IJPSR (2018), Volume 9, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 23 June, 2017; received in revised form, 05 June, 2018; accepted, 08 July, 2018; published 01 August, 2018

CYTOTOXICITY AND *IN-SILICO* STUDIES OF ANETHOLE IN TRIPLE NEGATIVE BREAST CANCER CELLS

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Keywords:

Triple negative breast cancer, Anethole, *In-silico*, Docking

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ABSTRACT: Triple negative breast cancer (TNBC) is a type of breast cancer in which receptors such as estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 are absent. Triple-negative breast cancer is a high risk breast cancer that lacks the benefit of specific therapy that targets these receptors. In the present study, anethole an aromatic compound from star anise was exposed to TNBC cell line MDA-MB-231 to assess its anticancer property. Cytotoxicity assay was done to determine and optimize the dose. DNA damage was analyzed by DNA fragmentation method. The use of targeted agents against TNBC such as poly-ADP-ribose and insulin like growth factor receptor were investigated in the present study. The drug-target interaction was investigated using *in-silico* docking studies. The *in-silico* study provides evidence for the interaction of anethole with the target proteins namely poly ADP-ribose polymerase (PARP) and insulin like growth factor receptor (IGFIR). This interaction is presumably vital in exerting the anticancerous activity. The present study clearly elucidates that anethole possesses anticancer activity against the TNBC.

INTRODUCTION: Triple-negative breast cancer is characterized by tumors that do not express estrogen receptor (ER), progesterone receptor (PR), or HER-2 genes and represents an important clinical challenge because these cancers do not respond to endocrine therapy or other available targeted agents. The metastatic potential in triple-negative breast cancer is similar to that of other breast cancer subtypes, but these tumors are associated with a shorter median time to relapse and death [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(8).3414-19

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(8).3414-19

Currently, there is no preferred standard form of chemotherapy for TNBC, and treatment should be selected as it is for other cancer subtypes. The use of targeted agents against triple-negative breast cancer is currently being investigated. Poly-ADP-ribose polymerase (PARP) inhibitors have recently shown very encouraging clinical activity in early trials of tumors arising in BRCA mutation carriers and in sporadic triple-negative cancers [2].

The Insulin-like growth factor (IGF) is involved in tumorigenesis and the proliferation, survival and migration of tumor cells. High circulating IGF-1 concentrations and low blood IGF binding protein concentrations are risk factors for several types of cancer including breast cancer. The IGF signalling axis has been shown to play critical role in progression and development of various cancers [3]. Hence, the target proteins such as PARP and IGFIR

were selected for *in-silico* studies. As chemotherapy has lot of side effects, natural products are gaining more importance. Anethole, an aromatic compound of star anise, has demonstrated both anti and pro-cancerous effects depending on the estrogen receptor statuses in individual cell lines. Anethole suppressed TNF-induced both lipid peroxidation and ROI generation, which may explain its role in suppression of inflammation and carcinogenesis.

Besides their anti-inflammatory property, anethole exhibit chemopreventive activities indicated by suppression of the incidence and multiplicity of both invasive and noninvasive adenocarcinomas [4]. The present study was aimed to analyze the anticancer activity of anethole in breast cancer cells and to investigate the mechanism of drug-target interaction using *in-silico* docking studies.

MATERIALS AND METHODS: Triple negative breast cancer MDA-MB-231 cell line was obtained from National Centre for Cell Science (Pune, India) and revived 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 25 cm^2 culture flasks with the growth condition maintained at 37 ºC and 5% CO_2 in an air jacketed CO_2 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% CO_2 and 95% humidity to monolayer and then subjected to various assays. Standard chemotherapeutic drug etoposide (100mg/ 5ml) was used for comparing the effect of the compound with the standard drug.

Measurement of Cytotoxicity:

Lactate Dehydrogenase Assay: Lactate dehydro-genase (LDH) can be used to assess cytotoxicity resulting from chemical compounds or environmental toxic factors. LDH is an enzyme widely present in cytosol that converts lactate to pyruvate. When plasma membrane integrity is disrupted, LDH leaks into culture media. Quantifying the level of LDH in the medium is carried out using LDH- Cytotoxicity assay kit.

