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IN VITRO ANTICANCER ACTIVITY OF *VITEX NEGUNDO* IN HeLa AND A549 CELL LINES

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Abstract

Cancer is one of the leading causes of morbidity and mortality around the world. The advent of modern drug therapies has undeniably improved cancer patients care. The effort for finding new anticancer agents with better efficacy and lesser side effects has been continued. Cancer chemoprevention with natural phytochemical compounds is an emerging strategy to prevent or cure cancer. Present study was aimed to investigate the antiproliferative activity of *Vitex negundo* on A549 cell line. The therapeutic effect of four different extracts (Petroleum ether, Ethyl acetate, Chloroform and Methanol) of *Vitex negundo* stem and leaf was studied A549 cell lines by MTT cell viability assay. When compared to leaf, stem of *Vitex negundo* showed potent cytotoxic activity against A549 cell line. Among the four solvent extracts of *Vitex negundo* stem, chloroform stem extract fraction showed significant cytotoxicity against A549 cell lines followed by methanolic stem extract fraction whereas ethyl acetate and petroleum ether extract had less cytotoxicity.

Keywords **:** *Anticancer, A549, Vitex negundo, apoptosis, MTT, cytotoxicity*

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Introduction

Cancer has been considered to be a global burden since ages. It is described as an uncontrolled proliferation and dedifferentiation of a normal cell. According to global cancer statistics, there'll be an estimated 18.1 million new cancer cases (17.0 million excluding nonmelanoma skin cancer) and 9.6 million cancer deaths (9.5 million excluding nonmelanoma skin cancer) in 2018. In both sexes combined, lung cancer is the most ordinarily diagnosed cancer (11.6% of the entire cases) and therefore the leading explanation for cancer death (18.4% of the entire cancer deaths), closely followed by female breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) for incidence and colorectal cancer (9.2%), stomach cancer (8.2%), and liver cancer (8.2%) for mortality. Lung cancer is that the most frequent cancer and therefore the leading explanation for cancer death among males, followed by prostate and colorectal cancer (for incidence) and liver and stomach cancer (for mortality). Among females, breast cancer is the most ordinarily diagnosed cancer and therefore the leading explanation for cancer death, followed by colorectal and carcinoma (for incidence), and the other way around (for mortality) (Bray et al. 2018).

Lung cancer is the leading explanation for cancer-related deaths worldwide in both men and women. It is categorized into two main histological groups: small cell lung carcinoma (SCLC, 15% of all lung cancers) and non-SCLC (NSCLC, 85% of all lung cancers). NSCLCs are generally subcategorized into adenocarcinoma, squamous cell carcinoma (SqCC), and large cell carcinoma. As depicted in the figure 1, WHO classification divides adenocarcinomas into adenocarcinoma in situ (AIS, preinvasive lesion), minimally invasive adenocarcinoma (MIA), or (overt) invasive adenocarcinoma supported the extent of invasiveness. SqCCs are classified into keratinizing SqCC, non-keratinizing SqCC, and basaloid SqCC. In the 2015 WHO classification, a new category of "neuroendocrine tumors" was established. Invasive neuroendocrine tumors comprise three subtypes: SCLC, large cell neuroendocrine carcinoma (LCNEC), and carcinoid tumor (typical/atypical) (fig:1) (Inamura 2017).

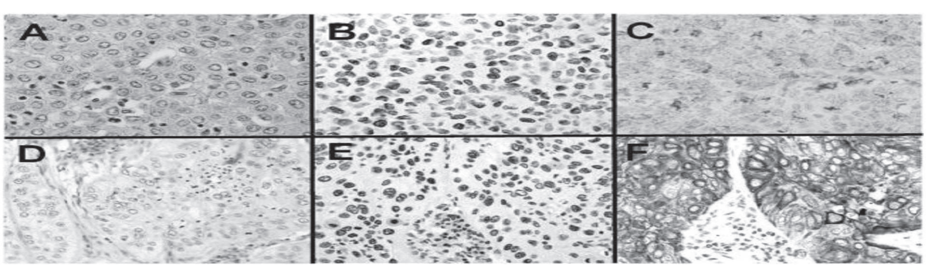


Fig 1: Solid adenocarcinoma (A–C) and non-keratinizing squamous cell carcinoma (SqCC) (D–F) (Lung cancer: understanding its molecular pathology and the 2015 WHO classification. *Frontiers in oncology*, *7*, 193).

