Antiproliferative and cytotoxic efficiency of various extracts from Datura stramonium against highly metastatic cervical cancer HeLa cell

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Abstract: The main aim of the present research study was to evaluate the antiproliferative activity against the highly metastatic cervical cancer of the different extracts prepared using solvent of different polarity of the seeds and leaves of Datura stramonium plant. The cytotoxic assay was evaluated using MTT assay. Significant morphological changes were observed in a dose dependent manner when treated with different extracts of D. stramonium. The current works shows promising result and gives a scope to further investigate the active principles for the much needed treatment against the metastatic cervical cancer.

Keywords: Datura stramonium, cervical cancer, extracts, seeds, leaves, metastatic

Introduction
Initially cardiac disease were once considered worldwide as one of the most responsible agent for the high mortality rate. But now with the increasing environmental pollution, exposure to harmful radiations, overuse of chemicals and stressful life style are some of the reasons for the increasing number of cancer patients globally. Human papilloma virus (HPV) which is a viral infection transmitted either by skin contact or is transmitted sexually. The viral infection appears in form of warts which is a skin or mucous membrane growth. The growth of warts can be on any part of the body ,finger, toes, necks, face and even on genital organs. There are nearly 100 varieties of HPV known, most of them are responsible only for warts but those appearing on the genital organs can lead to cancer. The strain HPV 16 and 18 which are transmitted sexually (STD) are responsible for nearly more than 50% high grade cervical cancer on women (1). The common symptoms include persistent pain in back and pelvic region, heavy bleeding during menstruation, foul smelling discharge, irregular or post menopause bleeding.

Women in the age of 35 to 47 are more prone for cervical cancer. Cervical cancer can be detected either by Pap smear test, vinegar solution test or by DNA test. The HPV vaccine has greatly helped in decreasing the mortality rate while the other mode of treatment include topical application of saliclyic acid, trichloroacetic and podofilox cream, surgery can be another option to remove the warts. The ideal therapy is to improve the immune system. According to WHO global strategies for eliminating cervical cancer is by spreading awareness among the women, encourage them for HPV test and promote vaccination against HPV.

India is a rich source of a variety of Medicinal plants and since ages these plants were being used in traditional folklore for the treatment of different ailments. The advantage in using and consuming medicinal plants is that they are devoid or have very low toxicity and side effects hence are considered much safer than synthetic drugs. Even the World Health Organisation has reported that around 82% of the people still depend upon drugs which are derived from natural sources.(2,3,4)

Besides having a rich heritage of medicinal plants there are a large variety of wild growing plants around us which have a varied class of secondary metabolites on literature survey it was found that even these wild plants have also been used in traditional folk lore for getting relief from various ailments. In search of new more effective cytotoxic principle against the highly metastatic cervical cancer chemical evaluation of Datura stramonium plant was carried out.

Datura stramonium belonging to the family Solanaceae is an annual herb, highly poisonous in nature found wildly growing around us. The plant material was collected from the local surrounding and authenticated. On literature survey it was found that inspite of being highly poisonous since ancient times the different part of the plant have been used for the cure of different ailments (5).

The table given below given an overview of the different medicinal properties exhibited by various parts of the plants.
Table – I
Pharmacological Properties | Part of the Plant | References
--- | --- | ---
Anti-inflammatory | Leaves & Seeds | 5
Antidiabetic | Leaves & Seeds | 6
Antiperspirant | Seed | 7
Antisthamatic | Whole Plant | 8
Antifeedant | Leaves & Seeds | 9
Analgesic | Seed | 10
Larvicidal | Leaves | 11
Antifungal | Whole Plant | 12
Nematicidal | Whole Plant | 13
Antioxidant | Seed | 14
Antimicrobial | Stem, Leaves | 15
Insecticidal | Seed | 16
Breast Cancer MCB-7 | Leaves & Stem | 17

