

Screening *In vitro* Anticancer Activity of *Alseodaphne semecarpifolia* Nees Stem Bark Extracts against some Cancer Cell lines

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ABSTRACT

Introduction: Cancer is considered as the prime lethal disease that affects different organs of the body. Even with the rapid developments in the medical sciences, there are no proper medicines to cure specific kind of cancer without side effects. The inhibition of tumour cell growth without side effects either by the use of herbal or synthetic drugs is considered as an important target in cancer therapy. In traditional medicinal system *A. semecarpifolia* stem bark is the prime source of herbal drug to treat lymphatic and skin cancers. **Objective:** The purpose of this study is to evaluate the anticancer potential of *A. semecarpifolia* stem bark extracts against some cancer cell lines. **Methods:** The *in vitro* anticancer activity was evaluated against DLA, EAC, HeLa, HepG2 and L929 cell lines by trypan blue dye exclusion assay and SRB assay. **Results:** The results of the anticancer activity revealed that, when compared to standard drug Cyclophosphamide, SBPEE and SBCE of *A. semecarpifolia* showed significant anticancer activity against DLA and EAC cell lines, without causing any toxicity to the normal mouse fibroblast cells L929. Whereas, none of the three extracts showed cytotoxicity against HeLa, HepG2 and L929 cell lines. **Conclusion:** The present study suggested that, SBPEE and SBCE possess significant cytotoxic activity against DLA and EAC cell lines, which confirms the traditional medicinal claim of *A. semecarpifolia* as a potent anticancer plant against lymphatic and skin cancer.

Key words: *Alseodaphne semecarpifolia*, Cell lines, Cell viability, Cytotoxicity, Carcinoma, Lymphoma.

INTRODUCTION

Cancer is a disease caused by loss of cell cycle control, in which there is an uncontrolled multiplication of the cells.¹ It is associated with abnormal and uncontrolled cell growth. It is a significant health problem worldwide, generally due to the lack of early detection methods. It is caused by both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutations that occur from metabolism). One of the prime requirements of cancer chemo preventive agent is elimination of damaged or malignant cells through inhibition of the rapid multiplication of cells or induction of apoptosis with very less or no toxicity to normal cells.² Controlling the rapid multiplication of the cancer cells without affecting the normal cells is a challenging task in the discovery of anticancer compounds.

Medicinal plants are the gift of the nature for the human beings to treat various kinds of cancers without causing side effects. The reason behind using them as a medicine is, they contain numerous phytochemicals of therapeutic value which are synthesised in the plant body at specific physical, chemical and biological stress conditions. Numerous plants continue to play a pivotal role in drug discovery and development and some of

which leads for the development of novel drugs. Hence, evaluation of cancer chemo preventive activity of medicinal plant is of utmost importance in drug discovery.

Alseodaphne semecarpifolia is a large evergreen tree belongs to the family Lauraceae.³⁻⁶ It grows up to 10-15 metres tall. The stem bark is dark and smooth. Leaves are alternate, usually clustered at the end of the branches.³⁻⁶ Flowers are small, yellowish-green, in lax panicles at the end of the branches and the fruit is ellipsoid, black, 1-2 cm long, blunt, smooth and seated on thick clavate pedicel.³⁻⁶ The flowering and fruiting season of this plant is from March to May.^{3,4} It is distributed in peninsular India and Sri Lanka, *A. semecarpifolia* is the only species from the genus *Alseodaphne*, which is found in Sri Lanka.⁷ In Malabar (Uttarakannada) and Malnad (Shivamogga) regions of Karnataka, it is commonly called as Mase/Mashe and Sehunda.⁸⁻¹¹ In southern states of India it is also known as, Nelthare in Kannada, Kanaipirandai, Attapattai and Arambaram in Tamil, Naaramamimidi in Telugu, Phudgus in Marathi and Mulaknaari in Malayalam.¹²

The ethno medicinal survey in the Malnad region of Karnataka revealed that stem bark of this plant is used to treat lymphatic and skin cancers, but there is no sufficient scientific data is available to defend its traditional claim as an anticancer plant. However, scientific investigations on *Alseodaphne*

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species is still rare and they are less explored phytochemically and pharmacologically.^{12,13} Hence, the present study was undertaken to investigate the anticancer property of the stem bark extracts of *A. semecarpifolia* against four human cancer cell lines, such as Dalton's Lymphoma Ascites (DLA), Ehrlich Ascites Carcinoma (EAC), human cervical cancer cell line HeLa, human hepatoma cell line HepG2. The normal mouse fibroblast cell line L929 was also evaluated in comparison with the standard drugs Cyclophosphamide and Adriamycin.