Measurement of DNA Damage:

DNA Fragmentation: DNA fragmentation which is the hall mark of apoptosis occurs at the late phase of apoptosis. The DNA isolated from anethole treated as well as untreated cells were subjected to agarose gel electrophoresis and observed for the electrophoretic pattern occurring during apoptosis. This was done by the method proposed by Yin *et al*., (1994) [5] with slight modifications.

***In silico* Studies on Anethole:**

PubChem: The PubChem Compound Database contains validated chemical depiction information provided to describe substances in PubChem substance. Structures stored within PubChem Compounds are pre-clustered and cross-referenced by identity and similarity groups. Anethole structure was retrieved from the PubChem Compound Database.

Selection of the Target Proteins: Triple negative breast cancer (TNBC) has a poor outcome due to the lack of beneficial therapeutic targets. PARP (poly ADP-ribose polymerase) inhibitors, insulin-like growth factors (IGFs), vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and G-protein coupled receptor (GPCR) are some of the targets used in clinical trials are trying to find out whether specific targeted therapy against triple-negative breast cancer. Hence, PARP and IGFs were selected for the docking studies.

Protein Data Bank: The Protein Data Bank (PDB) is a repository for the 3-D structural data of large biological molecules, such as proteins and nucleic acids. The structure of Poly ADP-ribose polymerase (PARP) and Insulin like growth factor receptor (IGF-1R) was retrieved from the protein data bank.

Molecular Docking: Molecular docking procedure consists of the following steps:

1. Ligand preparation
2. Protein preparation
3. Receptor grid generation
4. Ligand docking procedures

The docking was done in Standard precision mode (SP), which is appropriate for screening ligands of

unknown quality in large numbers. The docked protein and the ligands were viewed with Glide Pose Viewer. The images of the best docked poses of the ligand and the protein were saved as .jpg files.

Statistical Analysis: The parameters analyzed in the study were subjected to statistical treatment using Sigma Stat statistical package. All measurements were expressed as mean ± standard deviation. Statistical significance was determined by one-way ANOVA. Values of $p<0.05$ were considered significant.

RESULTS AND DISCUSSION:

Lactate Dehydrogenase Assay: LDH release has been considered as a very reliable marker of cell lysis due to membrane damage, indicating cyto-toxicity. LDH leakage assay is based on the release of the enzyme into the culture medium after cell damage. The effect of anethole on LDH release in MDA-MB-231 cells was assessed. The percent of LDH released into medium after 24 h treatment was 56.6% and in combination with etoposide it was 64.3%. A chart was plotted using the percentage of cytotoxicity in Y axis and samples in X axis. The result is shown in **Fig. 1**.

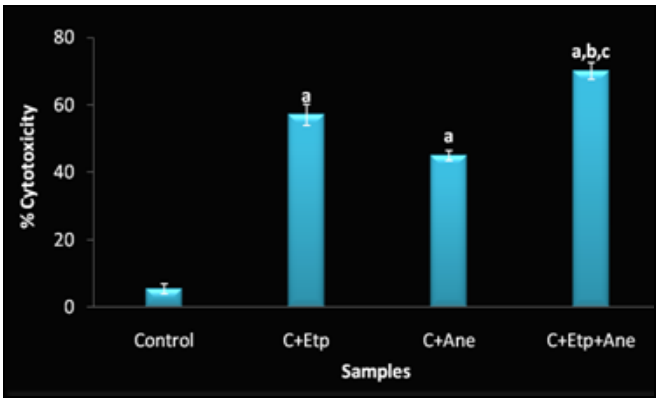

FIG. 1: EFFECT OF ANETHOLE ON THE SURVIVAL OF MDA-MB-231 CELLS BY LDH ASSAY

C - Control, Etp - Etoposide, Ane - Anethole. The values are mean ± Standard deviation of triplicates. a - Statistically significant ($p<0.001$) compared to untreated group. b - Statistically significant ($p<0.001$) compared to etoposide treated group. c - Statistically significant ($p<0.001$) compared to compound treated group

When anethole was combined with standard drug etoposide, cells released significantly higher levels of LDH into the medium due to over stress by mitochondrial dysfunction and showed that the more significant cytotoxicity to the MDA-MB-231 cells. Anethole treated groups alone show considerably slightly lower toxicity.