**Phytochemistry**
Datura stramonium contains sixty-four different types of tropane alkaloids. The major tropane alkaloids hyoscyamine and scopolamine and several minor tropane alkaloids have been identified in Datura species. The alkaloids scopoline, 3-(hydroxyacetoxy) tropane, 3-hydroxy-6-(2-methylbutyryloxy)tropane, 3a-tigloyloxy-6-hydroxytropane, 3,7-dihydroxy-6-tigloyloxytropane, 3-tigloyloxy-6-propionloxytropane, 3-phenylacetoxy-6,7- epoxy tropane, 3-phenylacetoxy-6-hydroxytropane, aponorscopolamine, 3a,6a-ditigloyloxytropane and 7-hydroxyhyoscyamine are reported for the first time by Berkov et al. [18]. Sterols and there derivatives [5.alpha.-ergosta-7, 22-dien-3.beta.-ol (16.53%), 3-hydroxycholestan-5-yl, acetate (14.97%), and 26,26-Dimethyl-5, 24(28)-ergostadien-3.beta.-ol (10.39%)] are the major constituents of essential oil of Datura stramonium. The primary biologically active substances in D. stramonium are the alkaloids atropine and scopolamine. The aqueous and ethanolic extract of the stem-bark of Datura stramonium contained alkaloids, saponins, tannins, steroids, flavonoids, phenols and glycosides while the following amino acids were isolated from the seeds Alanine, glutamate, phenylalanine, tyrosine. The tropane alkaloids were the important anticholinergic alkaloids isolated from Datura stramonium. The highest content of alkaloid are present in the vegetative and generative phases of leaves and capsules, respectively. Generally, the younger parts of plants contained more alkaloids than older ones. Alkaloid content decreased rapidly in leaves in the generative phase. Scopolamine was lowest (0.013%) in roots in the vegetative period, and then totally disappeared in the generative period. Atropine present in roots in both the vegetative (0.045%) and generative (0.056%) periods. Stems were rich in atropine (0.070%) but poor in scopolamine (0.023%) in both stages. The maximum contents of atropine are found in the stems leaves and seeds. The maximum contents of hyoscyamine and scopolamine are found in the stems and leaves of young plants, hyoscyamine being always the predominant component.

**Material and methods**
The plant material was collected from the local surrounding, the seed pods, leaves, stem were separated. The leaves were spread and left for air drying, while the seeds removed from the pods were also spread and left for air drying for at least a week with occasionally turning upside down, the completely dried leaves were also grinded to a fine powder. It was filled in a glass percolater and extracted first with methanol. The process was repeated at least three times after methanolic extraction. The residue was then again extracted with ethanol acid again the process was repeated three times. The combined methanolic and ethanolic extracts were separately concentrated under reduced pressure. The air dried seeds were grinded to a fine powder and was filled in a glass percolater. The seeds were first extracted with hexane. The process was repeated at least three times. The combined hexane extract was concentrated under reduced pressure to give the crude hexane extracts of seeds. Now the residue of the seeds were extracted with acetone. The process was separated at a minimum of three times, followed by extraction with ethanolic. The combined hexane extracts, acetone and ethanolic extracts were concentrated separately under reduced pressure.

**Test for Carbohydrates**
**Molish Test**
2ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1ml of concentrated sulphuric acid was added carefully along the sides of the test tube, formation of violet ring at the junction indicates the presence of carbohydrates.

**Fehling’s Test**
To 1 ml. of aqueous extract, 1 ml. of Fehling A and 1 ml. of Fehling B solution were added in a test tube and heated in the water bath for 10 minutes. Formation of red precipitate indicates the presence of reducing sugar.

**Benedict’s Test**

Equal volume of benedict's reagents and extract were mixed in a test tube and heated in the water bath for 5-10 minutes solution appears green, yellow, or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar.

**Barfoed’s Test**

1 ml. of extract and Barfoed’s reagent were mixed in a test tube and heated on water bath for 2 minutes, red color due to the formation of cupric oxide indicates the presence of reducing sugar.

