



***In Vitro* Analysis of Anethole as an Anticancerous Agent for Triple Negative Breast Cancer**

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ABSTRACT

Triple negative breast cancer (TNBC) is a type of cancer that has none of the three markers that are currently used for targeted chemotherapy. These markers are estrogen receptor (ER), progesterone receptor (PR) and HER2. In the present study anethole, an aromatic compound from anise and fennel has been tested with the TNBC cell line MDA-MB-231. Cytotoxicity assays were done to determine and optimize the dose. The events of apoptosis and DNA damage were analyzed by various staining techniques and comet assay respectively. The cytotoxicity assay (MTT assay) and cell viability assay (SRB assay) shows that anethole acts effectively against the cancer cell proliferation. Apoptotic cells were detected with DAPI, PI, AO/EtBr and Giemsa staining. The DNA damage was successively measured using comet assay. The present study clearly illustrated the phytochemical anethole possesses the anticancer activity against triple negative breast cancer.

Keywords: Anethole, DNA fragmentation, MTT assay, SRB assay, Triple-negative breast cancer.

INTRODUCTION

Cancer is considered as the second leading cause for death worldwide. Cancer can be characterized by the failure in the regulation of tissue growth results in the uncontrolled multiplication of the normal cells to form tumors which in further invades into nearby parts of the body.¹ Breast cancers are routinely classified by stage, pathology, grade and expression of estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor (Her2/neu). Current successful therapies include hormone-based agents that directly target these receptors.²

Triple-negative breast cancer (TNBC) is a heterogeneous subset of neoplasms that is defined by the absence of these targets.³ Approximately 15% of globally diagnosed breast cancers are designated as ER⁻, PR⁻ and Her2/neu⁻ negative.⁴ Hence there is an urge for the development of new anticancer drug for its treatment and prevention. Natural products from plants play a dominant role in the discovery of such new drugs.⁵ It has been estimated that about 60% of approved drugs were of natural origin.

Anethole, 1-methoxy-4-(1-propenyl) benzene, is the principle aromatic compound found in star anise. For the past decade, numerous studies have been conducted with anethole and its analogs to uncover the potential anti-cancer effects in natural spices. The purpose of this study was to determine the ability of anethole to selectively target the TNBC subtype of breast cancer cells, assessed by its effects on the growth, survival, and tumorigenesis of a representative panel of TNBC cells.

MATERIALS AND METHODS

Collection and culturing of cell lines

The MDA-MB-231 cell lines were collected from the National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in 20% Dulbeccos Minimal Essential Medium (DMEM) containing 10% foetal bovine serum (FBS). The culture medium was supplemented with antibiotic 1µl of Penstrep. The cultures were maintained in 25cm² culture flasks with the growth condition maintained at 37⁰ C and 5% CO₂ in an air jacketed CO₂ incubator.

Once a confluent monolayer was obtained the cells were removed by trypsinization and seeded in 6 well plates with cover slips placed in each well and in 96 well plates. The cells were allowed to grow by incubating in 5% CO₂ and 95% humidity to monolayer and then subjected to various assays.

Measurement of cell viability and cytotoxicity

The extent of cell survival and cytotoxicity of the compound was quantified using the MTT assay and anti proliferative effect by the SRB assay.

MTT Assay

The influence of anethole in cell proliferation of MDA-MB-231 and hence the IC₅₀ value of the compound was assessed by MTT assay.⁶

SRB Assay

The SRB assay provides rapid and sensitive method for measuring the cytotoxicity and metabolic activity of the cells in the presence and absence of etoposide, with or without anethole at different concentrations.⁷



Measurement of apoptotic index

We analyzed the apoptotic effect of anethole on MDA-MB-231 cells by Giemsa stain, acridine orange/ethidium bromide stain propidium iodide and DAPI stain.

