APOPTOSIS MEDIATED CELL GROWTH INHIBITION BY ISOPROPHL MYRISTATE IN BREAST CANCER CARCINOMA CELLS

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ABSRACT

Isopropyl myristate was isolated and purified from the endophytic fungi isolated from the leaves of the medicinal plant Enicostemma axillare(Lam.)Merr. Apoptotic activity was tested on human breast adenocarcinoma cancer (MCF-7) cell line. Initially, the cytotoxic effect of the compound on the cell line was analyzed by MTT assay. Further, DNA was visualized for formation of an oligo ladder, which is a characteristic feature of apoptosis. CASPASE-3 and CASPASE-9 proteins were quantified upon treatment with the purified compound. Dose dependent inhibition of cancer cells was observed with IC_{50} values of 71.58µg/ml. The inhibition of cancer cells was found to be apoptotic in nature based on fragmented DNA and upregulation of CASPASE-3 and CASPASE-9 proteins. Further, docking analysis was performed to determine the structure activity relationship between the compound and PI3K receptor.

Keywords: *Apoptosis, Enicostemma axillare, Isopropyl myristate, MTT, Docking.*

INTRODUCTION

Apoptosis or Programmed Cell Death (PCD) is a highly organized genetically regulated cell death process (Cotter and Madden, 2000). [1] It plays an important role in the maintenance of tissue homeostasis. Numerous identified anti-cancer compounds act by induction of apoptosis. For instance, taxol works by inducing apoptosis in various cancer cell lines such as breast cancer, glioblastoma, hepatoma and ovarian cancer. It activates apoptosis by pro-apoptotic stimuli targeting mitochondria, resulting in mitochondrial depolarization and activation of caspase enzyme, eventually leading to cell death (Broker *et al*., 2002; Huisman *et al*., 2002; Von Haefen *et al*., 2003; KH *et al*., 2005; Ehrlichova *et al*., 2005; Day *et al*., 2006; Janssen *et al*., 2007).[2-8]

In India, breast cancer is the most common cancer occurring in women and accounts for 27% of all cancers in women. For every 2 women newly diagnosed with breast cancer, one woman out of it dies in India (Ferlay et al., 2012; Bray et al., 2013). Overall, 1in28 women are likely to develop breast cancer during her lifetime.

The secondary metabolites obtained from plant source have played a leading role in generation of various novel compounds for exploitation in the pharmaceutical industry. Endophytes are one such organism which is known to colonize the healthy tissues of plant at certain or all stages of their life without causing any harm or apparent damage (Bacon and White, 2000; Petrini and Fisher, 1990). [9-10]They are known to produce

enormous amount of secondary metabolites which can be utilized as therapeutic agents (Schulze and Boyle, 2005; Tan and Zou, 2001). [11-12]

The medicinal plant used in the present study was *Enicostemma axillare* which belongs to the family Gentianaceae. In the Indian system of medicine, it has been used to treat various skin diseases, helminthiasis (Warrier *et al*., 1995) and tumors (Mudaliar, 2002). [13-14]The anticancer potentials of the plant have been examined in rodent and cellular models (Kavimani and Manisenthilkumar, 2000). [15] The methanolic extract has been proven to possess significant inhibitory effect on the growth of cancer cells (Krishna and Mohandass, 2014). [16] The plant is known to be a blood purifier and has been used in the treatment of Dermatopathy and Venereal infections (Pandikumar *et al*., 2011). [17]

In the present study, a compound isolate named Isopropyl myristate purified from the fungal exract obtained from the plant *Enicostemma axillare* was examined for its anti-cancer activity. MTT assay was performed to analyze the cytotoxicity against MCF-7 cell lines, followed by DNA fragmentation and Caspase quantification assay to analyze whether cell death process takes up the apoptotic pathway. Finally, docking analysis was performed to study the molecular interaction between the compound and breast cancer specific receptor PI3K.

MATERIALS AND METHODS

Human cancer cell lines and culture conditions

MCF-7 cells were procured from National Centre for Cell Sciences (NCCS), Pune, cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 100U/ml penicillin and 100U/ml of streptomycin in a 5%CO_2 atmosphere at 37°C.

