Study of Cytotoxic and Antimitotic Activities of *Solanum nigrum* by Using *Allium cepa* Root Tip Assay and Cancer Chemo preventive Activity Using MCF-7- Human Mammary Gland Breast Adenocarcinoma Cell Lines

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ABSTRACT

**Objective:** In the present study we have utilized the *Allium cepa* root tip meristem model to evaluate the cytotoxic and antimitotic activities of *Solanum nigrum*. The studies were extended to human cells using MCF-7-Human mammary gland breast adenocarcinoma cell lines. **Methods:** Preliminary antimitotic screening was done using *Allium cepa* root tip assay. The herbal powder obtained from plant part-dry leaves were extracted with various solvents. The antimitotic activity was analysed using *Allium cepa* root meristematic cells. Experiments were carried out with incorporation of folic acid in the extract. Folic acid inhibited the antimitotic activity of *S.nigrum* extract. **Findings:** The results obtained were compared with methotrexate-a known anticancer drug. Extracts of *S.nigrum* was found to be extremely effective in the prevention of cell proliferation of the mammary gland breast adenocarcinoma cell lines. **Discussion:** The pronounced antimitotic and anticancer activities of *S.nigrum* was due to its potential antioxidant property especially by the key role of phytochemicals such as polyphenols, steroidal saponin glycoside, alkaloids and flavonoids. Active principle sterol has been separated by TLC. **Conclusion:** These findings suggest that the promising antioxidant properties of the plant could be exploited in herbal preparations against oxidative stress, ageing, Ischemic heart disease in dissolving thrombus, microbial infections, hormone replacement therapy (HRT) and cancer justifying their use in traditional medicine.

Keywords: *Solanum nigrum*; *Allium cepa*; Mammary carcinoma fibroblast-MCF-7 cell lines; Antimitotic; Anticancer.

INTRODUCTION:
The “functional food” industry has produced and marketed foods enriched with bioactive compounds, but there are no universally accepted criteria for judging efficacy of the compounds or enriched foods. The lack of understanding bioactive compounds and their health benefits should not serve to reduce research interest but should instead encourage plant and nutritional scientists to work together to develop strategies for improvement of health through food¹.

Cancer is the uncontrolled growth of cells coupled with malignant behavior: invasion and metastasis. Cancer is thought to be caused by the interaction between genetic susceptibility and environmental toxins. In the broad sense, most
Chemotherapeutic drugs work by impairing mitosis (cell division), effectively targeting fast-dividing cells. As these drugs cause damage to cells they are termed cytotoxic. Some drugs cause cells to undergo apoptosis called "programmed cell death". The use of minerals and plant-based medicines are believed to date back to prehistoric medicine. In present work an attempt has been made to study the pharmacognosy of the traditional or tribal medicine i.e. *Solanum nigrum*, leaves of the plant are claimed to possess anti-tumor activity and hence, efforts has been taken to determine the anticancer activity.

Another possible mechanism of action reported for anticancer drugs is inhibition of DNA synthesis and thus prevention of cell division. Folic acid supplied from the diet is essential for the production of terahydrofolic acid (THF). The conversion of folic acid to THF is carried out by an enzyme folate reductase. Anticancer drugs compete with folic acid for this enzyme thus restricting the production of THF required for synthesis of DNA and consequently for cell replication. Cells which do not have adequate production of THF eventually die.

Plants have been used in traditional medicine for several thousand years. The knowledge of medicinal plants has been accumulated in the course of many centuries based on different medicinal systems such as Ayurveda, Unani and Siddha. In India, it is reported that traditional healers use 2500 plant species and 100 species of plants serve as regular sources of medicine. During the last few decades there has been an increasing interest in the study of medicinal plants and their traditional use in different parts of the world. Documenting the indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources.

Today according to the World Health Organization (WHO), as many as 80% of the world's people depend on traditional medicine for their primary healthcare needs. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases.