Giemsa staining

The morphological changes in the cells were followed in the presence and the absence of the anethole and/or the oxidant. The cells were fixed and stained with giemsa for 10 minutes and observed under the phase contrast microscope.⁸

Acridine orange/Ethidium bromide staining

Apoptotic cells were identified by AO/EtBr staining.⁹ The combination of AO/EtBr staining technique is used to differentiate apoptotic and normal cells.

Propidium iodide (PI) staining

The nuclear changes in the anethole treated MDA-MB-231 cells were observed by Propidium iodide staining.¹⁰

DAPI Staining

Apoptotic cells were detected with DAPI (4'-6'-diamidino-2-phenyl indole) staining technique.¹¹

Measurement of DNA damage

We analyzed the effect of anethole on DNA damage of MDA-MB-231 cells by single cell gel electrophoresis or comet assay.

Comet assay

Comet assay was performed according to protocol.¹² Approximately 1×10^5 MDA-MB-231 cells were seeded in 24-well tissue-culture plates and after 24 hr treated with various concentration of anethole in the presence and absence of etoposide for 24 hr. The comet tail lengths were analyzed by CASP software.

Statistical analysis

The parameters analyzed in all the phases of the study were subjected to statistical treatment using SigmaStat statistical package. All measurements were expressed as mean \pm standard deviation. Statistical significance was determined by one-way ANOVA. Values of $p < 0.05$ were considered significant.

RESULTS

Cancer is considered as the serious health problem worldwide and various natural compounds from the plants were analyzed for their anticancer effects by scientists. Medicinal plants represent a vast potential resource for anticancer compounds. The results obtained are presented below.

Cytotoxicity Assay

Anti proliferative effect of anethole was evaluated using MTT assay. The IC_{50} values of anethole concentrations that kill 50% of treated cell lines compared to untreated cells was found to be 50 μ M (Figure 1). The viability of the

cells drastically reduced in groups treated with anethole and etoposide. The effect of anethole treated group also showed a reduction in cell viability with less toxicity when compared with other groups (Figure 2). It indicates the anethole has anti proliferative activity towards MDA-MB-231 cell line at minimum concentration.

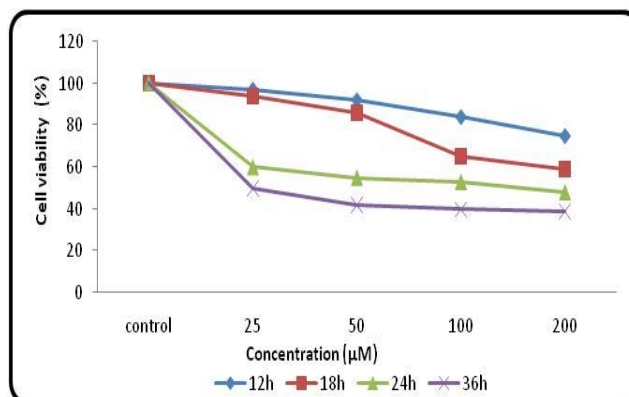


Figure 1: Time - dose dependent effect of anethole on proliferation of MDA-MB-231 cells by MTT assay. MDA-MB-231 cells were exposed to anethole (25 to 200 μ M) for 12h, 18h, 24h and 36h and cell viability was then determined by methyl thiazolyl tetrazolium assay (MTT). Data are the mean \pm SD of at least three independent experiments performed in triplicate.

The assay for cytotoxicity using SRB provides a sensitive method for measuring the viability of the cells. In the present investigation, Cells treated with etoposide and anethole extensively decrease the metabolic activity of cancer cells. The combined effect of drug and anethole was found to reduce the metabolic rate of the cells to much lower extent compared to the control suggesting its use along with standard drug (Figure 2).

