



## Research Article

### **BCL2 MEDIATED APOPTOTIC INDUCTION POTENTIAL OF THE DNOP ISOLATED FROM *PACHYGONE OVATA* (POIR.) MIERS EX HOOK. F. & THOMSON IN MCF-7 (HUMAN BREAST CARCINOMA)**

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#### ABSTRACT

Cancer targeted phytotherapy has been an area of research in recent times, owing to the high mortality rates due to this global malignancy. Cancer tissues are becoming resistant to synthetic chemotherapeutic agents leading to the evolution of Drug-resistant cancer cells and that has been a major reason for encouraging the phytotherapeutic research. In the current study, an effort has been made to understand the potent inhibitory potentials of the isolated bioactive component, di-n-octyl-phthalate (DNOP) from the Indian medicinal plant *Pachygone ovata*, on human breast carcinoma and elucidate its molecular mechanism of action. The cytotoxicity was determined at 70.8% at a concentration of 125µg/ml with an IC<sub>50</sub> of 42.47µg. DNA oligomers in multiples of 180bp were observed in the DNA fragmentation assay. CASPASEs 3 and 9 were found to be upregulated and BCL2 gene expression was observed to be downregulated. These results showed significant potential of the DNOP and the induction of BCL2 mediated apoptosis in cancer cells treated with the isolated compound.

**Keywords:** *Pachygone ovata*, Cancer, DNOP, Cytotoxicity, MCF-7, Caspase, BCL2

#### INTRODUCTION

*Pachygone ovata* (Poir.) Miers ex Hook. F. & Thomson (Menispermaceae) is a lofty climber indigenous to the sandy seashores of the Coromandel Coast of India<sup>1-3</sup>. In ancient times, plants were used to treat different kinds of diseases<sup>4</sup>. Natural products have played an important role in the development of drugs and drug leads for various diseases including cancer<sup>5</sup>. Plant-based medicine used in cancer research has many scientific evidences which are much safer and economic. The use of naturally occurring compounds with chemo-preventive properties attracted much interest in chemotherapy and treatment of cancers.

Among the worldwide female population, breast cancer is considered as a heterogeneous and significant cause for human death. Breast cancer causes due to heredity and non-genetic alterations that affect the regulation and function of genes<sup>6</sup>. It is a most frequent neoplasm<sup>7</sup> hence the search for new and effective drugs to treat the breast cancer is of central importance<sup>8</sup>.

The ability of cancer cells to disrupt apoptosis has been highly implicated in various malignancies and hence been exploited as an anticancer strategy. Owing to the fact that the apoptosis causes minimal inflammation and damage to the tissue, apoptotic cell death-based therapy has been the centre of attraction for the development of anticancer drugs. Increased understanding of the molecular pathways underlying apoptosis has enabled scientists to establish unique approaches targeting apoptosis pathways in cancer therapeutics. Apoptosis, a program of cellular suicide, is a form of programmed cellular death which occurs through activation of the cell-intrinsic suicide machinery and is considered an important mechanism in the action of many

anticancer drugs<sup>9-12</sup>. It's been suggested as a promising target for cancer chemotherapy<sup>13-15</sup>.

Hence, this study is carried out to investigate the effects of di-n-octyl-phthalate (DNOP), the compound extracted from *Pachygone ovata* on MCF-7 human breast cancer cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, its apoptotic induction effect by DNA fragmentation assay and activation of CASPASE cascade, followed by apoptotic gene expression elucidation.

#### MATERIALS AND METHODS

##### Sample preparation

Di-n-octyl-phthalate was identified and isolated from the plant *Pachygone ovata* using bioassay guided fractionation (Data not shown).

##### Cell line and culture conditions

In this study, the MCF-7 cell line (human breast cancer cells) was obtained from National Centre for Cell Sciences, Pune and cultured in DMEM containing 10% heat-inactivated fetal bovine serum. The cells were plated on tissue culture dishes at 37°C in a humidified 5% CO<sub>2</sub> incubator and cultured for 2-4 days until confluence was reached.

