

## Induction of apoptosis by the aqueous and ethanolic leaf extract of *Vitex negundo* L. in MCF-7 human breast cancer cells

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**Abstract-** The aim of this investigation was to evaluate the anti-proliferative potential of aqueous and ethanolic extract from *Vitex negundo* against human breast cancer cell (MCF-7). The aqueous and ethanol extract from *V. negundo* potently inhibited growth of MCF-7 in a concentration-dependent manner. *V. negundo* pretreatment resulted in deferential cell viability and IC50 value were observed in MCF-7 cell line but not in control cell line. The above result suggested that *V. negundo* has a potential benefits in breast cancer cells.

**Keywords-** Anti-cancer, anti-proliferative, breast cancer, cytotoxicity, *V. negundo*, MCF-7

### 1. Introduction

Cancer is the second leading cause of death worldwide and it is expected that around 26.6% of increase in the total number of cancer cases registered in India for the year 2008 [1] while in past years, cancer has been regarded mainly as a group of diseases afflicting the developed countries. Among the incidence of various forms of cancer is now rapidly rising worldwide, the breast cancer continues to be the most commonly diagnosed cancer [2]. The conventional treatment such as surgical resection, radiation therapy, and chemotherapy are not still satisfactory, prevention of this disease or at least stopping it at its inception is important. Because increasing evidence indicates that some plant components can suppress tumor development [3-5], identifying such factory may be an effective, non-invasive strategy for decreasing the incidence and severity of cancer. Medical plants have been used in Asian countries and interest in this area of research has recently increased in all over countries. *Vitex negundo* L. has been one of most widely used and well-documented medicinal plants for centuries [6]. As a traditional medicine, a number of pharmacological activities have been attributed to *V. negundo*, such as analgesic and anti-inflammatory activity [7], enzymes inhibition [8], nitric oxide scavenging activity [9], snake venom neutralization activity [10], anti-feeding activity [11], anti-radical and anti-lipoperoxidative [12], CNS activity [13], hepatoprotective activity [14], antibacterial activity [15], anti-fungal [16], larvicidal activity [17], anti-androgenic effects [18] mosquito repellent activity [19] and anti-diabetic effect [20]. However, in this study, we explored the possibility that extract of *V. negundo* have possesses anti-proliferative effects. We examined whether or not the crud extracts of *V. negundo* inhibit MCF-7 breast cancer cell growth and if the aqueous and ethanolic extract induce apoptosis.

### 2. Materials and methods

#### 2.1. Chemicals

All the chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) fetal bovine serum (FBS) was purchased from GIBCO. 3-(4, 5- Dimethyl thiazol-2yl)-2, 5-dimethyltetrazolium bromide (MTT), propidium iodide were purchased form Sigma chemical company (St. Louis, MO. USA).

#### 2.2. Collection of medicinal plant materials

The medicinal plant used in this study was collected from Ambattur, Chennai, Tamil Nadu, The plant was examined and identified by a botanist from Centre for Advanced Studies (CAS) in Botany, University of Madras, Guindy campus, Chennai, Tamil Nadu, India.

#### 2.3. Preparation of extracts

Excised *V. negundo* leaf was thoroughly washed with tap water followed by sterile distilled water twice, shade dried at room temperature and powdered by using mortal pestle. The powdered plant material was percolated with 500 ml of ethanol and aqueous (5% (w/v), the plant leaf extract was evaporated to dryness under reduced pressure and were stored at -20°C until used. To test the biological activity, the dried crude extracts were dissolved in dimethyl sulfoxide (DMSO) to concentration of 100 mg/ml stock solution; this was later mixed with the culture media (DMEM) to achieve the desired concentration.

#### 2.4. Cell lines and cell cultures

MCF-7 (human breast cancer cell line) was obtained from the National Centre for Cell Science (NCCS), Pune, India. Cells were cultured in DMEM (St. Louis, Mo, USA) supplemented with 10% (v/v) FBS, penicillin 100 µg/ml, streptomycin 20 µg/ml, kanamycin acid sulphate 20 µg/ml and 7.5 % sodium bi-

carbonate solution. The cells were maintained as monolayers in 25 cm<sup>2</sup> plastic tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Exponentially growing cells were used in all the experiments.