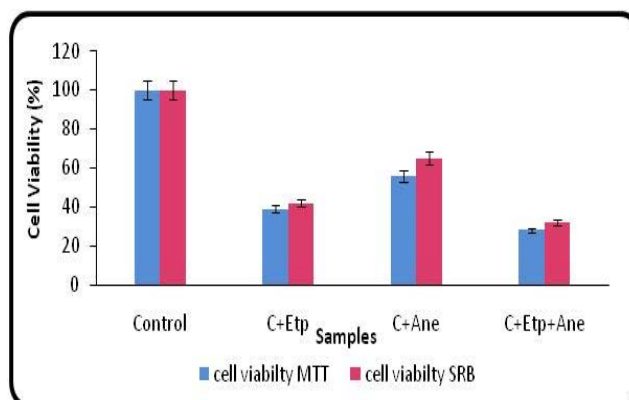


Figure 2: Effects of anethole on the survival of MDA-MB-231 cells by MTT assay and SRB assay

MDA-MB-231 cell lines were incubated with anethole in the presence and absence of etoposide for 24 hours. Cell viability was measured by MTT assays and SRB assays. The graph displays the mean \pm SD (standard deviation) of three independent experiments. The viability of control group is fixed at 100%.

Abbreviations: C - Control, Etp - Etoposide, Ane - Anethole.

Hussain analyzed viability and proliferation of diffuse large B cell lymphoma (DLBCL) by MTT assay and found that the cancer cell proliferation and viability was reduced in dose dependent manner on treatment with resveratrol, a stilbenoid present in the skin of red grapes.¹³ Our studies are in accordance with the above findings. These findings are also in agreement with¹⁴ who reported that an isolated flavonoid Quercetin from the rhizome of *Smilax china* showed appreciable antiproliferant activity in a rapidly multiplying human keratinocyte (HaCaT) cell line. Sumathi investigated the cytotoxic activity of latex of *Euphorbia antiquorum* on mouse spleen cells and found that latex milk extract was not toxic to normal cells.¹⁵

Measurement of apoptotic index

Morphological features of apoptotic cells include shrinkage, condensation of chromatin and cytoplasm, detachment of the cells from the neighbouring cells, fragmentation of the nucleus and membrane blebbing. These features were observed by Giemsa staining. Anethole and etoposide combinatorial drug treatment group showed more blebbing and cell shrinkage when compared with other groups. The Acridine orange /Ethidium bromide staining showed that the compound anethole induced apoptosis in MDA-MB-231 cancer cells. The results showed that the nuclear region of viable cells is uniformly green and that of the apoptotic cells are orange with bright dots corresponding to nuclear chromatin fragmentation.

Nuclear changes such as chromatin condensation around the nuclear membrane were noticed by propidium iodide and DAPI staining. The result showed that anethole along with etoposide induced membrane damage mediated cell death in MDA-MB-231 cells.

Calcitriol induced apoptosis associated morphological changes in human bladder squamous and transitional carcinoma (SCaBER and T24) cell lines.¹⁶ These results are in agreement with¹⁷ who reported that methanolic extract of *Prosopis cineraria* induce apoptosis in MCF-7 breast cancer cells. Similarly PI staining was used and identified increased apoptotic population in carvacrol treated human hepato cellular carcinoma cell line.¹⁸ Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria-dependent pathway in human osteosarcoma U-2 OS cells as examined by DAPI staining.¹⁹ The apoptotic ratio was calculated for each treatment group and presented in Figure 3.

Various staining like Giemsa, AO/EtBr, propidium iodide and DAPI proved that anethole induce apoptosis in the triple negative breast cancer cells (Figure 4).

Measurement of DNA damage

Comet assay

The results obtained by comet assay indicated the extent of DNA damage. By treating the MDA-MB-231 cells with anethole in the presence and absence of etoposide drug the extent of DNA damage was determined. The length

and area of the DNA tail induced by anethole was shorter and smaller when compared to etoposide treated cells. The comets were longer and larger when cells were treated with etoposide and anethole together. The quantitative comet assay data for DNA damage in MDA-MB-231 cells are shown in Figure 5.