##### MTT assay<sup>16</sup>

Cytotoxicity of DNOP was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the reduction of MTT into formazan dye by the

action of mitochondrial enzymes. The MCF 7 cells were plated in 96 well flat bottom tissue culture plates at a density of approximately  $1.2 \times 10^4$  cells per well and allowed to attach overnight at 37°C. The medium was then discarded and cells were incubated with different concentrations of the sample (25, 50, 75, 100 & 125 µg/ml) for 24 hours. After the incubation, medium was discarded and 100µl fresh medium was added with 10µl of MTT (5mg/ml). After 4 hours, the medium was discarded and 100µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm in a microtitre plate reader. Cyclophosphamide was used as a positive control. The toxicity in cells was expressed as IC<sub>50</sub>, which is a dose of cytotoxic compound where 50% viability is achieved. The IC<sub>50</sub> was determined using Graph Pad Prism software version 7.01.

### DNA fragmentation assay

To evaluate apoptotic oligonucleosomal fragmentation, genomic DNA was extracted using cell lysis buffer as described<sup>17</sup> after treating the MCF-7 cells with varying concentrations of DNOP. DNA samples were separated in 2% agarose gel electrophoresis at 50 V, 3 h and visualized with ethidium bromide using gel documentation system.

### CASPASEs quantification assay

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 DNOP, the cells were lysed using Lysis buffer (50mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1mM DTT, 100 mM EDTA). 100-200 µg protein (cellular extracts) was diluted in 50 µL cell lysis buffer for each assay. Cellular extracts were then incubated in 96-well microtiter plates with 5 µL of the 4mM p-nitroanilide (pNA) substrates, DEVD-pNA for 2 hr at 37°C. CASPASE activity was measured by cleavage of the above substrates to free pNA. Free pNA (cleaved substrates) was measured by absorbance at 405 nm in a microtiter plate reader. Relative CASPASE-3 and CASPASE-9 activity was calculated as a ratio of the absorbance of treated cells to untreated cells.

### RT-PCR analysis

Total RNA was isolated from cell specimens using ONE STEP-RNA Reagent (Biobasic Inc.) developed from the guanidinium thiocyanate-phenol method<sup>18</sup>. After the isolation, RNA was immediately reverse transcribed with EasyScript Plus™ Reverse Transcriptase. The primer sequences used for the PCR amplification were GAPDH, 5'-GGCCAACCGTGAAAAGATG-3' and 5'-GGATCTTCATGAGGTAGTCTGTGC-3'; BCL2, 5'-GGATTCATGACCAGGGAGTTCCTC-3' and 5'-GCGGTCTCCACTGAGAATAATGAC-3'. Agarose gel electrophoresis of PCR products was done using 1.5% agarose gel. The mixture was loaded to each well along with 1Kb Ladder as a reference. The gel was run at 50 V for 90 min and visualized in gel documentation (Figure 1).

## RESULTS

### Cytotoxicity of DNOP against MCF-7 cells

Cytotoxicity of DNOP, isolated from *Pachygone ovata* was investigated against MCF-7 by using MTT assay. Human breast cancer MCF-7 cells were treated with serial concentrations of DNOP (25, 50, 75, 100 and 125 µg/ml) for 24 hrs and subsequently measured for the cell viability. The viability of

MCF-7 cells exposed to DNOP and positive control declined in a dose dependent fashion. In Figure 2 the cytotoxicity of MCF-7 cells increased with increasing doses of DNOP, with the maximum cytotoxicity of 70.8% being observed at a concentration of 125µg/ml. The IC<sub>50</sub> of the compound was observed to be 42.47µg.