### 2.5. MTT assay

The effect of *V. negundo* on cells was determined with MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [21]. Briefly, the cells were plated at a density of 1×10<sup>4</sup> cells/well in 200 µl of culture medium containing 100, 200, 300, 400 and 500 µg/ml freeze-dried aqueous extract in 96-well microtiter plates. A stock solution of ethanolic extract was prepared in DMSO and diluted with the culture media to achieve final concentrations of 100, 200, 300, 400 and 500 µg/ml. The concentration of DMSO remained within the maximum permissible concentration of 0.1% in both control and treated samples were ensured. Each concentration of *V. negundo* was repeated in 10 wells. After incubation for the desired period of time at 37°C in a humidified incubator, cell viability was assessed. MTT assay (50 µl, 5 mg/ml in phosphate – buffered saline stock, diluted to a working strength of 1 mg/ml with media) was added to each well and incubated for 2 hr, after which the plate was centrifuged at 600 g for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. After removal of the medium, 0.1 ml of buffered DMSO was added to each well and plates were shaken. The absorbance was measured at 570 nm in an automated plate reader and percentage of growth inhibition was calculated using the following standard.

$$\text{Inhibitory rate \%} = \frac{\text{Absorption control} - \text{Absorption test}}{\text{Absorption test}} \times 100$$

$$\text{Absorption test}$$

### 2.6. Nuclear morphological examination

MCF-7 cells were plated at 5×10<sup>4</sup> cells/well into a six-well chamber plate. At >80% confluence, the cells were treated with leaf extract of *V. negundo* for 48 hr. The cells were washed with PBS fixed in methanol: acetic acid (3:1v/v) for 10 min and stained with 50 µg/ml of propidium iodide for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined under a fluorescent microscope (Carl Zeiss Axiolab inverted Microscope) and at least 1×10<sup>3</sup> cells were counted to assess apoptotic cell death [22].

### 2.7. DNA Fragmentation Assay

DNA preparation an agarose gel electrophoresis was carried out according to a method previously reported [23]. Cells were cultured in 100 mm dishes treated with extract as described above and extracted for 2 hr in an extraction buffer (50 mM of Tris, pH 7.5, 20 mM of EDTA and 1%

Nonidet P-40). Sodium dodecyl sulfate was then added to 1% and the mixture was incubated with 500 µg/ml of proteinase K for 2 hr at 42°C. The mixture was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and 2.5 volume of absolute ethanol. Equal amounts of DNA samples were electrophoresed on a 1.5% agarose gel in Tris-borate EDTA buffer and visualized by ethidium bromide staining under UV transilluminator.

### 2.7. Statistical analysis

All experiments were repeated three times and then data were shown as means ± standard deviation (S.D) of three assays. Student t- test was applied, and p<0.05 was considered as statistically significant.

## 3. Results

Aqueous and ethanolic extract of *V. negundo* leaves, resembled a dark brown colored paste and powdered which was found to be highly soluble in water, chloroform and ethanol. The plants may be considered as a biosynthetic laboratory, not only for primary chemical compounds such as carbohydrates, proteins and lipids that are utilized as food, but also for a multitude compounds like glycosides, tannins, alkaloids, volatile oils etc., that exert a physiological effect. The compounds that are responsible for therapeutic effect are usually secondary metabolites. The systemic study of the crude drug embrace through consideration of primary and secondary metabolites derived as a result of plant metabolism. Active metabolites of plants contribute for the widely varying physiological functions inhibited by them. In order to understand the effect of aqueous and ethanolic extract of *V. negundo* on breast cancer cell, experiments were conducted using cultured human breast carcinoma cells. Results of the viability test were measured using MTT spectrophotometric assay. This method is based on the quantification of purple-colored formazan, which was formed by the reduction of MTT [3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazoliumbromide]. The reduction of MTT is proportional to the number of active mitochondria in the live cells. Dose-dependent inhibition of MCF-7 cells was observed at different concentrations (100-500 µg/ml) of aqueous and ethanolic extract of *V. negundo* treatment. At 200 and 300 µg/ml, inhibition of MCF-7 cells was found to be 50.45% aqueous (viability) and 50.61% ethanolic extract (viability) respectively, at 24 hr “Fig (1 A)”. Further, the maximal cytotoxic activity at a concentration of aqueous and ethanolic extract of *V. negundo* treatment, at 500 µg/ml, inhibition of MCF-7 cell was found to be 36.44% aqueous (viability) and 39.04% ethanolic (viability) respectively, at 48 hr “Fig (1 B)”. The morphological observation of MCF-7