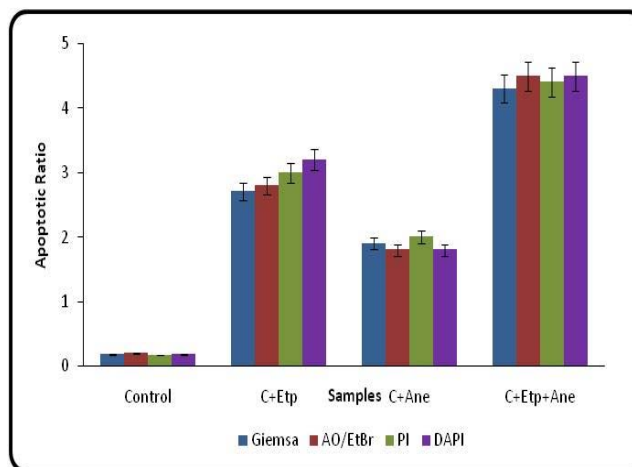
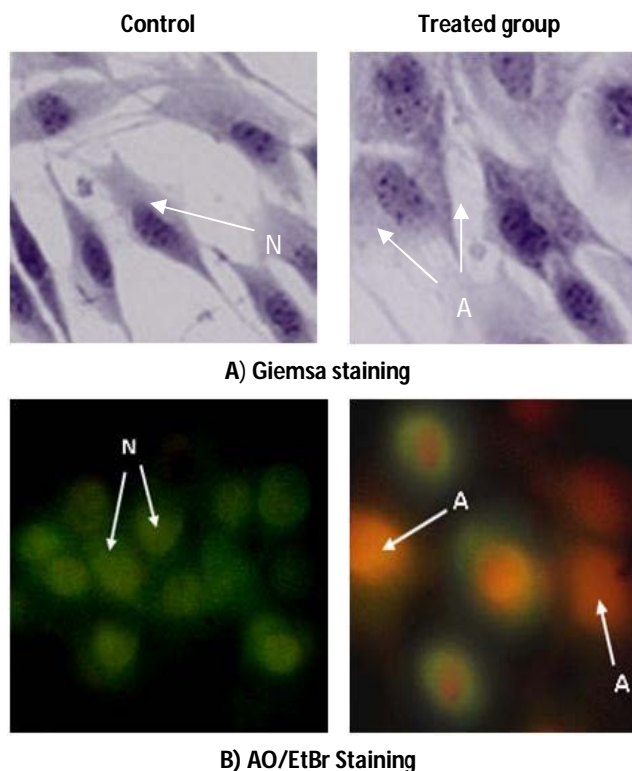
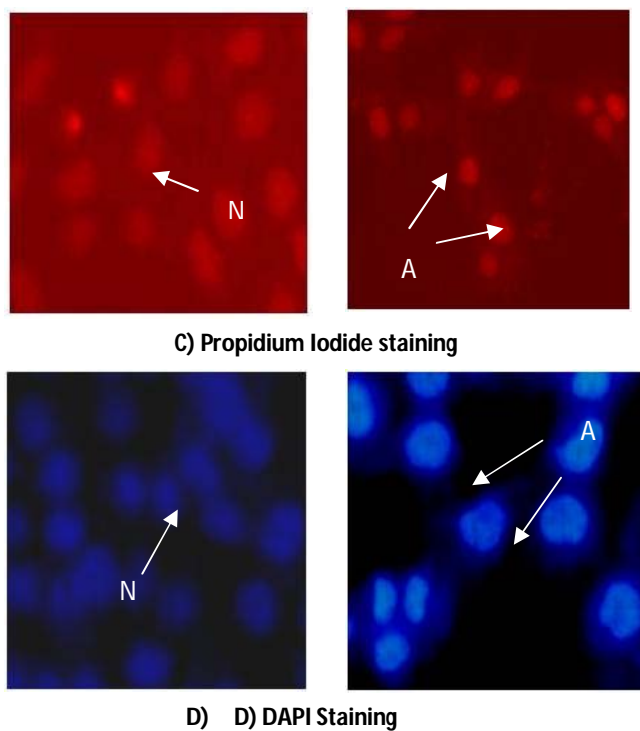


Figure 3: Effects of anethole on the apoptotic ratio of MDA-MB-231 cells by Giemsa, AO/EtBr, Propidium iodide and DAPI staining. Graph showing the % of apoptotic cells induced by the treatment with anethole by various staining on MDA-MB-231 cancer cell lines. The graph displays the mean \pm SD (standard deviation) of three independent experiments, *-significant at $p < 0.001$ vs Control, ** c-significant at $p < 0.001$ Vs anethole treated group.