The most important cause of lung cancer is exposure to tobacco smoke through active or passive smoking (Alberg and Samet 2003). At least 60 carcinogens are present in tobacco smoke that may be categorized into three groups: polycyclic aromatic hydrocarbons, nitrosamines, and aromatic amines. Additionally, nicotine, a precursor of many tobacco carcinogens, also promotes lung tumorigenesis (Catassi et al. 2008). Metabolic activation of tobacco components, like the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone (NNK), generates metabolites that bind DNA and promote adduct formation. The persistence of DNA adducts leads to somatic mutations in oncogenes and tumor suppressor genes like K-Ras and TP53, which are critical to the formation of lung cancer. Although the genotoxic effects of tobacco carcinogens are well established, tobacco components also can promote lung tumorigenesis through modulation of signal transduction pathways that regulate cell proliferation, transformation and survival.

A549 cells are adenocarcinomic human alveolar basal epithelial cells, and constitute a cell line. A549 cells, as found within the lung tissue of their origin, are squamous and liable for the diffusion of some substances, like water and electrolytes, across alveoli. If A549 cells are cultured in vitro, they grow as a monolayer; adherent or attaching to the culture flask. The cells are able to synthesize lecithin and contain high levels of unsaturated fatty acids, which are important to take care of membrane phospholipids. A549 cells are widely used as a type II pulmonary epithelial cell model for drug metabolism and as a transfection host.

Chemotherapy has been used for cancer treatment already for almost 70 years by targeting the proliferation potential and metastasising ability of tumor cells. Despite the progress made in the development of potent chemotherapy drugs, their toxicity to normal tissues and adverse side effects in multiple organ systems as well as drug resistance have remained the major obstacles for the successful clinical use. Cytotoxic agents decrease considerably the quality of life of cancer patients manifesting as acute complaints and impacting the life of survivors also for years after the treatment. Toxicity often limits the usefulness of anticancer agents being also the reason why many patients discontinue the treatment. The use of phytochemicals not only reduces adverse side effects but improve the effectiveness of chemotherapeutics. Various plant-derived compounds improve the efficiency of cytotoxic agents, decrease their resistance, lower and alleviate toxic side effects, reduce the risk of tumour lysis syndrome, and detoxify the body of chemotherapeutics. The personalised approach using various phytochemicals provides thus a new dimension to the standard cancer therapy for improving its outcome in a complex and complementary way (Sak 2012).

Materials and Methods

Plant Material

- Leaves and stem of Vitex negundo was collected from a botanical garden in Kaloor, Ernakulam.
- The leaves and stem were shade dried at room temperature for 10 days.
- The dried leaves and stem were then powdered by using mixer grinder and used for extraction.

Plant Extraction

- Around 25g of dried and coarsely powdered leaves and stem of Vitex negundo were used for extraction in 4 different solvents with sequential polarity gradient - Petroleum Ether, Ethyl acetate, Chloroform and Methanol (non polar to polar solvents) by using soxhlet apparatus.

- In soxhlet extraction, the powdered plant material were taken in a chamber of soxhlet extractor in between two cotton plugs and the extractor was placed onto a flask and heated at the boiling point of the solvent.

- After boiling, the vapors of the solvent were condensed by a condenser provided with cold water supply and were allowed to fill in the soxhlet chamber. When the solvent was filled till the level of siphon arm, it was collected in the still pot.

- This cycle was repeated till the complete extraction of the plant components was dissolved in the solvents. After many cycles, the desired compound was concentrated in the distillation flask. The purpose of using this system was that instead of many portions of warm solvent being passed through the sample, just one batch of solvent was recycled.

- After extraction, the solvent was removed from the extract by allowing the solvent to vaporize at room temperature.

Media Preparation

DMEM media was purchased from Gibco, Grand Island, N.Y, USA. The media powder was dissolved in 1 litre autoclaved and cooled, double distilled water within the biosafety cabinet. The supplements - HEPES and Sodium bicarbonate were added as per the manufacturer's recommendations and vacuum driven filter sterilized through a 0.22 µm pore sized Polyvinylidene fluoride or polyvinylidene difluoride

(PVDF) filter membrane (Millipore, USA) in a sterile filtration unit (Tarson, India). This stock media is stored in amber bottles at 40C and was supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 10 ml/l1X antibiotic- antimycotic solution to prepare the working media as and when required.