### DNA fragmentation analysis

MCF-7 cells were treated with the DNOP was subjected to DNA fragmentation analysis to understand the apoptotic induction potentials. The observation of formation of oligonucleosomal DNA fragments in multiples of 180bp confirmed the activation of apoptosis in compound treated cells. (Figure 3)

### CASPASE quantification assay

To determine the mechanism underlying the apoptosis, CASPASE quantification assay was performed which showed the exposure of cells in dose-dependent manner had increased the activity of the CASPASEs in treated MCF-7 cells. The CASPASE 3 was found to be upregulated by 1.404 and CASPASE 9 was upregulated by 1.401 (Table 1 & 2).

### RT-PCR analysis

RT-PCR was performed to determine the comparative expression of BCL2 gene in cells treated with DNOP and untreated cells. The isolated RNA, when visualized using Agarose gel electrophoresis was found to be intact and viable. The cDNA obtained was amplified by PCR for the BCL2 gene. A constitutively expressed gene namely GAPDH, was chosen in order to assess the quality of PCR. Results showed the amplification of BCL2 gene and GAPDH gene at ~700 bp and ~450bp respectively. While BCL2 was found to be downregulated, the expression of GAPDH was found to be consistent in both the treated and untreated cells.

## DISCUSSION

Most cancer chemotherapy agents currently in clinical use have originated from plants or are analogs of plant-derived compounds<sup>19,20</sup>. A large number of chemotherapeutic agents are derived from natural plant products and they can induce apoptosis in cancer cells<sup>21</sup>. Globally breast cancer is one of the primary leading cancer affecting women. Incidence of breast cancer is on the rise in Asian countries like India and Japan<sup>22</sup>.

MTT assay has been employed by various researchers to understand the viability of cells after treatment with candidate drugs<sup>16</sup>. In the current study, Di-n-octyl-phthalate (DNOP) was isolated from the Indian medicinal plant *Pachygone ovata*, based on its preliminary anti-cancer studies. This compound was understood to have toxicity against Human Breast Cancer cell lines MCF-7, by MTT assay.

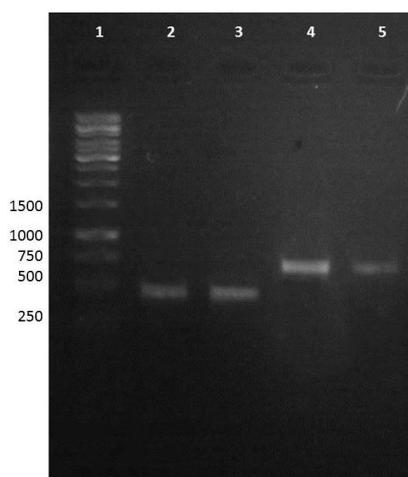
Apoptosis is a critical pathway and occurs in development, immunological competence, and homeostasis<sup>23</sup>. It is a form of self-regulated cell death and is hence preferred over necrosis as a form of drug induced cell death in treating diseases<sup>24,25</sup>. DNA fragmentation has a characteristic biochemical phenomenon occurring during apoptosis that involves the activation of specific endonucleases which cut the linker DNA between nucleosomes. This is elucidated by analyzing the isolated DNA from DNOP treated cells on an agarose gel and the observation of a DNA laddering pattern in multiples of 180bp, confirming the induction of apoptosis.

**Table 1: CASPASE 3 quantification activity of untreated and sample treated cells**

CASPASE 3	OD at 405 nm	Relative activity
Control	0.350±0.01	1
25	0.379±0.01	1.084
50	0.392±0.01	1.123
75	0.409±0.004	1.170
100	0.444±0.01	1.272
125	0.491±0.01	1.404

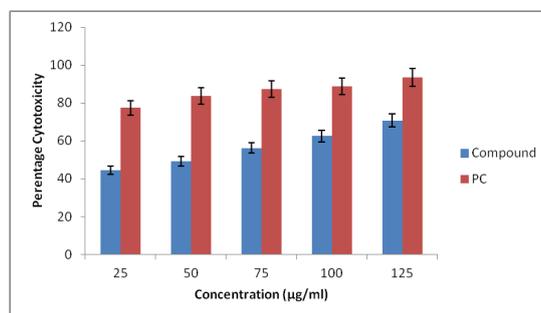
**Table 2: CASPASE 9 quantification activity of untreated and sample treated cells**