MATERIALS AND METHODS

Collection and identification of plant sample

The stem bark of *A. semecarpifolia* Nees was collected from Karigudda, Aaneguli village, Sagara thaluk and Shivamogga district of Karnataka, India. The plant was identified and authenticated by taxonomist Dr. Y. L. Krishnamurthy, Professor, Department of post graduate studies and research in Applied Botany, Kuvempu University and the voucher specimen was maintained in the Kuvempu University herbaria (No. KUBP78).

Preparation and extraction of the sample

The freshly collected stem bark was washed under running tap water to remove the soil and other dust particles and air dried in the laboratory condition at room temperature for 45 days. The dried stem bark was ground mechanically into fine powder and subjected to sequential soxhlet extraction for 48 h. The extract was concentrated using rotary evaporator and desiccated until further usage.

In vitro cytotoxicity study

Cell lines

Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) tumour cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala, India. HeLa, HepG2 and L929 cell lines were obtained from ACTREC, Khargar, Navi Mumbai and they were grown in RPMI 1640 medium containing 10% FBS and 2 mM L-glutamine. For the present experiment, the cells were inoculated into 96 well microtiter plates in 100 μ L, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37°C, with 5% CO₂, 95% air and 100% relative humidity for 24 h prior to the addition of plant extracts.

Trypan blue dye exclusion assay

Aleodaphne semecarpifolia stem bark extracts and the standard drug cyclophosphamide were used for *in vitro* cytotoxicity study by trypan blue dye exclusion assay, against Dalton's Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) tumour cells.

Trypan blue dye exclusion assay was carried out according to the protocol described by Strober, 1997.¹⁴ The tumour cells were washed thrice with PBS. Cell viability was determined by trypan blue dye exclusion technique. Viable cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of the plant extracts and standard drug cyclophosphamide and the volume was made up to 1ml using PBS. Control tube contained only cell suspension. These assay mixtures were incubated for 3 h at 37°C. Further cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2 to 3 min and loaded on to a haemocytometer. Dead cells take up the blue colour of trypan blue while live cells do not.

SRB assay

A. semecarpifolia stem bark extracts and the standard drug adriamycin were used for *in vitro* cytotoxicity study by SRB assay, against HeLa, HepG2 and L929 cell lines.

SRB assay was carried out according to the protocol described by Vanicha and Kanyawim, 2006.¹⁵ The stem bark extracts were initially solubilised in DMSO at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use. At the time of extract addition, an aliquot of frozen concentrate (1 mg/ml) was thawed and diluted to 100 μ g/ml, 200 μ g/ml, 400 μ g/ml and 800 μ g/ml. Aliquots of 10 μ l of these different extract dilutions were added to the appropriate microtiter wells containing 90 μ l of medium, resulting in the required final extract concentrations *i.e.* 10 μ g/ml, 20 μ g/ml, 40 μ g/ml and 80 μ g/ml.

After the addition of the plant extracts, plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 μ l of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 μ l) at 0.4% (w/v) in 1% acetic acid was added to each of the wells and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base and the absorbance was read on plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells \times 100. Using the six absorbance measurements [time zero (Tz), control growth (C) and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels.

RESULTS

In trypan blue dye exclusion assay, the cytotoxicity study of different extracts showed that, SBPEE at the concentration of 200 μ g/ml, significantly controlled the growth of cancer cell lines DLA and EAC with 98.33 ± 1.15 and $96.33 \pm 3.51\%$ inhibition respectively. SBCE showed moderate cytotoxic effect with 78.66 ± 1.52 and $83.33 \pm 3.21\%$ of cell death against DLA and EAC cells. Whereas, the cytotoxic effect of SBEE was found to be very less, *i.e.* 11.66 ± 2.08 and $14.33 \pm 2.51\%$ against DLA and EAC cells respectively (Figures 1 and 2).