**Test for proteins and Amino acids**

1. **Biuret's Test**: The extract was treated with 1 ml. of 10% sodium hydroxide solution in a test tube and heated. A drop of 0.7% copper Sulphate solution was added to the above mixture. The formation of violet or pink color indicates the presence of protein.

**Tests for Glycosides**

**Keller–Killiani Test**: To 2 ml. of test solution, 3 ml of glacial acetic acid and 1 drop of 5% ferric chloride solution were added in a test tube. Add carefully 0.5ml concentrated sulphuric acid by the side of the test tube. Formation of blue color in the acetic acid layer indicates the presence of cardiac glycosides.

**Tests for Alkaloids**

To the extract, dilute hydrochloric acid was added, shake it well and filtered with the filtrate, the following tests were performed.

1. **Mayer's Test**: To 2-3 ml. of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of white or creamy precipitate indicates the presence of alkaloids.

2. **Dragendroff's Test**: To 1-2 ml of filtrate, few drops of Dragendroff’s reagent were added in a test tube. Formation of red precipitate indicates the presence of alkaloids.

3. **Hager's Test**: To 1-2 ml. of filtrate, few drops of Hager's reagents were added in a test tube. Formation of yellow color precipitate indicates the presence of alkaloids.

4. **Wagner's Test**: To 1-2 ml of filtrate, few drops of Wagner's reagents were added in a test tube. Formation of reddish brown precipitate indicates the presence of alkaloids.

**Tests for Flavonoids**

1. **Lead Acetate Test**: The extract was treated with few drops of lead acetate solution. Formation of yellow colored precipitate indicates the presence of flavonoids.

2. **Alkaline Reagent Test**: The extract was treated with few drops of sodium hydroxide separately in a test tube. Formation of intense yellow color, which becomes Color less on addition of few drops of dilute acid, indicates presence of flavonoids.

**Tests for Triterpenoids and steroids**

1. **Salkowski Test**: The extract was treated with chloroform and filtered. The filtrate was added with drop of concentrated sulphuric acid, shaken and allowed to stand if lower layer turns red, sterol is present. Presence of golden yellow layer at bottom indicates the presence of triterpenoids.

**Tests for Tannins and Phenolic Compounds**

1. **Ferric Chloride Test**: Some amount of extract was dissolved in distilled water, to this solution 2 ml. of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of phenolic compounds.

2. **Lead Acetate Test**: Some amount of extract was dissolved in distilled water. To this solution few drops of lead acetate solution was added. Formation of white precipitate indicates of phenolic compounds.

3. **Dilute Iodine Solution Test**: To 2-3 ml. of extract, few drops of dilute iodine solution were added. Formation of transient red color indicates presence of phenolic compounds.