human breast cancer cells were stained with propidium iodide and morphological examination were observed under the fluorescence microscope. The aqueous extracts treatments were significantly observed the partial apoptosis occurs at the concentration of 300 µg/ml at 48 hr when compared with control "Fig. (2 D)". But in ethanolic extract, the partial apoptosis was observed at the concentration of 200 µg/ml at 48 hr of incubation compared to control "Fig. (2 B)". Maximum apoptosis was observed in cells incubated with higher concentration (500 µg/ml) of both aqueous and ethanolic leaf extract "Fig. (2 C & E)". It may be due to the growth inhibitory effect of aqueous and ethanolic extract of *V. negundo*. To determine whether or not the extract induces apoptosis of MCF-7 cells, we performed a DNA fragmentation assay. DNA isolated from cells treated with 200 µg/ml and 300 µg/ml of extract pattern that is characterize the apoptotic cell death after 48 hr "Fig. (3 B & C)". Maximal apoptosis was recorded after 48 hr exposure of MCF-7 cells to 500 µg/ml of both aqueous and ethanolic leaf extract. These results suggest that *V. negundo* was induced and augmented the apoptosis in cancer cell line MCF-7 treated with both aqueous and ethanolic leaf extract "Fig. (3 E & F)".

#### 4. Discussion

The rationale for selection of *V. negundo* for evaluation of its anti-cancer activity is that it has been used widely for many years as a traditional folk medicine and it has been shown to exert effects on various human illnesses. To date, several bioactive compounds from various medicinal plants have been identified that show anti-oxidant, anti-inflammatory and in some instances anti-cancer effects [24]. In our study, the aqueous and ethanolic extracts of *V. negundo*, rich in plant constituents may cause cell growth inhibition and induce apoptosis differentially in cancer cells. Apoptosis is a well-identified biological response exhibited by cells after suffering DNA damage and is a useful marker for screening compounds for subsequent development as possible anticancer agents [25]. In this study, MCF-7 human breast cancer cells were treated with aqueous and ethanolic extract of *V. negundo*. The cell growth inhibitory effects of this plant crude extract support to exert their anti-cancer effects in vitro. Similarly, these results are consistent with previous studies [26, 27] and previously reported studies indicated that glycosidic compounds are possessing potent anti-carcinogenic properties in vivo [28, 29]. These compounds are able to inhibit cancer initiation, possibly through the induction of hepatic detoxifying enzymes as well as through their anti-oxidant properties. Flavonone glycons have anti-proliferative activities against many human cell lines like OCM-1, MCF-7 and HT-29

[30]. In our present investigations have demonstrated the *V. negundo* crude extract possesses significant anti-cancer properties and causes selective growth inhibition and apoptosis in cancer cells. We observed the maximum growth inhibitory effects (49.39%) on MCF-7 human breast cancer cells at 300 µg/ml of aqueous extract and 200 µg/ml in the case of ethanol extract. In our experiments, we cannot attribute the differences in biological effects observed between the ethanolic and aqueous *V. negundo* extract. In the present investigation, the anti-cancer activity of *V. negundo* extract as a whole. Our data suggests that these extracts may show the greater effects than the individual constituents present in plant extracts. On the basis of the findings of this study, we are currently undertaking extensive experiments to characterize and identify bioactive compounds from *V. negundo* and their anti-cancer effects on cancer cell, MCF-7 human breast cancer cells in vitro. An important investigation leads to the possibility that synergistic combination of biologically active agents may exist naturally in plants, and to investigate whether such combinations may be clinically useful for anti-cancer and apoptotic agents.

#### Acknowledgements

The authors acknowledge the National Centre for Cell Science (NCCS), Pune for providing cancer cell lines.