The IC₅₀ value of the SBPEE against DLA cells is 48.07 ± 3.05 μ g/ml, whereas it is 47.16 ± 3.51 μ g/ml against EAC cells. The IC₅₀ value of the SBCE against DLA cells is 80.64 ± 1.15 μ g/ml, whereas it is 86.20 ± 2.64

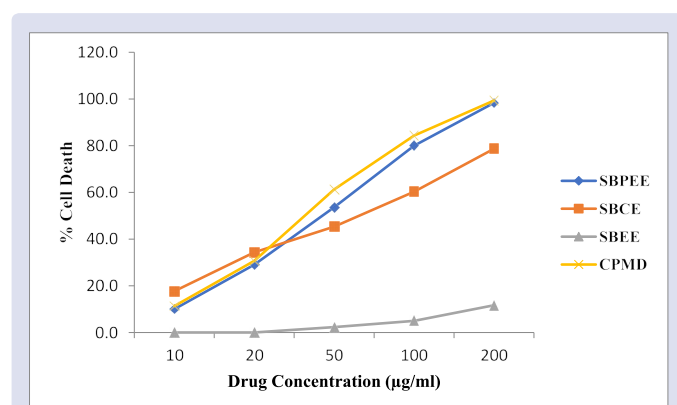


Figure 1: *In vitro* cytotoxic effect of *Aleodaphne semecarpifolia* stem bark extracts against DLA cell line.

$\mu\text{g/ml}$ against EAC cells. Therefore, the extracts showed toxicity in a dose dependent manner.

In SRB assay, none of the three extracts tested have exerted toxicity against human cervical cancer cell line HeLa, human hepatoma cell line HepG2 and a normal mouse fibroblast cells L929 (Figures 3-5). All the three extracts showed very less control of cell growth against these cell lines when compared to standard drug Adriamycin.

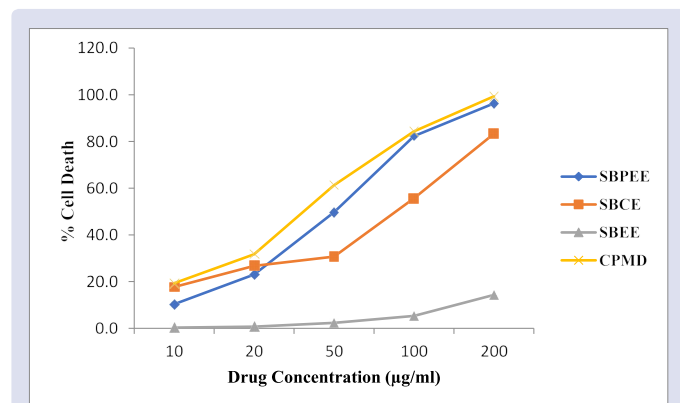


Figure 2: *In vitro* cytotoxic effect of *Alseodaphne semecarpifolia* stem bark extracts against EAC cell line.

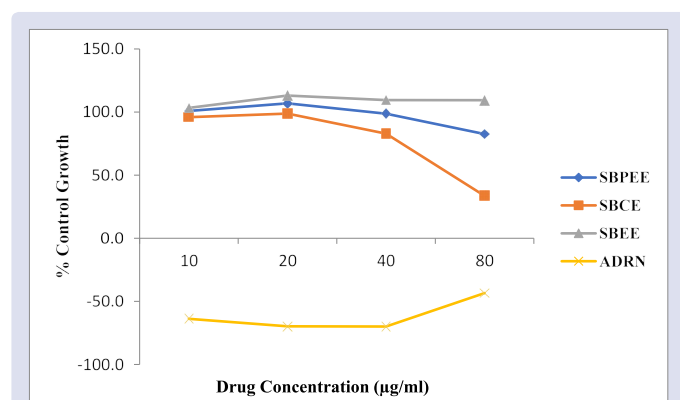


Figure 3: *In vitro* cytotoxic effect of *Alseodaphne semecarpifolia* stem bark extracts against HeLa cell line.

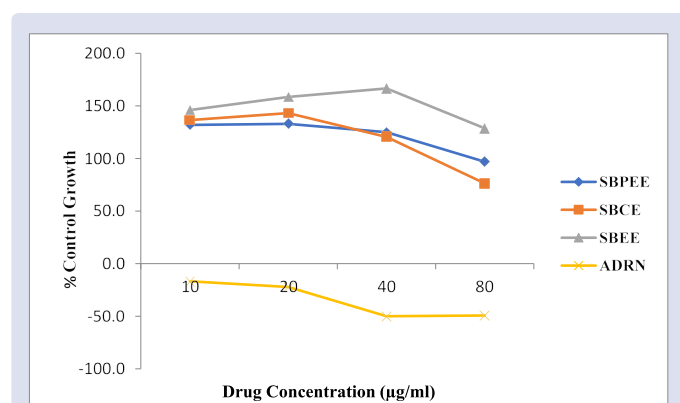


Figure 4: *In vitro* cytotoxic effect of *Alseodaphne semecarpifolia* stem bark extracts against HepG2 cell line.