CASPASE 9	OD at 405 nm	Relative activity
Control	0.350±0.01	1
25	0.387±0.004	1.108
50	0.412±0.01	1.180
75	0.422±0.01	1.206
100	0.464±0.01	1.328
125	0.490±0.01	1.401

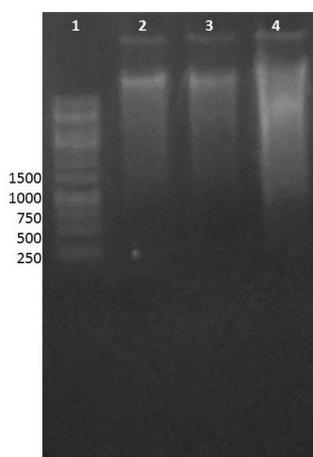


**Figure 1 RT- PCR – Bcl2.**

**Lane1:** 1 kb DNA Ladder, **Lane 2:** GAPDH expression in control cells, **Lane 3:** GAPDH expression in treated cells, **Lane 4:** Bcl2 expression in control cells, **Lane 5:** Bcl2 expression in treated cells.



**Figure 2: Percentage Cytotoxicity of sample and positive control (Cyclophosphamide)**



**Figure 3 DNA fragmentation analysis.** Lane 1 – 1 kb ladder; Lane 2 – DNA from cells treated with 25 µg sample; Lane 3 – DNA from cells treated with 50 µg sample; Lane 4 – DNA from cells treated with 100 µg sample

In mammalian cell lines, apoptosis has been known to follow through two major pathways: the extrinsic pathway, which is triggered by the stimulation of pro-apoptotic cell surface receptor signaling. The other is the intrinsic pathway, which involves a cascade of reactions that lead to the activation of CASPASEs<sup>26,27</sup>. CASPASEs are produced as proforms, which are proteolytically cleaved and activated during apoptosis. The activation of CASPASE-3 in treated cancer cells during apoptosis results in PARP cleavage and activation of CASPASE activated DNase (CAD), subsequently causing DNA fragmentation, which is one of the characteristic feature of apoptosis mechanism<sup>28,29</sup>. Our results demonstrated that the compound had induced intrinsic apoptosis in the MCF-7 cells by enhancing the cleavage of CASPASE-9 and CASPASE-3.

BCI-2 suppresses apoptosis in a variety of cell systems including factor-dependent lympho hematopoietic and neural cells. It regulates cell death by controlling the mitochondrial membrane permeability and appears to function in a feedback loop system with CASPASEs. BCI-2 also inhibits CASPASE activity either by preventing the release of cytochrome c from the mitochondria and/or by binding to the apoptosis-activating factor (APAF-1). In the current study, the expression of BCI2 was found to be downregulated thereby confirming the induction of intracellular apoptosis.

Phthalates are reported to have antimicrobial and other pharmacological activities<sup>30</sup>. The compound DNOP is one such phthalate, which is rarely studied and explored. DNOP is extensively used as plasticizer in many biomedical applications. Our study further established the potency of DNOP to induce apoptosis in MCF-7 human breast cancer cells. This result is highly significant owing to the fact that this is the first report of the isolation of DNOP from *P.ovata* plant. The presence of bioactive compounds justifies the use of plant for various ailments including cancer studies. In conclusion, the study demonstrates that *Pachygone ovata* (Poir.) Miers ex Hook. F. & Thomson has potent anti-tumor agents and antioxidant compounds against the human breast carcinoma cells. These secondary metabolites could serve as a lead for discovering novel anti-cancer drugs in the future.

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