Abbreviations: C - Control, Etp – Etoposide, Ane – Anethole.





N-Normal cells, A- Apoptotic cells

Figure 4: Effect of anethole on MDA-MB-231 cells by various staining techniques

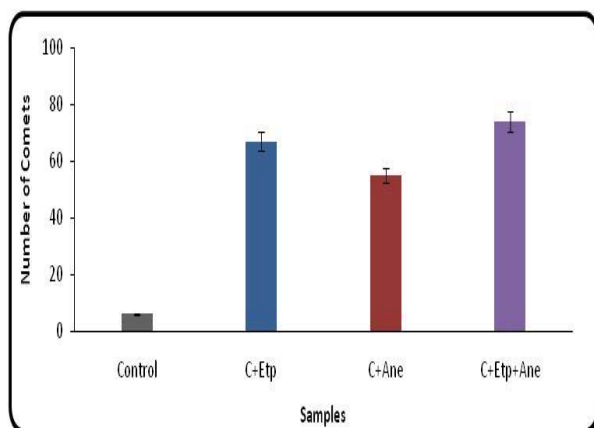


Figure 5: Effects of anethole on DNA damage of MDA-MB-231 cells by comet assay

DNA damage index of MDA-MB-231 cells lines treated with anethole analyzed by the comet assay. The graph displays the mean \pm SD (standard deviation) of three independent experiments,

*-significant at $p < 0.001$ Vs Control, **-significant at $p < 0.001$ Vs anethole treated group.

Abbreviations: C - Control, Etp – Etoposide, Ane – Anethole

The results showed more DNA strand breaks (longest tail length) with the combinatorial treatment of anethole and etoposide and least damage of DNA with anethole (Figure 6). These results are in agreement with²⁰ who reported that coronaridine exhibited greater cytotoxic activity in the laryngeal carcinoma cell line Hep-2 and also caused minimal DNA damage by inducing apoptosis in cell lines at

high concentrations. The results from the comet assay confirmed that quinacrine caused DNA damage in a dose-dependent manner in MCF-7 cells.²¹

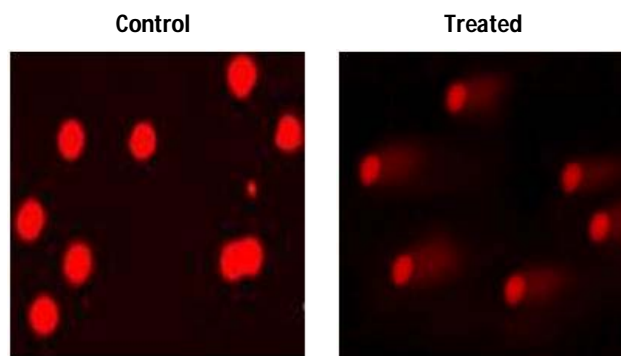


Figure 6: Effect of anethole on the induction of DNA in MDA-MB-231 cells lines. Images of control, anethole treated MDA-MB-231 cells showing comet formation.

In summary, the result data clearly indicated that the anethole can exert significant effects on cell proliferation, cytotoxicity and apoptotic reactions in triple negative breast cancer cells.

CONCLUSION

In conclusion, our results demonstrated that phytochemical anethole has the compound anethole have potential apoptotic activity and also promise to act as new drug candidate for triple negative breast cancer MDA-MB-231 cells. It can be used to treat triple negative breast cancer as a phyto therapeutic agent. It also justifies and validates the combination of standard chemotherapeutic drug with plant compound anethole to improve anticancer therapy.

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