*Solanum nigrum* Linn. (Family Solanaceae) is commonly used in the traditional medicine as a remedy for treating various diseases. The berries possess various medicinal properties such as sedative, diaphoretic, diuretic, hydragogue, expectorant and are useful in the disease of liver, heart and eyes and is also effective against piles, fever and dysentery. The leaves are used to heal open wounds and are known to possess hypotensive effect. The berries has been used in the treatment of stomach ulcers in the folk medicine in South Africa, European, China and through out India. The fruits of *S. nigrum* have been reported to play an adjuvant role in the hepatoprotective property. Inhibition of lipid peroxidation and free radical scavenging activity has been suggested as a possible mechanism of action. Previous studies have demonstrated that aerial parts of *S.nigrum* have the ability to decrease the secretion of gastric acid, pepsin level and stimulate mucus secretion.

The antimitotic activity was screened using *Allium cepa* root meristamatic cells which have been used extensively in screening of drugs with antimitotic activity. The roots of all plants have distinguished regions, one of them being the region of cell division that lies beyond the root cap and extends a few mm after that. Cells of this region undergo repeated divisions. The fate of cell division is higher in this region compared to that of the other tissues. This region is called the meristamatic region (meristos: divided). This division is similar to the above mentioned cancer division in humans. Hence, these meristamatic cells can be used for preliminary screening of drugs with anticancer activity. Even though doubts can be raised about extrapolation of...
results from plant tissue to animals and finally to humans, Khilman has noted that plant cells are 1000 times more resistant to colchicines which is a potent anti-carcinogen and which acts by inhibiting the microtubule formation. Thus, it is possible that chemicals that affect plant chromosomes will also affect animals.

**MATERIALS AND METHODS:**

**Collection and processing of plant material:** The plant *Solanum nigrum* was collected in Chennai, Tamil Nadu, India. The identification and nomenclature of the plant was based on The Flora of Presidency of Madras and The Flora of Tamil Nadu Carnatic. They were later verified at Botanical Survey of India, Southern Circle, Coimbatore, India. All the preserved specimens were deposited at the Herbarium of Entomology Research Institute, Loyola College, Chennai. The freshly collected leaves were washed and air dried in the shade at room temperature. Dried leaves and dried leaflets were taken separately and powdered for extraction. Fresh leaves were homogenized with solvent and then extracted. *Allium cepa* bulbs (red variety) were purchased from the local market and stored for the entire study.

**Chemicals:** Carmine stain and solvents were procured from Sigma Aldrich, New Delhi, India. Eagles minimal essential medium for cell proliferation, MTT, RPMI-1640 were purchased from LGC Promochem India Pvt. Ltd. Bangalore, India.

**Preparation of plant extracts:** The plant material (leaves) was air dried in the laboratory at room temperature. It was then powdered and extracted with hot water by boiling for 30 minutes to get the aqueous extract. The extract obtained was concentrated and dried under controlled temperature (60°C). The dried powder was successively extracted with 70% ethanol, then with chloroform. Finally it was concentrated and made up to particular volume. Extraction with each solvent was done in a water bath for 60min with a reflux condenser. Each time before extracting with the next solvent the marc was dried in an air oven below 50°C. Each extract was concentrated and evaporated to dry extract. Extracts of desired concentrations were prepared for further study using these dried extracts.

**Antimitotic activity:** This activity was evaluated using *A. cepa* root meristematic cells. *A. cepa* were sprouted in tap water for 48 hr at room temperature. The bulbs that developed uniform root were used for

Plate: 1
the experiment. These roots were treated with above prepared extracts of 10 concentrations. Water was used as medium/vehicle dilution. The different fractions used have been mentioned in Table 1. A blank with water was used as control. Methotrexate was used as a standard control. After 3hr of treatment, the root tips were fixed with fixing solution of acetic acid and alcohol. Squash preparations were made by staining the treated roots with acetocarmine stain. The mitotic index was calculated as

\[
\text{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100.
\]

The aqueous and organic extracts were also subjected to preliminary phytochemical characterization, which revealed the presence of the phytochemicals-alkaloids, phenols, flavonoids, sterol, saponin glycosides, reducing sugars, proteins, cardio active aglycones and cardinolides, saponin glycosides. Folic acid added to the solution of methotrexate, aqueous extract and organic extract of \textit{S. nigrum}. A similar experiment was undertaken to find out the probable mechanism of action through which the extracts and methotrexate act. Squash preparations made as above from the treated roots were observed.