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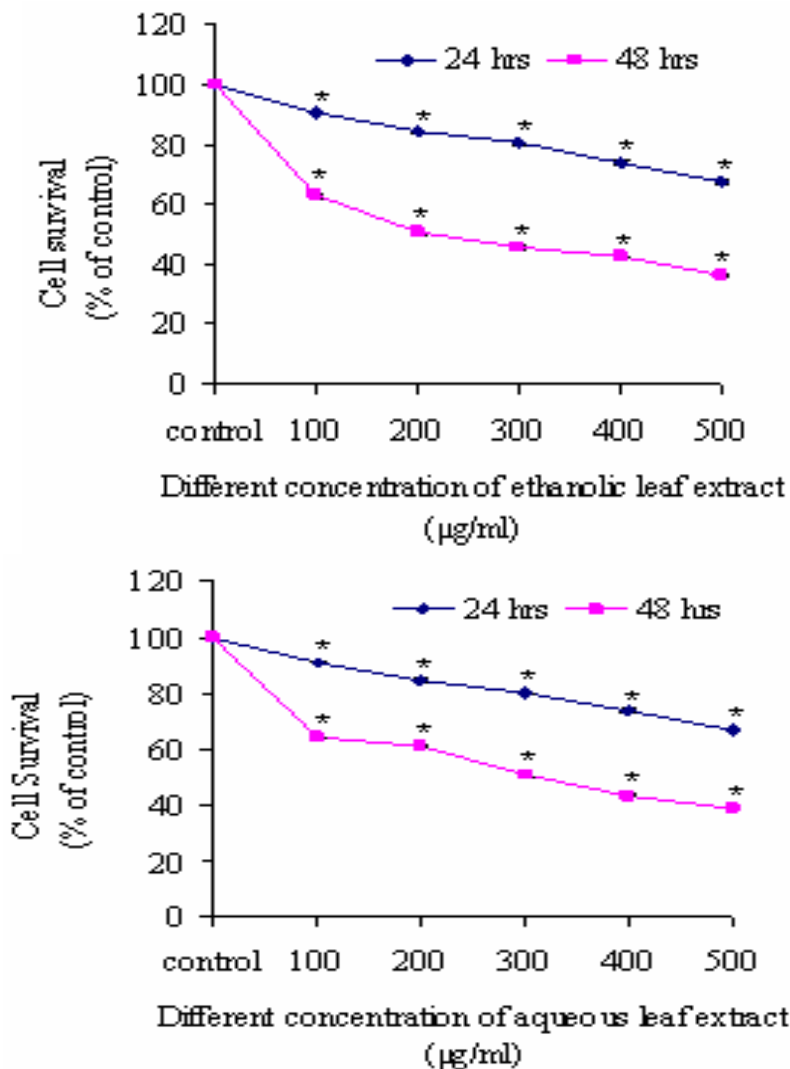


Fig. 1- Effect of *V. negundo* at different concentration of aqueous and ethanolic extract on the growth of MCF-7 cells. A- Ethanolic; B- Aqueous. Cell survival was measured using MTT assay and expressed as percentage of viable cells of treated samples to untreated control samples. Data are represented by mean  $\pm$  SD of three independent experiments each performed in tetrads.