Cell Lines

The cells used for the study were A549 cell line. They were procured from the Cell Repository of the National Center for Cell Science (NCCS), Pune. The cells were adherently cultured in DMEM working media. The cells were grown in incubation at 370C in a humidified 5% CO2 atmosphere (Heal Force CO2 incubator, Shanghai). The cultures were observed in an inverted microscope (Leica DM1L, Germany) and maintained with a media replacement of twice weekly. On reaching confluency they were subcultured to fresh cell culture flasks.

Preparation of Test compounds

Vitex negundo (10 mg) was dissolved in 50µL Dimethyl Sulfoxide (DMSO) by thorough vortexing in separate tubes and mixed with 950µL of Phosphate Buffered Saline and then diluted to different concentration(100µg/ml,200µg/ml &300µg/ml) and standard control 5-FU (50µg/ml) with DMEM medium containing 1% (v/v) FBS for cell culture experiments. The compound was transferred to the eppendorf tube and filtered using 0.22µm Durapore syringe filters (Millipore, Bedford, MA, USA) to the fresh eppendorf tube. The final concentration of DMSO for all treatment was 0.1% (v/v).

MTT ASSAY

Five thousand cells/well were seeded in 96-well plates DMEM with 10% FBS. After 24 hours of incubation at the above mentioned culture conditions, the cells were exposed to three different concentrations of plant extract (100µg/mL200µg/mL and 300µg/mL). 5-FU (50µg/mL) was used as positive control and an untreated group was used as negative control. All reaction groups were taken in triplicates. Following 24 hour incubation with the test compounds, MTT reagent was added in a 10% equivalent volume to that of the total culture volume and further incubated for 4 hours. After the incubation, solubilization solution in an equal volume to that of the culture volume was added and kept aside for 30 minutes in dark at room temperature. Finally absorbance was read at 570nm in an ELISA reader (BioRadiMark microplate reader, USA).

Calculation of Percentage of Cell Viability:

From the absorbance of the various groups, the percentage activity was calculated applying the following formula:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of Treated}}{\text{Absorbance of Control}} \times 100\%$$

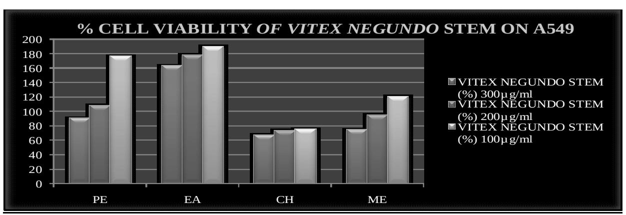
Results

Cytotoxicity Analysis -MTT ASSAY

Cytotoxic effect of Petroleum Ether, Ethyl Acetate, Chloroform and Methanol extracts of Vitex negundo on A549 cell lines were assessed by MTT assay. Leaf and stem parts were used for the assays. The results of the assay are depicted in graph 1 and 2.

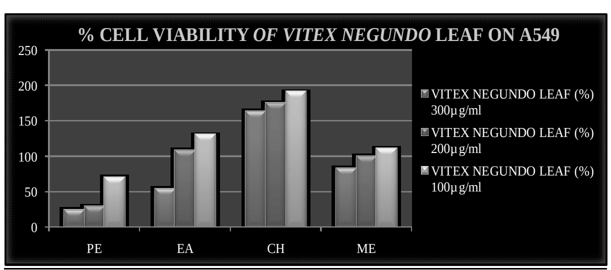
1. Percentage of Cell viability on A549 Cell Line

a) *Vitex Negundo Stem*



Graph 1: Graph showing % of cell viability of *V.negundo* stem in PE, EA, CH and ME extracts at 3 different concentrations (300µg/mL, 200µg/mL and 100µg/mL).

b) *Vitex Negundo Leaf*



Graph 2: Graph showing % of cell viability of *V.negundo* leaf in PE, EA, CH and ME extracts at 3 different concentrations (300µg/mL, 200µg/mL and 100µg/mL).