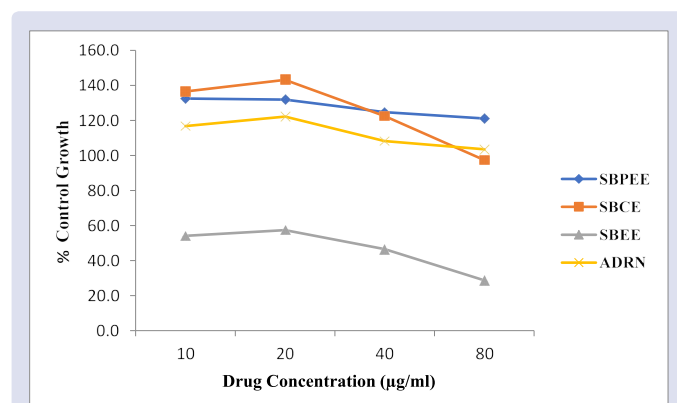


Figure 5: *In vitro* cytotoxic effect of *Alseodaphne semecarpifolia* stem bark extracts against L929 cell line.

DISCUSSION

Anticancer activity is the ability of the natural, synthetic or chemical agents to suppress or prevent carcinogenic progression. Several cytotoxic therapies in ayurveda include herbal remedies as they are the potent medicines in the traditional system and majority of the tumour cells are treated by herbal supplements. One of the best approaches for searching anticancer agents is the selection of medicinal plants based on their ethno medicinal value.¹⁶

Cancer always need to be diagnosed at its earlier stage, later stages of cancer will be hard to cure. Now a day's *surgery*, chemotherapy and radiotherapy are the standard methods employed for the treatment of cancer, although they are not fully effective. Efforts have been made to make the cancer treatment more suitable and agreeable, herbal drugs have been employed because they have reported with very low side effects, when compared to synthetic drugs.¹⁷

The tumour cells used in this study were DLA, EAC, HeLa, HepG2 and L929. Dalton's Lymphoma Ascites is a transplantable, poorly differentiated malignant tumour which appeared originally as lymphocytes in a mouse.¹⁸ Ehrlich Ascites Tumour is a rapidly growing carcinoma with very aggressive behaviour.¹⁹ HeLa is the oldest and most commonly used human cell line. It was derived from cervical cancer cells taken in 1951 from Henrietta Lacks, a patient who died of cancer. The cells taken were cultured and labelled as 'HeLa', in 1953, HeLa cells were the first human cells successfully cloned.²⁰ HepG2 is a perpetual cell line which was derived from the liver tissue of a 15 years old Caucasian American male individual with a well differentiated hepatocellular carcinoma. HepG2 cells are the suitable *in vitro* model system for the study of polarized human hepatocytes. Because of their high degree of morphological and functional differentiation *in vitro*, HepG2 cells are used as models for the *in vitro* anticancer studies.²¹ L929 cells are the normal mouse fibroblast cells.

Trypan blue is an essential dye, it will not enter into the cell wall of the plant cells that are grown in the culture.²² Trypan blue dye exclusion assay measures the cell viability based on the number of dead cells stained by trypan blue and unstained viable cells. SRB assay measures the cell density based on the measurement of cellular protein content, it is an efficient and highly cost-effective method for screening cytotoxicity.

Alseodaphne semecarpifolia is a potent source of secondary metabolites.²³⁻²⁵ Secondary metabolites from several plants exert numerous effects on tumour formation, they protect normal cells of the

body against adverse effects of anti-cancer therapies.²⁶ As *Aleodaphne semecarpifolia* is a potent source of certain secondary metabolites these secondary metabolites may protect normal cells against certain side effects of cancer treatment.

Among three extracts tested, SBPEE showed highest activity indicating its potent cytotoxic property against DLA and EAC tumour cells without affecting the normal mouse fibroblast cells L929. The amount of SBPEE required to cause 50% cell death against DLA and EAC cells is almost similar to standard drug cyclophosphamide, whereas the amount of SBCE required to cause the 50% cell death against DLA and EAC cells is twice the value of standard drug cyclophosphamide. None of the three extracts tested have showed toxicity against HeLa, HepG2 and L929 cell lines. Therefore, the results of this study supported the significant *in vitro* anticancer activity of *A. semecarpifolia* SBPEE and SBCE against solid and ascites tumour cells.