Measurement of DNA Damage:

DNA Fragmentation: DNA fragmentation into repeated oligonucleosomal fragments is the best-characterized biochemical markers of apoptosis. In the present study the extent of DNA damage by fragmentation was quantified in MDA-MB-231 cells exposed to etoposide in the presence and absence of anethole for 48 h. The extent of DNA damage was documented using gel documentation system. The integrated density value (IDV) of the bands is listed in **Table 1**.

TABLE 1: INTEGRATED DENSITY VALUE (IDV) OF THE BANDS IN THE AGAROSE GEL OF DNA FRAGMENTATION

Sample	IDV of the bands	
	Control	Etoposide
No compound	86420	43623
		40827
Anethole	65326	30273

Sankaranarayanan *et al*., (2013) [6] observed that lupeol compound induced DNA fragmentation and induced apoptosis in human lung cancer cells (A-549). DNA fragmentation of human bladder squamous and transitional carcinoma cell lines bladder cells (SCaBER and T24) was detected after exposing to calcitrol for 24 h. It was clearly observed in the SCaBER and T24 cells, whereas untreated control cells did not provide any ladders[7] as observed in our present study.

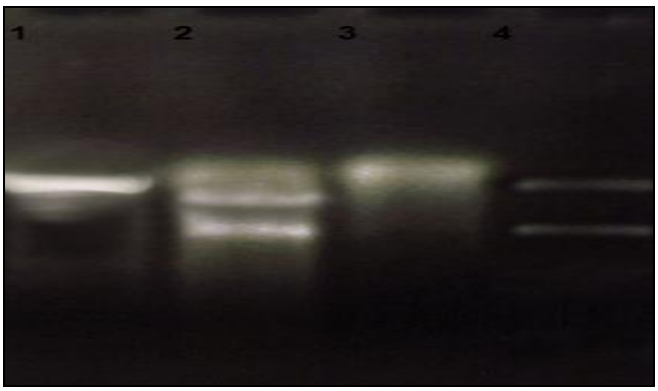

PLATE 1: DNA FRAGMENTATION BY AGAROSE GEL ELECTROPHORESIS

Lane- 1 Control, 2 Cells + Etoposide 3, Cells + Anethole, 4 Cells + Etoposide + Anethole

***In-silico* Studies:** *In-silico* screening of the ligands and/or of the receptors has become an essential tool to facilitate the drug discovery process but

compound collections are needed to carry out such *in-silico* experiments. The compound, anethole, was subjected to *in silico* studies for its efficacy against the target proteins present on the triple negative cancer MDA-MB-231 cells. The target proteins Poly (ADP-ribose) polymerase (PARP) and Insulin-like growth factor receptor (IGF1R) were selected. The 3D structures of the target proteins were obtained from the Protein Data Bank and the structures were refined using the protein preparation wizard module. The molecular docking and ADME studies were performed to characterize the active components.

ADME Studies: To be an effective drug, a compound not only must be active against a target, but also possess the appropriate ADME (Absorption, Distribution, Metabolism and Excretion) profile necessary to make it suitable for use as a drug. QikProp 3.0 module of Schrödinger predicts physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. The QikProp results of ligand, anethole is given in **Table 2**.

The QikProp results for anethole showed that the compound does not violate the Lipinski's rule of five, which is a refinement of drug likeness and is used to predict whether a chemical compound will have pharmacological or biological activity as an orally active drug in humans. The percentage of human oral absorption of anethole in gastro-intestinal tract is very high. The lipophilicity is expressed as the partition co efficient P in octanol/ water. Anethole is found to be lipophilic, indicating good absorption and distribution. Therefore, the compound anethole possesses good pharmacological activity. It was then subjected to docking using Glide.

