

## RESEARCH ARTICLE

# Oleuropein Induces Apoptosis Via the p53 Pathway in Breast Cancer Cells

Zeinab Korany Hassan<sup>1,2\*</sup>, Maha Hussein Elamin<sup>1</sup>, Sawsan Ali Omer<sup>1</sup>, Maha Hassan Daghestani<sup>1</sup>, Ebtessam Salah Al-Olayan<sup>1</sup>, Mai AbdelRahman Elobeid<sup>1</sup>, Promy Virk<sup>1</sup>

### Abstract

**Background:** Breast cancer is a major health problem worldwide. Olive oil induces apoptosis in some cancer cells due to phenolic compounds like oleuropein. Although oleuropein has anticancer activity, the underlying mechanisms of action remain unknown. The study aimed to assess the mechanism of oleuropein-induced breast cancer cell apoptosis. **Materials and Methods:** p53, Bcl-2 and Bax gene expression was evaluated by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in luminal MCF-7 cells. **Results:** Oleuropein-induced apoptosis was accompanied by up-regulation of both p53 and Bax gene expression levels and down-regulation in Bcl2. **Conclusions:** Oleuropein induces apoptosis in breast tumour cells via a p53-dependent pathway mediated by Bax and Bcl2 genes. Therefore, oleuropein may have therapeutic potential in breast cancer patients by inducing apoptosis via activation of the p53 pathway.

**Keywords:** Apoptotic pathway - Bax gene - breast cancer - Bcl2 gene - oleuropein - p53 gene - semi-quantitative RT-PCR

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### Introduction

Breast cancer is a leading cause of deaths with increasing rate in developing countries (Saad, 2011). Therapeutic bioactive nutritional agents come into view to have importance in preventing and treating diseases. Olive tree is important in medicine due to its phenolic content (Visioli et al., 2002) e.g. oleuropein. In breast cancer cells, oleuropein decreases cell viability (Menendez et al., 2007), acts as an anti-metastatic agent (Hassan et al., 2012), and induces cell apoptosis (Campolo et al., 2013; Elamin et al., 2013; Oi-Kano et al., 2013; Pasban-Aliabadi et al., 2013). Oleuropein inhibits cell proliferation via delaying the cell cycle at S phase and up-regulating cyclin-dependent inhibitor p21 (Elamin et al., 2013).

Apoptosis is cell death to remove aberrant cells. The milestone in considering the effectiveness of chemopreventive agent is the ability to induce apoptosis as a mechanism for tumor suppression (Choudhuri et al., 2002). The mitochondrial apoptotic pathway is initiated within the cell in which pro-apoptotic proteins are released from the mitochondria to activate caspase proteases triggering apoptosis (Lessene et al., 2008).

The p53, tumor suppressor gene, is aberrant in most cancers (Hollstein et al., 1991) and mediates apoptosis in response to DNA damage (Das et al., 1999). In normal cells, p53 arrests cells, reduces genetic instability and

allows the cells repair before progressing through the cell cycle (Taylor et al., 1999), but apoptosis induced by p53 is necessary for eliminating aberrant cells. The Bax, pro-apoptotic gene, is up-regulated in some systems during p53-mediated apoptosis. The up-regulation of Bax gene and down-regulation of Bcl-2 expression are established during apoptosis (Moon et al., 2007). The p53 signaling pathway is implicated in the transcriptional activation of Bax in apoptosis (Li-Weber, 2009). The pro- and anti-apoptotic genes are important in the fate of a cell. As the effect of oleuropein on apoptotic genes is not studied, this study aimed to investigate the mechanism of oleuropein-induced human breast cancer cell apoptosis.

### Materials and Methods

MCF-7 cells were obtained from ATCC and were cultured following the instructions of the company. MCF-7 was maintained in RPMI-1640 (GIBCO, USA), L-glutamin 1%, 10% fetal bovine serum (FBS), 1% antibiotic/anti-myotic (penicillin/streptomycin) (Sigma Aldrich, USA). Cells were maintained at 37°C in humidified incubator with 5%CO<sub>2</sub>. Cells were treated with 100µM or 200µM concentration of Oleuropein. The incubation period and oleuropein concentrations were determined according to our previous study (Elamin et al., 2013). The untreated cells were used as control. All

<sup>1</sup>Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia, <sup>2</sup>Department of Cancer Biology, National Cancer Institute, Cairo University, Cairo, Egypt \*For correspondence: [hildahafez@hotmail.com](mailto:hildahafez@hotmail.com)

treated and untreated cells were incubated for 72h and then collected for the gene expression studies

**Gene expression by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)**

**RNA extraction:** Total RNA was extracted from the Oleuropein -treated and control cell line with TRIzol (Gibco BRL), in accordance with the manufacturer’s instructions. Concentrations and purity of RNA were quantified spectro-photometrically by measuring A260 and A280; the ratio A260/A280 of pure RNA is approximately 1.8.

**Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR):** Total RNA isolated from treated and untreated cells was analyzed for anti-apoptotic Bcl-2 gene and pro-apoptotic Bax and p53 genes by semi-quantitative reverse transcriptase-PCR. cDNA was prepared from total RNA as described in manufacturer’s protocol (Invitrogen, USA). Reverse transcription using oligo-dT primers in a 20µl total volume reaction mixture using a superscript system (Invitrogen, USA) and PCR were performed as previously(Huang et al., 2012), with an endogenous control gene (GAPDH).

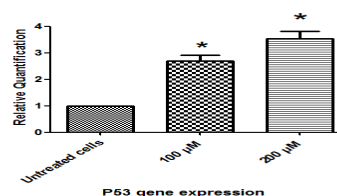
The primer sequences for PCR were as follows: 5’-TG TGTGTGGAGAGCGTCAACC-3’ and 5’-TTCAGAGACAGCCAGGAGAAATC-3’ used for Bcl-2; 5’-TCAGGATGCGTCCACCAA GAA-3’ and 5’-TCCCCGAGGAAGTCCAATGTC-3’ for Bax; 5’-AGCG ATGGTCTGGCC CCTCC-3’ and 5’-GCGCCGGTCTCTCCCAGGA-3’ for p53; and 5’-ATTCAACGGCACAGTCAAGG-3’ and 5’-GCAGAAGGGGCGGAGATGA-3’ for GAPDH internal control. Amplification was done at the temperature of 94°C for 5min, 25 cycles of 94°C for 30s, 58°C for 30s and 72°C for 45s, and 72°C for 10min (Huang et al., 2012). Experiments were performed in triplicate. Prior to amplification of each gene, normalization was carried out with endogenous control gene GAPDH. Aliquots of the PCR reaction were subjected to electrophoresis on 2% agarose gels and PCR fragments were visualized by ethidium bromide staining and photographed on gel documentation system. mRNA gene expression of the housekeeping gene was used as a quality control for the samples showing equal cDNA in all samples.

**Statistical analysis**

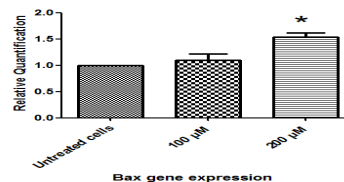
All experiments were performed in triplicate and analyzed by one way ANOVA (Graphpad prism 5) for significant differences. The p values of <0.05 were considered statistically significant. The data are presented as the mean±SD.

**Results**

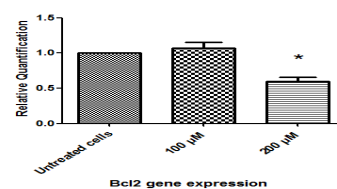
The p53 is targeted by a broad range of agents in apoptosis. The Bcl-2 families have a regulatory effect on the anti- and pro-apoptotic genes to determine the cells fate. To determine the mechanisms of oleuropein-induced apoptosis, we investigated the gene expression levels of the anti-apoptotic Bcl-2 and pro-apoptotic Bax and p53



**Figure 1. The Effect of Oleuropein on p53 Gene Expression on MCF 7 Cells.** Each bar represents the mean ± S.D. \*p<0.05, statistically significantly compared with untreated control



**Figure 2. Bax Gene Expression in MCF 7 Cells Treated with Oleuropein.** Columns, mean (n=3); bars, SD. \*p<0.05, statistically significantly compared with untreated control



**Figure 3. Bcl-2 Gene Expression in MCF7 Cells Treated with Oleuropein.** Columns, mean (n=3); bars, SD. \*p<0.05, statistically significantly compared with untreated control

in MCF-7 breast cancer cells.

*The effect of Oleuropein on p53 gene expression level*

Oleuropein significantly increased the gene expression level of p53 by 2.5 folds in the MCF-7 cells treated with 100µM oleuropein, while in the cells treated with 200µM oleuropein, the p53 gene expression level was increased significantly by 3.5 folds compared to the untreated cells. On the other hand, the up-regulation of p53 gene expression by 200 µM oleuropein was not significant compared to the 100 µM oleuropein concentration as shown in Figure 1. Our observation showed that oleuropein induces MCF-7 cell apoptosis in a p53-dependent pathway.