A similar cytotoxicity studies of the *Euphorbia neriifolia* latex acetone extract against DLA and EAC cells showed IC₅₀ value of 51 and 82 µg/ml.²⁷ In the present study *A. semecarpifolia* SBPEE has highest anticancer activity; it showed IC₅₀ value of 48.07 ± 3.05 µg/ml and 47.16 ± 3.51 µg/ml against DLA and EAC cells respectively. Cytotoxicity study of *Thespesia populnea* chloroform leaf extract against DLA and EAC cells showed 100% and 95% inhibition and *Hygrophilla schulli* hexane leaf extract showed 94% and 98% inhibition against DLA and EAC cells respectively at 200 µg/ml.²⁸ In the present study *A. semecarpifolia* SBPEE showed 98.33 ± 1.15 and 96.33 ± 3.51% inhibition against DLA and EAC tumour cells respectively at a concentration of 200 µg/ml.

CONCLUSION

The present investigation paves way for pharmacological research in future and for the discovery of new sources of drugs from these phytochemicals. It provides strong evidence that *A. semecarpifolia* SBPEE and SBCE has *in vitro* anticancer activity against DLA induced solid tumour and EAC induced ascites tumour cells. Hence, the present study supported the traditional claim of *A. semecarpifolia* stem bark extract to cure lymphatic and skin cancers. Further validation is required to evaluate the effects of *A. semecarpifolia* SBPEE and SBCE on cell cycle and apoptotic genes.

Even though the anticancer activity of these extracts is fairly weak against HeLa and HepG2 cell lines, the results presented here will prove useful for further research aimed at identifying molecules associated with fewer side effects that may be effective against certain cancers.

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CONFLICTS OF INTEREST

Authors declare that, there are no conflicts of interest for this study.

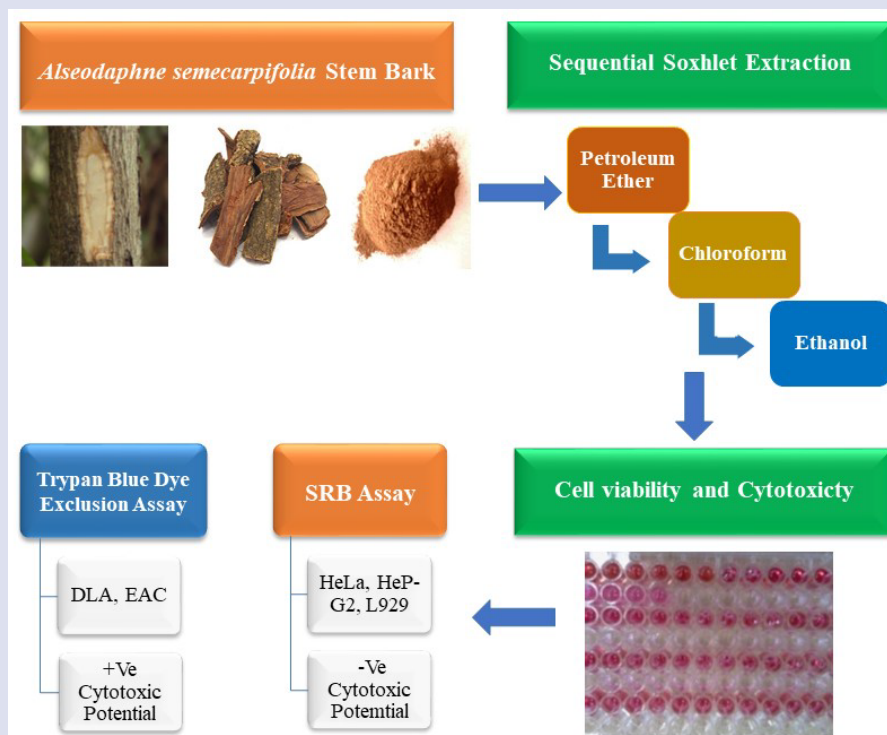
ABBREVIATIONS

ACTREC: Advanced Centre for Treatment, Research and Education in Cancer; ADRN: Adriamycin; CPMD: Cyclophosphamide; DLA: Dalton's Lymphoma Ascites; DMSO: Dimethyl Sulfoxide; EAC: Ehrlich Ascites Carcinoma; FBS: Fetal Bovine Serum; IC₅₀: Inhibitory Concentration 50; PBS: Phosphate Buffered Saline; RPMI 1640: Roswell Park Memorial Institute 1640 Medium; SBPEE: Stem Bark Petroleum Ether Extract; SBCE: Stem Bark Chloroform Extract; SBEE: Stem Bark Ethanol Extract; SRB: Sulphorhodamine B.

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GRAPHICAL ABSTRACT



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