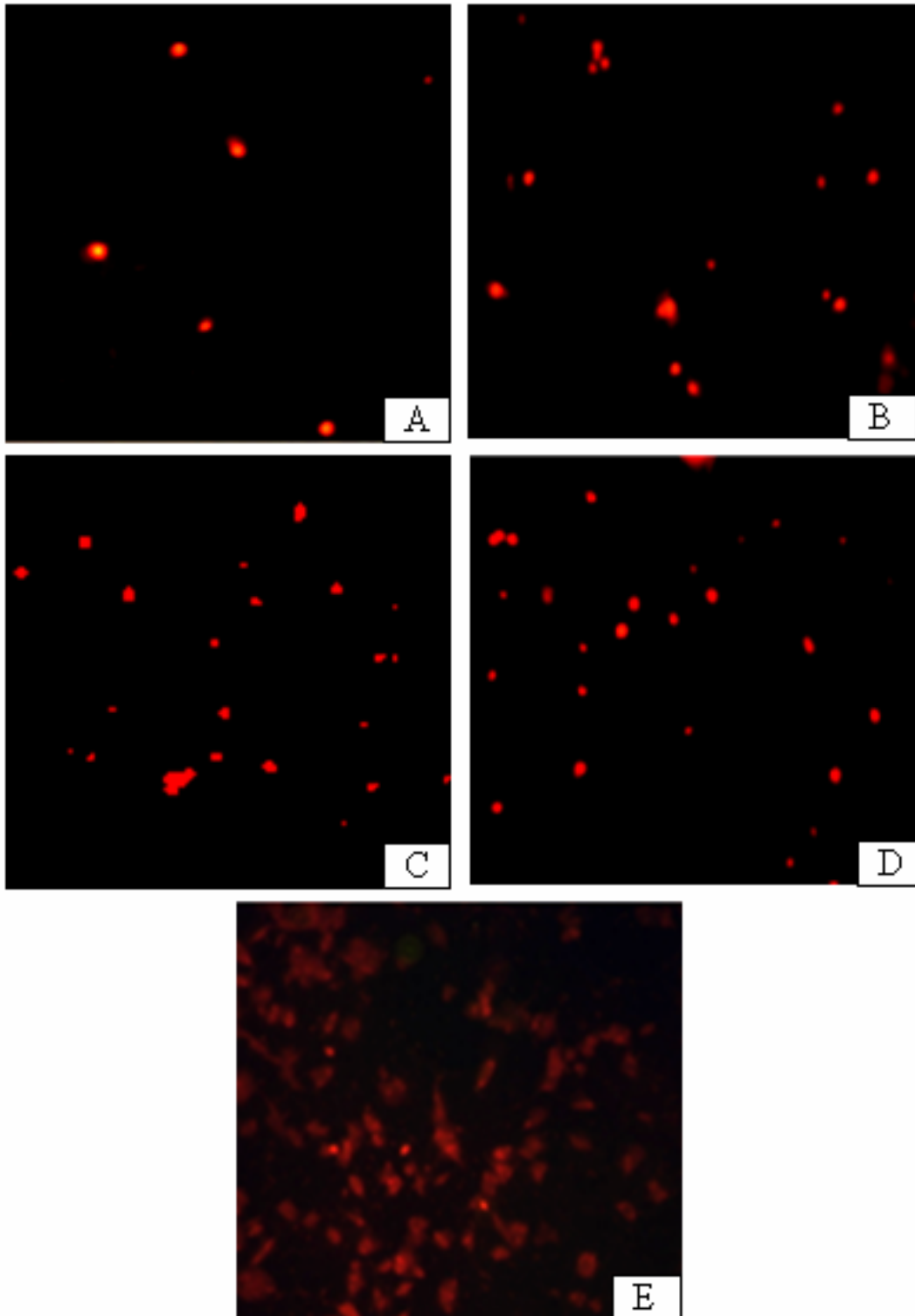


Fig. 2-Morphological changes and the number of apoptotic nuclei in MCF-7 after treatment with *V. negundo* for 48 hr. A-Control; B-200 and C-500 µg/ml of ethanolic leaf extract and D- 300; E - 500 µg/ml of aqueous leaf extract after 48 hr of incubation respectively.

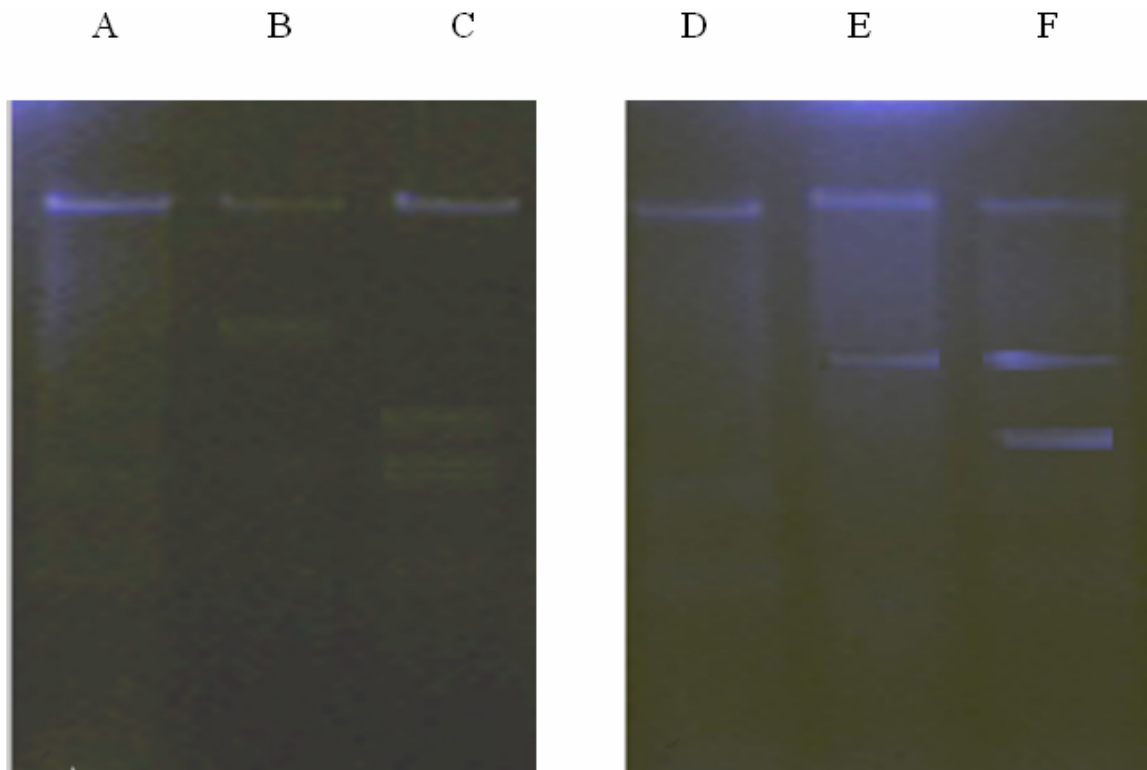


Fig. 3- DNA fragmentation in aqueous and ethanolic extract of *V. negundo* treated with MCF-7 cells. A & D- Control; B & C- *V. negundo* at 200 & 500 µg/ml ethanolic leaf extract and E & F -*V. negundo* at 300 & 500 µg/ml aqueous leaf extract for 48 hr of incubation respectively.