TABLE 2: ADME RESULTS OF THE LIGANDS USING QikProp

S. no.	Descriptors	Standard values	Ligand values
1	Molecular weight (Da)	130.0-725.0	148.204
2	Number of hydrogen bond donors	0.0/6.0	0.0
3	Number of hydrogen bond acceptors	2.0-20.0	0.75
4	QP log P for octanol/water	-2.0/6.5	3.140
5	Apparent Caco-2 Permeability (nm/sec)	<25 poor, >500 great	9906
6	Apparent MDCK Permeability (nm/sec)	<25 poor, >500 great	5899
7	Lipinski rule of 5 Violations	(maximum is 4)	0
8	% Human oral absorption in GI (±20%)	(<25% is poor)	100%
9	Qualitative model for human oral absorption	(>80% is high)	high

Molecular Docking Using Glide: The ligand anethole was docked to the prepared proteins with Glide in standard precision mode. A correlation was calculated by Glide score. For the prediction of results mainly four parameters are considered namely G-score, Glide energy, H-bonds and Good interactions, which indicates the binding affinity of ligand towards receptor.

Poly (ADP-ribose) Polymerase (PARP) and Insulin-like Growth Factor Receptor (IGF1R): The target proteins present in the triple negative breast cancer cells Poly (ADP-ribose) polymerase (PARP) and Insulin-like growth factor receptor (IGF1R) were docked with anethole.

The binding affinity of anethole with the target proteins and the top ranked poses generated by Glide SP docking are given in **Table 3**. The docking efficiency and the molecular interactions showing good contacts of anethole with PARP and IGF1R are depicted in **Fig. 2**, **3**, **4**, and **5** respectively.

Table 3 shows that anethole showed good glide score of -5.31 and -5.74 with targets PARP and IGF1R respectively. The ligand also possessed a good minimum energy, among which anethole docked with PARP possessed the highest minimum energy of -32.94. Anethole showed more good contacts with the PARP protein than.

TABLE 3: GLIDE SP DOCKING OF THE LIGANDS WITH THE TARGET PROTEIN PARP AND IGF1R

S. no.	Descriptors	Ligand docked with	
		PARP	IGF1R
1	GLIDE score	-5.31	-5.74
2	Energy (kcal/mol)	-32.94	-30.83
3	Pose number	68	61
4	Conformation number	1	1
5	Good contacts	179	173
6	H Bonds	0	0

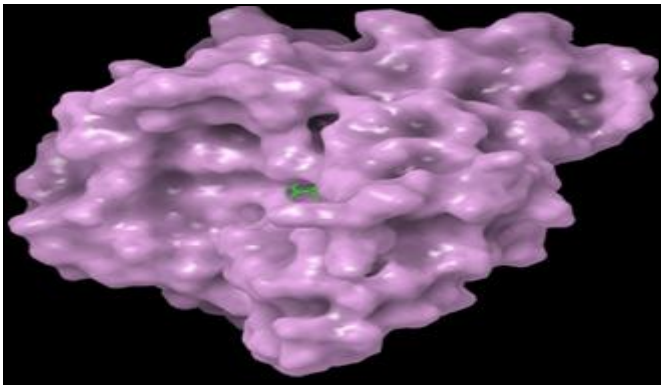

FIG. 2: DOCKING OF ANETHOLE WITH POLY (ADP-RIBOSE) POLYMERAS (PARP)

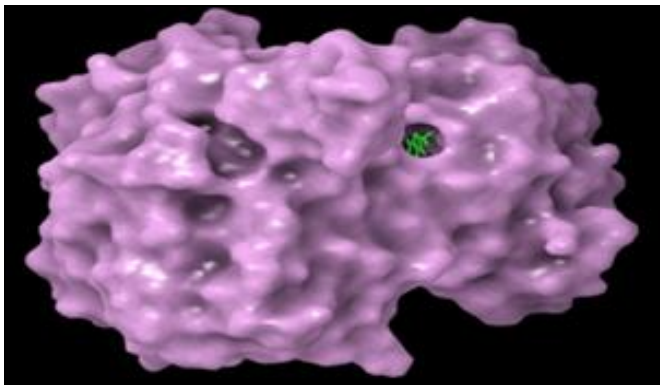

FIG. 4: DOCKING OF ANETHOLE WITH INSULIN-LIKE GROWTH FACTOR RECEPTOR (IGF1R)