From the results, it is shown that Vitex negundo stem extract showed more cytotoxicity than the leaf. In comparison with PE, EA, CH and ME stem extract, CH and ME showed more cytotoxicity against A549 cell lines. A549 cell lines exhibited maximum apoptotic activity in chloroform stem extract in all the three concentrations 300µg/mL, 200µg/mL and 100µg/mL followed by methanolic stem extract in 300µg/mL and 200µg/mL. Whereas 300µg/mL petroleum ether stem extract show minimal amount of anticancer activity and ethyl acetate stem extract showed no cytotoxicity. Among Vitex negundo leaf extract, PE (300µg/mL and 200µg/mL) extract showed high amount of cytotoxicity, 300µg/mL of EA and ME extract showed substantial amount of cytotoxicity whereas CH extract showed proliferative activity. In some of the extracts the values exceeded beyond hundred which is assumed to be because of certain metabolites in the plant extract that aid in the proliferation of cells.

Discussion

Chemotherapy is one of the principle modes of cancer treatment. However, drug resistance occurs sooner or later in these tumors and the majority of the patients develop progressive disease. The ever increasing side effects of chemotherapeutic drugs have necessitated the use of natural phytochemicals as an alternative for cancer treatment. There are significant benefits of combining phytochemicals with chemotherapy, either as complementary or adjuvant treatment. They are also used in different treatment regimens that combine chemotherapy, radiation treatment and surgery. Phytochemicals are relatively less toxic and is effective at all stages of cancer. Diverse phytochemicals and various botanical formulations have been characterized as agents that possess potential to regulate apoptosis. Apoptosis is a process by which cells undergo programmed cell death under certain physiological or pathological conditions. Apoptosis and its related signaling pathways have a profound effect on the progression of cancer; therefore apoptosis is targeted in the treatment of various tumors (Pfeffer and Singh 2018).

The present study indicates that the *Vitex negundo stem* extract are potential cytotoxic agents against A549 cell line. Among the various stem extracts of Vitex negundo, the highest cytotoxic activity was observed in CH and ME treated cells followed by PE extract. A549 cell lines exhibited maximum apoptotic activity in chloroform stem extract in all the three concentrations 300µg/mL, 200µg/mL and 100µg/mL followed by methanolic stem extract in 300µg/mL and 200µg/mL. Whereas 300µg/mL petroleum ether stem extract show minimal amount of anticancer activity and ethyl acetate stem extract showed no cytotoxicity.

It is reported that Vitex negundo contain several types of compounds, such as volatile oils, lignans, flavonoids like flavones, luteolin -7 glucoside, casticin, iridoid glycosides, terpenes (triterpenes, diterpenes, sesquiterpenes) and steroids (Chandramu et al. 2003; Sathiamoorthy et al. 2007), alkaloid nishindine, and constituent like Vitamin C, carotene, gluco-nonital, benzoic acid, β-sitosterol and C-glycoside (Husain et al. 1992). Seeds contain hydrocarbons, β-sitosterol, benzoic acid and pthalmic acid (Husain et al, 1992), anti-inflammatory diterpene, flavanoids, artemetin, and triterpenoids (Chawla et al, 2004 Jul 1). Fatty acids, β-sitosterol, vanillic acid, p-hydroxybenzoic acid and luteolin have been isolated from the bark (Husain et al., 1992). Stem bark yields leucoanthocyanidins (Chopra 1956), p-hydroxybenzoic acid and β-sitosterol (Meena et al. 2011). It is reported that ethanolic leaf extract of Vitex negundo contain various metabolites like 5-hydroxy-3, 6, 7-trimethoxy-2-(3, 4-dimtoxypheny)-4H-chrome-4-on and 5, 7-dihydroxy-2-(3, 4-dihydroxyphenyl)-4H-chromen4-one. The methanolic extract also contains, Negundoside, Agnuside, and Vitegnoside (Meena et al. 2011). This in turn shows that the cytotoxic and apoptotic activity exhibited in the present study by the stem and leaf extracts of *Vitex negundo* might be attributed to the individual or combined activity of the identified phytochemicals.

Conclusion

In conclusion, this study demonstrates the antiproliferative activity of the *Vitex negundo* extracts in human lung cancer cell line, A549. Furthermore, the results states that the different extracts of *Vitex negundo* suppress cell growth by inducing apoptosis. Among the various extracts of *Vitex negundo stem* and leaf, stem extract inhibited the proliferation of A549 cell line. Among different extracts, chloroform and methanol extract of *Vitex negundo stem* showed apoptotic activity in a dose-dependent manner followed by ethyl acetate. Whereas, PE leaf extract showed significant antiproliferative activity against A549 cell line. Nevertheless, further studies are required to comprehend the different mechanisms by which the fractions of the chloroform and methanolic extract of Vitex negundo stem exert its cytotoxic and apoptotic effects on HeLa cells.

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