*The effect of Oleuropein on Bcl-2 and Bax genes expression level*

In the MCF-7 cells treated with 100µM oleuropein no significant differences were observed in the Bcl-2 and Bax genes expression levels compared to the untreated cells. However, at 200µM of oleuropein, the Bax gene expression was up-regulated significantly compared to both the untreated control cells and the others treated with 100µM oleuropein as shown in Figure 2. Whereas Bcl-2 gene expression level was down-regulated significantly in the cells treated with 200µM oleuropein by 0.6 folds compared to both the untreated cells and the others treated with 100µM oleuropein as shown in Figure 3. In the cells treated with 200µM oleuropein the Bax/Bcl-2 ratio was higher than that of both the cells treated with 100 µM oleuropein and the untreated cells.

## Discussion

In spite of early diagnosis, radiation and chemotherapy, breast cancer is still a leading cause of cancer death (Jemal et al., 2011). Some studies have shown that certain cancers, are relatively low in the Mediterranean basin compared to other countries. Nutritional agents come into view to have importance in cancer treatment. Therefore, there is need to explore new anti-cancer drugs. Some epidemiological studies showed correlation between olive products consumption and incidence of breast cancer. Due to its little mild side effects, oleuropein is required as one of the phenolic compounds in olive with a powerful effect on inhibiting the proliferation and migration of cancer cells (Sirianni et al., 2010; Santiago-Mora et al., 2011). Oleuropein can also target steps in cancer progression (Abe et al., 2011). Nowadays, oleuropein has garnered interest due to its ability to inhibit proliferation, metastasis and promote apoptosis in several tumor cell lines (Hassan et al., 2012; Zare et al., 2012; Elamin et al., 2013; Kostomoiri et al., 2013; Oi-Kano et al., 2013; Parzonko et al., 2013). In our previous study, oleuropein showed cytotoxic effect on the luminal MCF-7 cells via the induction of the mitochondrial apoptotic pathway (Elamin et al., 2013). However, the effect of oleuropein on the pro- and anti-apoptotic genes is not studied.

The proapoptotic activity of p53 is due to its transactivation capabilities through the trans-activation of target genes correlated to apoptosis, or can directly activate the transcription of genes known to promote apoptosis (Fridman et al., 2003). The p53 is a transcription factor that mediates cell cycle arrest and apoptosis (Struckhoff et al., 2010). In the current study, oleuropein treatment significantly increases p53 gene expression level in MCF-7 cells treated with 100 $\mu$ M oleuropein compared to untreated cells. However, in the cells treated with 200 $\mu$ M oleuropein the expression level was further increased significantly from the untreated cells. The current observation showed that oleuropein induces MCF-7 cell apoptosis in a p53-dependent pathway. The tumor suppressor p53 is mutated in 30% of breast cancers (Borresen-Dale, 2003).

There are two major apoptosis signaling pathways, mitochondrial and death receptor pathway. The p53 gene is mediated apoptosis via the ability to control transcription of proapoptotic members of the Bcl-2 family. These include the 'multidomain' Bcl-2 family member Bax (Fridman et al., 2003). Bcl-2 is an apoptosis inhibitor and Bax is an apoptotic protein. The anti-apoptotic protein Bcl-2 mainly inhibits the mitochondrial pathways (Huang et al., 2012). In the current study, the MCF-7 cells treated with 100 $\mu$ M oleuropein showed no significant difference in the Bcl-2 and Bax genes expression compared to the untreated cells. With increasing the oleuropein concentration, the level of Bax gene expression was up-regulated significantly from both the untreated cells and the others treated with oleuropein 100 $\mu$ M. Whereas Bcl-2 gene expression level was down-regulated significantly in the MCF-7 cells treated with oleuropein 200 $\mu$ M. The change in the ratio of Bax/Bcl-2 is recognized to initiate caspase signaling (Kirkin et al., 2004). In the current study, the cells treated

with 200 $\mu$ M oleuropein showed Bax/Bcl-2 ratio higher than that of both the cells treated with oleuropein 100 $\mu$ M and the untreated cells. Similarly in previous study, the up-regulated P53 directly promotes Bax expression that destroys the integrity of the mitochondria to induce apoptosis (Chipuk et al., 2004).

In conclusion oleuropein induced apoptosis in tumor cells via a p53-dependent pathway mediated by Bax and Bcl2 genes. Therefore, oleuropein may have possible therapeutic potential in breast cancer patients by inducing apoptosis in cells via activation of p53 pathway.