Invitro cytotoxic activity of the purified compound using MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5 Diphenyltetrazolium Bromide] assay

The cytotoxic effect of the purified compound on MCF-7 cell line was assayed using MTT assay. Briefly, the cells were seeded in 96 well plates at a density of 1×10^4 cells/well for 24h. Subsequently, the fungal crude extracts dissolved in 0.4% DMSO, were added at different concentrations and incubated for 24h. Post incubation, MTT reagent (5mg/ml) was added and incubated for 4h in dark. The blue MTT formozan precipitate formed was solubilized in DMSO and measured spectrophotometrically at 570nm in an ELISA plate reader scanning spectrophotometer. Cells grown in culture media alone or with appropriate concentrations of DMSO were used as controls. Absorbance data were converted into % cell inhibition according to the following equation:

Cell inhibition = {(Abs. value of control- Abs. value of sample)/Abs value of control} x100%

The correlation of cell death and extract concentration was then analyzed by using a line regression test. The extract concentration required to inhibit cancer cell growth by 50% (IC_{50}) was then calculated from the dose-response curve (Mossman, 1983, Lin and wang, 1991; Hasan *et al*., 2014). [18-20]

DNA Fragmentation assay

Formation of an oligo ladder upon DNA fragmentation is a characteristic of apoptosis. To analyse the DNA laddering, MCF7 cells were seeded in 24 well plates and kept in CO_2 incubator. Cells were treated by the compound in three different concentrations (50µg/ml, 100µg/ml, and 200µg/ml) for 48h. At the end of incubation period, the total DNA was isolated from the cells. DNA samples were electrophoretically separated on 2% agarose gel containing ethidium bromide (0.4µg/mL). DNA was visualized by a UV (302nm) transilluminator (Emmy and Rogakou *et al.*, 2000; Gavrieli *et al.*, 1992). [21-22]] Quercitin treatment was used as positive control, while untreated cells were used as control.

CASPASEs quantification assay for the compound

CASPASEs activities were determined by chromogenic assays using CASPASE-3 and CASPASE-9 activation kits according to the manufacturer's protocol (Calbiochem, Merck). After treating with designated compounds, the cells were lysed using Lysis buffer (50mM HEPES, 100mM NaCl, 0.1% CHAPS, 1mM DTT, 100mM EDTA). Lysates were centrifuged at 10000rpm for 1minute. The supplements (cytosolic extract) were collected and protein concentration was determined by the Bradford Assay using BSA as a standard. 100-200µg protein (cellular extracts) was diluted in 50µL cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5µL of the 4mM p-nitroanilide (pNA) substrates, DEVD—pNA (caspase- 3 activity) for 2hr at 37°C. CASPASE activity was measured by cleavage of the above sub substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405nm in a microtiter plate reader. Relative CASPASE-3 and 9 activities were calculated as a ratio of the absorbance of treated cells to untreated cells.

Docking analysis

Automated docking studies were performed using the genetic algorithm GOLD (Gareth Jones, *et al*., 1997). [23] To construct a structure activity relationship, the mechanism of action of the compound along with the molecular target of compound had to be understood. Molecular docking analysis was exploited to study the interaction of the isolated compound with its anti-cancer molecular target. The compound was used as the subject in this study, along with PI3K receptor as the molecular target. The molecular docking analysis was conducted using GOLD software. During docking process, a maximum of 10 different conformations was considered for the drug. The conformer with highest binding score was used for further analysis (Nissink, *et al*., 2002). [24]

RESULTS

The compound isolated from the endophtic fungi obtained from *Enicostemma axillare* was subjected to MTT assay. It was observed that the compound had a cytotoxicity of 61.13% at a concentration of 125µg/mL. The IC_{50} value for the compound Isopropyl myristate was calculated to be 71.58µg/mL.

CASPASEs 3 and 9 were quantified in MCF7 cells treated with Isopropyl myristate. An upregulation in the level of the CASPASEs 3 and 9 was observed (Table 3, Fig. 2). DNA laddering was observed in the cells

treated with 100µg of the compound, indicating the activation of apoptosis leading to death of the treated cancer cells.

Table 1: The cytotoxicity percentage values of the purified compound and positive control in MCF7 cells (in µg/mL) is tabulated.

Concentration (µg/mL)	Cytotoxicity %	
	MCF7	
	Compound	Positive Control
25	39.06	59.36
50	46.56	67.57
75	50.97	81.04
100	55.94	96.12
125	61.13	98.61

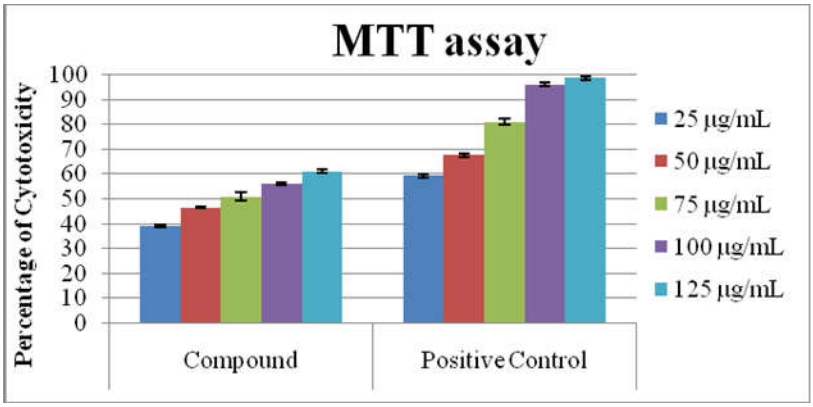

Figure 1: The percentage of cytotoxicity of compound and Positive Control against MCF7 cells.