**Phytochemical Screening**

Table 1: Phytochemical investigation of Datura stramonium seeds extract
<table>
<thead>
<tr>
<th>S. No.</th>
<th>Experiment</th>
<th>Ethanolic extract</th>
<th>Acetone extract</th>
<th>Hexane extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1.1</td>
<td>Mayer’s reagent test</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
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<tr>
<td>1.2</td>
<td>Wagner’s reagent test</td>
<td></td>
<td></td>
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<tr>
<td>1.3</td>
<td>Hager’s reagent test</td>
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<td>+ve</td>
<td>+ve</td>
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<tr>
<td>1.4</td>
<td></td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
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<tr>
<td>2.</td>
<td>Carbohydrates</td>
<td>Dragendorff’s Test</td>
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<td>+ve</td>
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<tr>
<td>2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2.2</td>
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<td></td>
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<tr>
<td>3.</td>
<td>Test for Reducing Sugar’s</td>
<td>Molish’s test</td>
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<td>+ve</td>
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<tr>
<td>3.1</td>
<td></td>
<td>Barfoed’s test</td>
<td></td>
<td></td>
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<tr>
<td>3.2</td>
<td></td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
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<tr>
<td>4.</td>
<td>Flavonoids</td>
<td>Fehling’s test</td>
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<td>4.1</td>
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<td>4.2</td>
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<tr>
<td>5.</td>
<td>Glycosides</td>
<td>Benedict’s test</td>
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<td>+ve</td>
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<td>5.1</td>
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<tr>
<td>6.</td>
<td>Tannins and Phenolic compounds</td>
<td>Alkaline reagent test</td>
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<td>+ve</td>
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<td>6.1</td>
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<tr>
<td>6.2</td>
<td>Lead acetate test</td>
<td>-ve</td>
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<td>6.3</td>
<td></td>
<td></td>
<td>-ve</td>
<td>+ve</td>
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<tr>
<td>7.</td>
<td>Test for Proteins and Amino Acids</td>
<td>Killer- Killiani test</td>
<td>-ve</td>
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<td>7.1</td>
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<td>8.</td>
<td>Test for Triterpenoids and Steroids</td>
<td>Ferric chloride test</td>
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<td>8.1</td>
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<td></td>
<td>Lead Acetate test</td>
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<td></td>
<td>Dilute Iodine Solution Test</td>
<td>+ve</td>
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<td></td>
<td>Ninhydrin test</td>
<td>-ve</td>
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<tr>
<td></td>
<td>Biuret test</td>
<td>-ve</td>
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<td>Salkowski test</td>
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Thin layer chromatography: Thin layer chromatography (TLC) was carried out for all the extracts of seed and leaves of D. stramonium.

Evaluation of seed extract of Datura stramonium (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) mediated cytotoxicity in cervical cancer HeLa cells

Reagents and chemicals

MTT (3-4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) were obtained from Sigma (St. Louis, MO, USA). Eagle’s minimum essential medium (EMEM), Fetal bovine serum (FBS), Trypsin–EDTA solution, Phosphate buffered saline (PBS), and the antibiotic-antimycotic solution was acquired from Gibco, U.S.A.

Cell culture

Cervical cancer HeLa cell lines were acquired from the National Centre for Cell Science (NCCS, Pune), and they were grown in Eagle's Minimum Essential Medium with 10% FBS and 1% antibiotic-antimycotic solution. The cell culture plates were kept in a CO2 incubator at 37°C with 5% CO2 humidity to maintain standard culture conditions.

Methodology

The MTT dye was used in the cell viability assay to evaluate the cytotoxicity of the plant extract (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A), on cervical cancer HeLa cells. A 96-well plate was used to culture the cells (5x10^3 cells/well) for 24 hours. Subsequently, the cells were treated with varying concentrations (100–400µg/ml) of plant extract (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) for 24 hours. After incubation, 10µl of MTT solution (5 mg/ml) was added to each well and again incubated for a further 3 h at 37°C. After incubation purple color formazan crystals were observed. To dissolve these purple formazan crystals, 100µl of dimethyl sulfoxide (DMSO) was added. Through the use of a microplate reader, the absorbance was determined at 590nm (Bio-Rad, USA). In comparison to the untreated control, the percentage of cell viability was calculated.

Morphological analysis of plant extracts-treated cervical cancer cells

Phase contrast microscopy was used to analyze the morphological changes in cervical cancer HeLa cell lines that had been treated with plant extract. In a short while, cells were seeded for 24 hours at a density of 5x10^3 cells per well. After that, cells were treated with plant extract at various doses (100–400µg/ml), and they were then incubated for an additional 24 hours. The morphology of HeLa cells was then examined using a FLoid Imaging station (ThermoScientific, USA).

Statistical analysis

Each experiment was carried out in triplicate, and the findings were given as the mean ± SEM. One-way ANOVA was used for the statistical analysis (significant differences from the control are indicated by ns>0.05, *p 0.05, **p 0.01, and ***p 0.001).