**Cell proliferation assay:**

MCF-7- Human mammary gland breast adenocarcinoma cell lines were obtained from the American type culture collection and grown in the Minimal essential medium Eagles with L-glutamine and Earle’s Basal salt solution adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1.mM non-essential amino acids and 1 mM sodium pyruvate and supplemented with 0.01 mg/ml calf insulin - 90% and 10% fetal calf serum in a humidified atmosphere of 5 % CO2 at 37°C.

The effect of \textit{S. nigrum} on cell viability and growth was determined using MTT (3-4.5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) colorimetric assay [using a commercially available kit]. The compounds were dissolved in water as stock solution (1000×) and then diluted with RPMI-1640 for cell culture experiments. All solutions were prepared fresh on the day of testing. Human mammary gland breast adenocarcinoma cells were seeded at a density of 1×10^4 per well in a 96 well plate.

After 24 hr, fresh medium was added containing aqueous extract of \textit{S.nigrum} at concentration of

\[0, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} \text{ and } 10 \text{ mg/ml. After 4 days of incubation, in medium containing extract of S.nigrum cells were further incubated for 4 hr with the metabolic substrate, tetrazolium to formazan which was detected spectrophotometrically at 540 nm with multiwell spectrophotometer (ELISA Reader, Biotek Instruments Inc., Burlington,VT).}

**Preparative TLC:** Slurry of silica gel GF_{254} was made in distilled water. This slurry was then applied on glass plates (12.5 x 12.5cm) with the aid of a TLC spreader to obtain preparative silica gel plates having thickness of about 0.5mm. The plates were dried in an oven at 105°C and activated 2hr before use.

**Sample preparation and application:** Aqueous extract (1mg/ml) was spotted on the plate. The plates were developed using in solvent system. The plates were visualized and the spots were marked. Then the results were compared with standard sterol.
Statistical analysis: The data was subjected to statistic analysis using analysis of variance followed by appropriate post-hoc tests. P<0.05 was considered as significant.

RESULTS:

Effect of aqueous extract of S.nigrum on mitotic activity: Antimitotic activity of aqueous extract was comparable to the activity of methotrexate (Table 1). The activity of organic extract was less than that of the aqueous extract. A one way ANOVA showed that there was a significant effect of treatment on mitotic activity. Post-hoc analysis using the Newman-Keuls test showed that the activity of all the different extracts were significant when compared with water (control). The aqueous extract showed lowest mitotic index i.e. highest activity amongst all the different extracts.

Table 1

Table 1 shows the antimitotic activity of different extracts and methotrexate. It was observed that the extract decreases the mitosis of A.capa root tips. The phase was differentiated in each case and it was observed that the number of non-dividing cells increase with an aqueous extract than organic extract. A one-way ANOVA showed that there was a significant decrease in the mitotic index by aqueous and organic extracts of S.nigrum (F=1266.2; P<0. 001) Post-hoc analysis using Newman-Keuls test showed that the aqueous extract was the most effective and it showed more or less similar effect to that of standard methotrexate-anticancer drug, which was not significantly different from the referred drug (q=0.7016; P>0.05). The cell divisions were differentiated and number of cells in each phases of cell division i.e. either prophase, metaphase, anaphase, or telophase were recorded. Thus, the number of cells entering prophase decreased. Since the cells do not enter prophase, further stages of cell division also decrease. Newman-Keuls test showed that the aqueous extract was the most effective. It was observed that the aqueous fraction showed better activity than organic fraction.

Effect of folic acid on antimitotic activity of S.nigrum and methotrexate: Analysis of data using a 3-way ANOVA showed that there was a significant effect of the pretreatment with folic acid on the antimitotic activity of S.nigrum and methotrexate.(F=144.65). The mitotic index increased when folic acid was added to the aqueous, organic extracts of S.nigrum and methotrexate solution which otherwise reduce the mitotic activity in the absence of folic acid. This however, did not increase with increase in folic acid concentration suggesting that the effect was not dose-dependent. By comparing the mitotic index of methotrexate and S.nigrum, it was observed that incorporation of folic acid increased the mitotic index significantly in case of methotrexate, but not so in case of S.nigrum. Post-hoc analysis of the data showed that folic acid inhibited the anti-mitotic activity of methotrexate to a greater extent as compared to S.nigrum.
DISCUSSION:

The result from the study showed that the aqueous extract of *S. nigrum* had excellent anti-mitotic activity that was comparable to the activity of methotrexate. Maximum numbers of non-dividing cells were observed. Methotrexate-anticancer drug competitively inhibits dihydrofolate reductase (DHFR), an enzyme that participates in the tetrahydrofolate synthesis. Methotrexate acts specifically during DNA and RNA synthesis, and thus it is cytotoxic during the S-phase of the cell cycle. Logically, it therefore has a greater toxic effect on rapidly dividing cells such as malignant and myeloid cells\(^{26,27}\). The addition of folic acid inhibited the antimitotic activity of *S. nigrum* significantly\(^3\), but does not completely inhibit the activity of methotrexate.

Folic acid is essential for the production of tetrahydrofolic acid (THF) which is in turn, required for synthesis of DNA and consequently for cell replication. Hence the dividing cells were high compared to that of the methotrexate treated cell.\(^28\)

Aqueous extract of *S. nigrum* was also effective in reducing the cell viability of MCF-7-Human mammary gland breast adenocarcinoma cell lines that may be acting following the same mechanisms as those in the *Allium cepa* meristamatic cells. By virtue of this, if the extract is administered in humans it may prevent cell proliferation by directly combining with cell receptors/enzymes and eliciting signals or cell apoptosis.

Phytochemical characterization of the different extracts revealed the presence of the phytochemicals- Indole alkaloids, polyphenols, flavonoids, sterol, saponin glycosides, reducing sugars, proteins, cardio active aglycones and cardinolides. Aqueous fraction contains steroids along with other polar constituents. Though, the probability of steroids extracted in a polar solvent was low, these steroids occurred as aglycones of the saponin glycosides after the glycosides hydrolyzed. There might be other polar compounds in the total extracts that might be acting synergistically with steroids of *S. nigrum*. The most common phytosterol are B-sitosterol, campesterol and stigmasterol\(^{29}\). *S. nigrum* is having these phytosterol. An important group of antioxidant, not previously mentioned, includes the sterol\(^{30}\). Phytosterol may offer protection from chemically induced colon cancer.\(^31\) Phytosterol has effect on apoptosis. The rate of tumor growth is dependant upon a balance between the rates of cell proliferation and apoptosis. Apoptosis is a programmed cell death, as influenced by phytosterol.\(^{32,33}\) Hence, the sterols from *S. nigrum* must be contributing to the anticancer potential of the herb.

The aqueous extract of *S. nigrum* seems to prevent prophase stage in cell division where DNA duplication occurs. Methotrexate is a known anticancer drug that inhibits DNA synthesis. When folic acid was supplemented to the methotrexate and the total aqueous extract, it was seen that mitotic index increased. Thus, it may be suggested that the extract may be acting through the pathway inhibiting tetrahydrofolic acid and hence folic acid is required for DNA synthesis that arrest cell division. Methotrexate is known as
anticancer drug which compete with folic acid for the enzyme reductase\textsuperscript{25,26}. The total aqueous extract of \textit{S.nigrum} may also be competing with folic acid thus inhibit the DNA synthesis. Hence, addition of folic acid increases the mitotic index due to the availability of folic acid. However, the mitotic index does not increase significantly in case of \textit{S.nigrum} as compared to that of methotrexate. This may be because the extract may be mediating its effects through other mechanisms also. The extract binding with different cell proteins are responsible for cell division. This effect may be due to steroidal glycosidic alkaloid or steroidal alcohol.\textsuperscript{32,33}

The finding of this study indicate that \textit{S.nigrum} is a promising source of steroidal glycosidic alkaloid or steroidal alcohol, polyphenols, flavonoids, iodole alkaloid and FRSA, which have the ability to modify the physiological function of cells and hence act as anti-cancer drugs to arrest the proliferation of cancer cells. The extract shows commendable antioxidant activity which also may be one of the contributing factors to its anticancer potential. In the present study extracts of \textit{S.nigrum} was tested for the antitumor activity and it showed most effective inhibition of MCF-7 Human mammary gland breast adenocarcinoma cell proliferation. Apoptosis is a critical molecular target by dietary bioactive agents for the prevention of cancer. The potential use of \textit{S.nigrum} as therapeutic agent holds great promise as the isolation of one or more cytotoxic chemicals from crude extract and the judicious use of such chemicals can control the progression of cancer and also can prevent the formation of tumour in individuals who are highly susceptible to developing a tumour.

