cell types in clonogenic assay. It thus appears that although more viable cells are present even at higher concentrations of water extract using viability assay (IC$_{50}$ 18.2) these cells were perhaps unable to proliferate further. Unfortunately, clonogenic assay could not be successfully performed on Vero cells. The clones of Vero cells although were visible under microscope, they could not be easily counted. This was perhaps due to typical morphology of these cells. The clones grew from single cells, but migrated very fast coalescing with the neighboring clones (Data not shown). The clones may perhaps be identifiable using soft agar. A plastic surface was however, preferred in this study as the efficiency of cloning on plastic surface is much better than cloning on soft agar and would thus give a true picture of the ability of single cells to proliferate and form a clone.

Dysregulation of apoptosis is one of the important factors implicated in the etiology of cancer. Control of cancer growth depends upon the ability of cancer cells to undergo apoptosis. Most of the chemotherapeutic drugs including vinblastin, etoposide, adriamycin and methotrexate exert their effect by induction of apoptosis in tumor cells. The main participants of the cascade of reactions that lead to apoptosis are a family of cystein proteases called caspases. Activation of initiator caspase-8 and an effector caspase 3 enzyme were assessed in this study to evaluate the effect of Bryonia extract in inducing apoptosis in cell lines. The results of caspase assay conducted after treatment of SiHa and Vero cells with IC$_{50}$ concentration of Bryonia aqueous leaf extract are indicated in Table 4. About 5.56 fold increase in caspase-3 activities as compared to the control untreated cells were observed in SiHa cell line. Whereas 4.4 fold increase in caspase-3 activity was seen in Vero cell line after treatment with 4.2 times more concentration of the extract. About 5.0 fold and 2.6 fold increase in caspase-8 activity was also detected in SiHa and Vero cell lines, respectively. An increase in caspase activity after treatment with Bryonia extract clearly indicates induction of apoptosis in cells. This data suggest that the action of Bryonia leaf extract in inducing cell death in treated cells could be through induction of apoptosis. Moreover, the fold increase in caspase...
activity appeared more in cancer cells compared to Vero cells treated with 4.2 times concentration of Bryonia extract. These results are in agreement with cytotoxicity tests where the IC50 value on SiHa cells was 20 µg/mL as compared to Vero cell line where the IC50 was 86 µg/mL.

Activity of caspase-3, which is an effect of caspase involved in both mitochondrial as well as death receptor pathways of apoptosis was expected in drug induced cytotoxicity of cancer cells. The activation of caspase-8, an initiator enzyme of death receptor pathway was rather unexpected in cells treated with Bryonia extract. Activation of caspase-8 has however also been shown to activate the mitochondrial apoptotic pathway. Further investigations are necessary to elucidate the mechanism of cell death induced by Bryonia extract.

Methanol and chloroform extracts though showed cytotoxicity to cancer cells, aqueous extract exhibited maximum cytotoxic effect. Its cytotoxic activity in particular, to Mcf-7 cell line was better than SiHa cells. The IC50 value of 18.20 µg was in defined limits of active extract according to National Cancer Institute guidelines. Much higher concentration of extract was required to produce same effect on normal cells (P>0.001).

In vivo antitumor activity of methanol extract of leaves has been reported by Sivakumar et al. They have shown inhibitory effect on tumor volume, viable cell count and enhancement in time of appearance of tumor in mice. In our study, aqueous extract appeared to be even better than methanol extract to produce selective cytotoxicity to human cancer cells. This finding is consistent with one of the earlier reported observation that the plant has high level of toxicity when it is fresh and toxicity declines with dehydration of plant (unpublished).

The reference anticancer drugs tested in our laboratory were needed in much lesser concentration than Bryonia extracts to produce cytotoxic effect on cells. Obviously, the standard drugs are highly purified as compared to the crude leaf extract used by us. However, IC50 concentration for normal cells too was lower. In comparison, the aqueous extract demonstrated much better ability to discriminate between cancer and normal cells. Also, the fraction of non-responding cells at higher concentrations of the drug was more with standard drug compared to water extract of leaves.

**Conclusion**

The present investigations thus suggest that the aqueous extract of B. laciniosa leaves have cytotoxic activity to cancer cells which is worth considering for further exploitation. Cancer cell death appears to be due to induction of apoptosis, perhaps through mitochondrial pathway. This should be explored further on panel of different cancer cell lines. Additional studies are also needed to identify active constituents of plant and their biological activity. A systematic bioassay guided extraction of all the parts of the plant may serve to be important for improved efficiency of the drug.
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