Results

Seed extracts of Datura stramonium (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) inhibited the proliferation of cervical cancer HeLa cells

The MTT test was first used to evaluate the cytotoxicity potential of seed extract of Datura (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) in HeLa cells. Our findings demonstrated that treating HeLa cells with seed extract of Datura (Dsl-1) for 24 hours caused a dose-dependent decrease in percent cell viability, which was measured to be 86.53 ± 3.099 %, 67.78 ± 2.9 %, 47.12 ± 3.5 % and 29.32 ± 3.2 %, at the doses of 100, 200, 300 and 400µg/ml, respectively (Figure 1a to 1e). While treating with Dss-1H the percent cell viability of the HeLa cells was found to be 80.85 ± 2.8 %, 68.93 ± 2.4 %, 47.70 ± 2.5 % and 27.7 ± 2.7 % at the doses of 100, 200, 300 and 400µg/ml, respectively, 24 hours. Similarly, the cervical cancer cells were treated (100-400µg/ml) with Dss-3 EtOH for 24 hours, indicating a more substantial decrease in cell proliferation (73.54 ± 4.72, 57.97 ± 4.09, 34.35± 3.70 and 21.29 ± 3.93 %). Similarly, the percent cell viability of plant extract (Dsl-2 and Dss-2A) was also found to be decreased upon treatment with the same concentration (100-400µg/ml) for 24 hours, which were 79.47 ± 3.6 %, 66.31 ± 3.20 %, 50.52 ± 3.08, 33.15 ± 3.215 and 81.32 ± 3.0 %, 67.17 ± 1.19 %, 38.77 ± 2.99, 15.15 ± 3.2% respectively, as compared to control.

The IC50 value of plant extracts (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A), were 275.8 ± 1.037µg/ml, 271.4 ± 1.066µg/ml, 208.7 ± 1.066µg/ml, 283.3 ± 1.057µg/ml and 240.7 ± 1.049µg/ml respectively, at 24 hours. As a consequence, the data above demonstrated that the plant extract has enough potential to inhibit cervical cancer cell proliferation in a dose-dependent manner.

Fig 1a: Sample 1: Dsl-1
Sample 2: Dss1-H

Fig: 1b
Fig 1c and 1d: Sample 3 – Dss-Et-OH and Sample 4 – Dsl-2
**Figure 1a-e:** The effect of plant extracts (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2, and Dss-2A) against HeLa cervical cancer cells. (A) The percentage of cell viability after 24 hours of treatment with various concentrations of plant extracts (100–400 g/ml). The data are the mean ± SEM of three separate tests that were carried out in triplicate. Significant differences from the control are indicated by ns > 0.01, *p 0.01, **p 0.001, and ***p 0.0001. (B) Displays the IC50 of a plant extract (Dsl-1, Dsl-1H, Dsl-3 EtOH, Dsl-2, and Dsl-2A) at 24 against HeLa cervical cancer cells.

**Plant extract (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) induced morphological changes in cervical cancer cells**

HeLa cells treated with plant extract (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) displayed significant morphological changes in a dose-dependent manner (100–400 µg/ml) on phase contrast images (Figure 2 a). HeLa cells that had been treated with plant extracts also showed increased cell dissociation and cytoplasmic shrinkage. Under a microscope, untreated cells displayed ongoing cell proliferation and unaltered cell shape as shown in figure 2a-b. As a consequence, the outcome demonstrated that plant extract had cytotoxic effects in cervical cancer Hela cells.
Fig 2 a:
Figure 2a-b:

Phase contrast images of HeLa cells that were given either a vehicle or different dosages (100µg/ml to 400µg/ml) of plant extract (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) for 24 hours. (Magnification 20X; Scale bar 100µm). The images shown are representative of three independent experiments performed in triplicate.

Conclusion
The present study demonstrates that the seed extracts of Datura stramonium (Dsl-1, Dss-1H, Dss-3 EtOH, Dsl-2 and Dss-2A) have cytotoxic and anti-proliferative properties against cervical cancer HeLa cells. Especially, the ethanolic extract has significant anti-proliferative potential which has been proven by percent cell viability and morphological change. Overall, our findings suggest that seed extracts of Datura stramonium may have the potential to be an effective anti-proliferative agent against cervical cancer.

References