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Table 1: Antimitotic activity after treatment of A.copa roots with aqueous, organic extracts of Solanum nigrum and methotrexates

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Different solutions used for treatment</th>
<th>% of Non-dividing cells</th>
<th>% of dividing cells</th>
<th>Mitotic Index</th>
<th>Mitotic Index</th>
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<td></td>
<td></td>
<td>P  M  A  T</td>
<td></td>
<td>Avg</td>
<td>SD</td>
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<tr>
<td>1</td>
<td>Water (control)</td>
<td>14  78  5  2  1</td>
<td>86</td>
<td>86.2</td>
<td>0.8367</td>
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<tr>
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<td>Methotrexate (Standard)</td>
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<td>43</td>
<td>43</td>
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<tr>
<td>3</td>
<td>Aqueous extract</td>
<td>57  39  2  1  1</td>
<td>43</td>
<td>43.4</td>
<td>1.817</td>
</tr>
<tr>
<td>4</td>
<td>Organic Extract</td>
<td>44  50  2  3  1</td>
<td>56</td>
<td>56</td>
<td>1.225</td>
</tr>
</tbody>
</table>

Table 1: Antimitotic activity after treatment of A.copa roots with aqueous, organic extracts of Solanum nigrum and methotrexates

P-Prophase, M-Metaphase, A- Anaphase, T-Telophase
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<td>Organic Extract</td>
<td>44</td>
<td>50 2 3 1</td>
<td>56</td>
<td>56 1.225</td>
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Table 2: Antimitotic activity after treatment of *A. cepa* roots with aqueous extract of *S. nigrum* + folic acid, organic extract of *S. nigrum* + folic acid and methotrexate + folic acid

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Different solutions used for treatment</th>
<th>% of Non-dividing cells</th>
<th>% of dividing cells</th>
<th>Mitotic Index</th>
<th>Mitotic Index</th>
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<td>Methotrexate + Folic acid</td>
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<td>67 5 3 3</td>
<td>78</td>
<td>77.8 0.8367</td>
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<td>24</td>
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<td>75.8 0.8367</td>
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P-Prophase, M-Metaphase, A- Anaphase, T-Telophase
Plate: 1 Treatment of Allium cepa roots with different extracts of Solanum nigrum and solutions

Plate 2: Separation of Sterol by TLC

1. Standard sterol
2. Aqueous extract of Solanum nigrum
3. Organic extract of Solanum nigrum
Plate 3: Different stages of mitosis of Allium cepa roots after treatment with water
Plate-4: Antimitotic activity of aqueous extract of S.nigrum and the stages of cell division
Plate-5: Antimitotic activity of organic extract of S.nigrum and the stages of cell division
Plate-6: Antimitotic activity of methotrexate, methotrexate + folic acid and the stages of cell division
Plate 7: Antimitotic activity of aqueous leaf, aqueous leaf + folic acid extracts of S. nigrum and the stages of cell division – Plate a and Plate b
Plate 8: Antimitotic activity of organic leaf, organic leaf+ folic acid extracts of S. nigrum and the stages of cell division (Plate a and Plate b)
Plate-9: Normal MCF-7 Human mammary gland breast adenocarcinoma cell lines – showing confluent monolayer (20X magnification)
Plate 10: Anticancerous effect of aqueous dry leaf extract of S. nigrum on MCF-7 cell lines 12 hrs post infection showing cell rounding (40X magnification)
Plate-11: Anticancerous effect of aqueous dry leaf extract of S. nigrum on MCF-7 cell lines -24 hrs post infection showing cell rounding (40X magnification)
Plate 12: Anticancerous effect of aqueous dry leaf extract of S. nigrum on MCF-7 cell lines - 32 hrs post infection showing cell growth inhibition and prevention (40X magnification)
Plate-13: Effects of aqueous dry leaf extract of S.nigrum on MCF-7 cell lines -38hrs post infection complete cell death and depopulation (40X magnification)