Table 2: The IC_{50} value of compound in MCF7 cells (in µg/mL).

	Compound
IC_{50}(µg/mL)	71.58

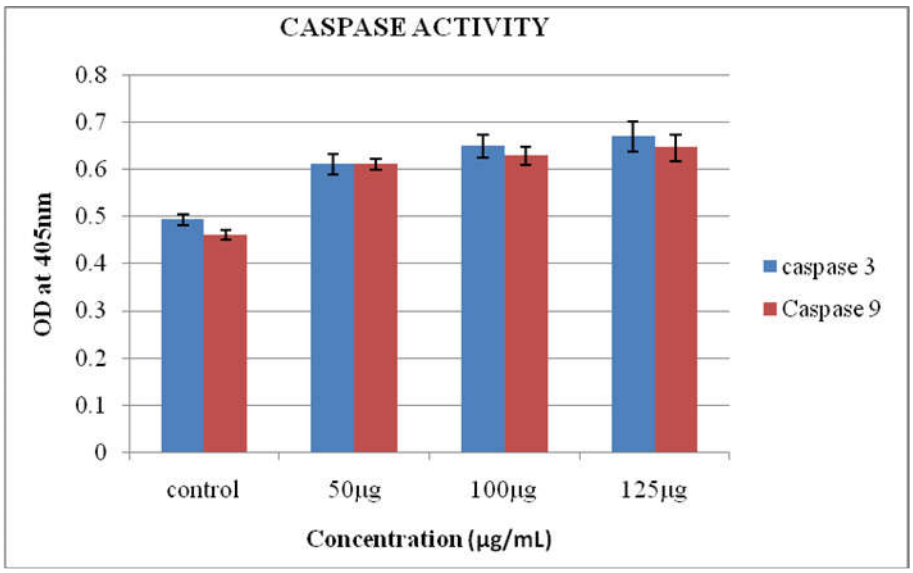

Figure 2: Caspase 3 and caspase 9 was quantified by mesuring the absorbance at 405nm.

Table 3: The relative fold increase in the caspase activity of both 3 and 9.

Relative caspase activity				
	control	**50µg**	**100µg**	**125µg**
Caspase 3	1	1.2381	1.3184	1.3599
Caspase 9	1	1.3257	1.3647	1.4017

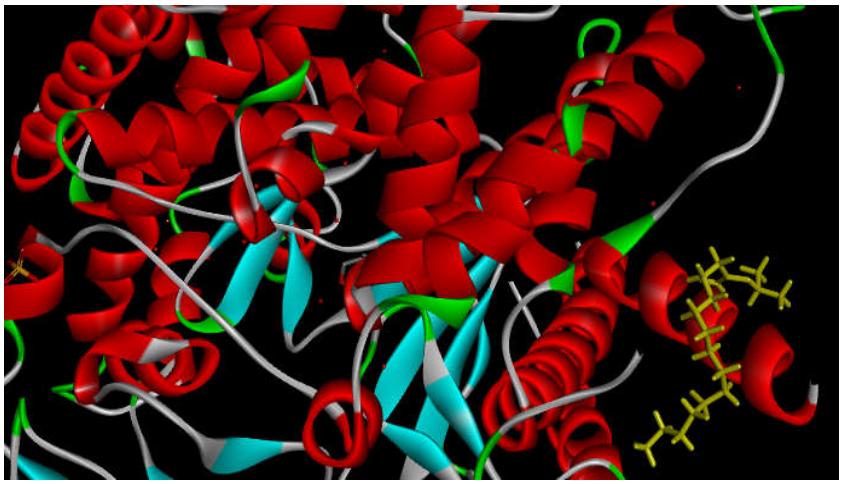

Figure 5: The ligand Isopropyl myristate bound with the receptor PI3K gamma.