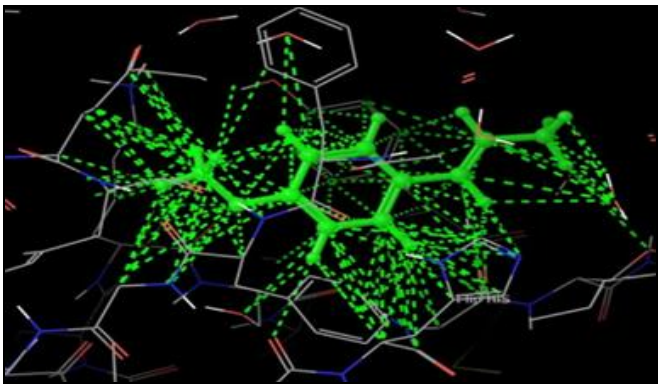

FIG. 3: MOLECULAR INTERACTION OF ANETHOLE WITH POLY (ADP-RIBOSE) POLYMERASE (PARP) SHOWING GOOD CONTACTS

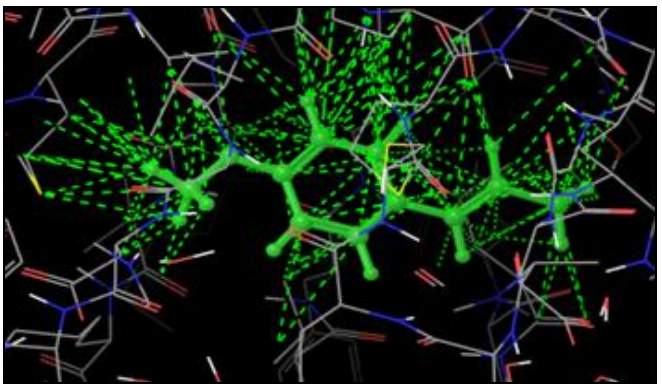

FIG. 5: DOCKING OF ANETHOLE WITH INSULIN-LIKE GROWTH FACTOR RECEPTOR (IGF1R) SHOWING GOOD CONTACTS

IGF1R protein. The ligand was found to possess good docking efficiency with both the target protein molecules namely PARP and IGF1R, revealing their importance to influence the activity of proteins. Drug discovery is a time-consuming, expensive, and interdisciplinary process whereas advances in computational techniques and hardware solutions have enabled *in silico* methods to speed up lead optimization and identification [8]. ADME screening was carried out in order to understand the pharmacokinetic behaviour of reported derivatives using descriptors like human intestinal absorption (HIA), Lipinski rule of five, blood brain barrier (BBB) penetration, hepato-toxicity, aqueous solubility and inhibition probabilities [9].

The molecular docking studies with quercetin and its analogues into the binding cavity of iNOS (Nitric oxide synthases) inducible showed the analogues have more favourable interaction than quercetin and could be a potential lead molecule as anticancer compounds [11]. The p53-binding domain of mortalin was used as receptor to screen 9000 drug-like compounds from ZINC database using molecular docking program. It was observed that three drug-like molecules ZINC01019934, ZINC00 624418 and ZINC00664532 were adequate to interrupt stability of p53-mortalin complex and warrant for anticancer agent. Our study revealed that the compound anethole possessed good docking scores and reasonable stability. The ADME profile supports the bioavailability of the compounds. Thus, our results provide evidence for the interaction of anethole with the target proteins PARP and IGF1R and induce the apoptotic activity in triple negative breast cancer MDA-MB-231 cells. This interaction is presumably vital in exerting the anticancerous activity.

CONCLUSION: In conclusion, our results demonstrated that anethole has potential apoptotic activity and also promise to act as new drug candidate for triple negative breast cancer cells (MDA-MB-231). 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.

ACKNOWLEDGEMENT: Nil

CONFLICT OF INTEREST: Nil

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How to cite this article:
Sumathi S, Poornima A, Muthukumari D and Padma PR: Cytotoxicity and *in-silico* studies of anethole in triple negative breast cancer cells. Int J Pharm Sci & Res 2018; 9(8): 3414-19. doi: 10.13040/IJPSR.0975-8232.9(8).3414-19.