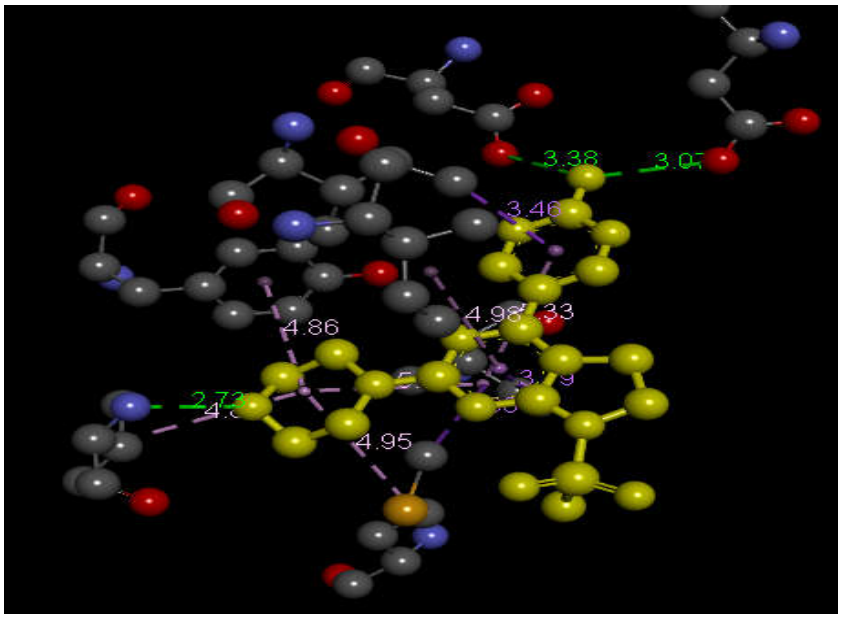

Figure 6: The bond configurations of bound ligand.

Table 4: The fitness profile of the ligand Isopropyl myristate.

Ligand name	Fitness	S(hb-ext)	S(vdw-ext)	S(hb-int)	S(int)
Isopropyl myristate	**31.83**	**3.34**	**28.6**	**0**	**-10.83**

DISCUSSION

Fungal endophytes have gained special attention due to their potential to produce metabolites which can prove to be of pharmacological interest and the role that they play in regulating the ecological systems at different trophic levels. Endophytic fungi from plants are well known sources of bioactive secondary metabolites (Schulz *et al.*, 2002). [25] They are reported to be reservoirs of novel compounds with distinct bioactivities (Tan *et al.*, 2001). [26] Nevertheless, majority of the plants have not been exploited for their endophytes. This indicates that there are boundless opportunities to discover novel fungal forms, taxa and biotypes.

The secondary metabolites of endophytic organisms include alkaloids, steroids, terpenoids, quinones, lignans, phenols, flavonoids, aliphatic compounds, etc. The phytochemicals within the endophytes could be a potential source for medicinal and industrial use and they are also considered to be a potential source of precursors in the development of synthetic drugs (Sadananda et al., 2011). [27] The main aim of the study is to develop new therapeutic drugs with high efficacy and low toxicity. There is an increasing effort to characterize and identify the endophytic fungi isolated from medicinal plants.

The purified fungal isolate was examined for its level of cytotoxicity by MTT assay. The mechanism of cell death induction was studied by quantifying the amount of caspases. They are present in the mammalian cells as latent enzymes that get activated during apoptosis and hydrolyze specific cellular proteins once they are activated, thereby disassembling the cells (Saunders et al., 2000). [28]

The isolated compound was evaluated for its potential to induce apoptotic cell death by fragmentation of DNA upon drug treatment in cancer cells. Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 basepairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis (Alexei, Basnakian and Jill James, 1994). [29] Upon the treatment of MCF7 cells with the compound for 48hours, DNA was isolated and subsequently, agarose gel electrophoresis was performed. DNA ladders appeared in the case of treated cells whereas the control cells did not show any DNA fragmentation. The degradation of DNA down to oligonucleosomal fragments is a late event of apoptosis. Thus it can be inferred that cell death occurs by apoptosis cell. Taxol is an important anticancer drug widely used for treatment of ovarian and breast cancer that acts by inducing apoptotic cell death. Agents that suppress or inhibit the proliferation of cancer cells by induction of apoptosis might prove to be a useful and alternative approach to cancer chemotherapy which may prevent unfavorable side effects and resistance (Shafi, et al., 2009). Wang, (2011)[30] also reported that defects occuring along apoptotic pathways play a vital role in carcinogenesis and therefore, apoptosis targeted novel treatment strategies may be used to treat various types of cancer. Therefore, there is potential for the compound Isopropyl myristate to serve as novel-anti-cancer drug that specifically target and induce cancer cells to apoptosis.

In silico approach was adopted to predict the interaction of the purified fungal isolate with the breast cancer specific receptor. The structure-activity relationship was utilized to develop novel derivative natural compound with high anti-proliferative activity. This part of research aims to determine the model of interactions between the natural compound isopropyl myristate with anti-proliferative molecular target by molecular docking analysis.

CONCLUSION

The current study focuses on the anti-cancer potentials of the compound Isopropyl myristate, purified from the endophyte of *Enicostemma axillare*. The inhibitory effect of tumor proliferation is due to the apoptotic cell death characterized by activation of caspases and fragmentation of DNA of the treated cells.

These observations clearly indicated that apoptosis was induced via mitochondrial mediated intrinsic pathway in breast cancer cells MCF7 treated with Isolpropyl myristate. Hence, the purified fungal isolate from *Enicostemma axillare* proves be a potent source for an anti-cancer compound.

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