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## References

- Abe R, Beckett J, Nixon A, et al (2011). Olive oil polyphenol oleuropein inhibits smooth muscle cell proliferation. *Eur J Vasc Endovasc Surg*, **41**, 814-20.
- Borresen-Dale AL (2003). TP53 and breast cancer. *Hum Mutat*, **21**, 292-300.
- Campolo M, Di Paola R, Impellizzeri D, et al (2013). Effects of a polyphenol present in olive oil, oleuropein aglycone, in a murine model of intestinal ischemia/reperfusion injury. *J Leukoc Biol*, **93**, 277-87.
- Chipuk JE, Kuwana T, Bouchier-Hayes L, et al (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science*, **303**, 1010-4.
- Choudhuri T, Pal S, Agwarwal ML, Das T, Sa G (2002). Curcumin induces apoptosis in human breast cancer cells through p53-dependent Bax induction. *FEBS Lett*, **512**, 334-40.
- Das T, Sa G, Sinha P, Ray PK (1999). Induction of cell proliferation and apoptosis: dependence on the dose of the inducer. *Biochem Biophys Res Commun*, **260**, 105-10.
- Elamin MH, Daghestani MH, Omer SA, et al (2013). Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells. *Food Chem Toxicol*, **53**, 310-6.
- Fridman JS, Lowe SW (2003). Control of apoptosis by p53. *Oncogene*, **22**, 9030-40.
- Hassan ZK, Elamin MH, Daghestani MH, et al (2012). Oleuropein induces anti-metastatic effects in breast cancer. *Asian Pac J Cancer Prev*, **13**, 4555-9.
- Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991). p53 mutations in human cancers. *Science*, **253**, 49-53.
- Huang KF, Zhang GD, Huang YQ, Diao Y (2012). Wogonin induces apoptosis and down-regulates survivin in human breast cancer MCF-7 cells by modulating PI3K-AKT pathway. *Int Immunopharmacol*, **12**, 334-41.
- Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.
- Kirkin V, Joos S, Zornig M (2004). The role of Bcl-2 family members in tumorigenesis. *Biochim Biophys Acta*, **1644**, 229-49.
- Kostomoiri M, Fragkouli A, Sagnou M, et al (2013). Oleuropein, an anti-oxidant polyphenol constituent of olive promotes alpha-secretase cleavage of the amyloid precursor protein (A $\beta$ APP). *Cell Mol Neurobiol*, **33**, 147-54.
- Lessene G, Czabotar PE, Colman PM (2008). BCL-2 family antagonists for cancer therapy. *Nat Rev Drug Discov*, **7**,

- Li-Weber M (2009). New therapeutic aspects of flavones: the anticancer properties of Scutellaria and its main active constituents Wogonin, Baicalein and Baicalin. *Cancer Treat Rev*, **35**, 57-68.
- Moon DO, Lee KJ, Choi YH, Kim GY (2007). Beta-sitosterol-induced-apoptosis is mediated by the activation of ERK and the downregulation of Akt in MCA-102 murine fibrosarcoma cells. *Int Immunopharmacol*, **7**, 1044-53.
- Oi-Kano Y, Kawada T, Watanabe T, et al (2013). Oleuropein supplementation increases urinary noradrenaline and testicular testosterone levels and decreases plasma corticosterone level in rats fed high-protein diet. *J Nutr Biochem*, **24**, 887-93.
- Parzonko A, Czerwinska ME, Kiss AK, Naruszewicz M (2013). Oleuropein and oleacein may restore biological functions of endothelial progenitor cells impaired by angiotensin II via activation of Nrf2/heme oxygenase-1 pathway. *Phytomedicine*, **20**, 1088-94.
- Pasban-Aliabadi H, Esmaili-Mahani S, Sheibani V, et al (2013). Inhibition of 6-hydroxydopamine-induced PC12 cell apoptosis by olive (*Olea europaea* L.) leaf extract is performed by its main component oleuropein. *Rejuvenation Res*, **16**, 134-42.
- Saad ED (2011). Endpoints in advanced breast cancer: methodological aspects and clinical implications. *Indian J Med Res*, **134**, 413-8.
- Santiago-Mora R, Casado-Diaz A, De Castro MD, Quesada-Gomez JM (2011). Oleuropein enhances osteoblastogenesis and inhibits adipogenesis: the effect on differentiation in stem cells derived from bone marrow. *Osteoporos Int*, **22**, 675-84.
- Sirianni R, Chimento A, De Luca A, et al (2010). Oleuropein and hydroxytyrosol inhibit MCF-7 breast cancer cell proliferation interfering with ERK1/2 activation. *Mol Nutr Food Res*, **54**, 833-40.
- Struckhoff AP, Patel B, Beckman BS (2010). Inhibition of p53 sensitizes MCF-7 cells to ceramide treatment. *Int J Oncol*, **37**, 21-30.
- Taylor WR, Agarwal ML, Agarwal A, Stacey DW, Stark GR (1999). p53 inhibits entry into mitosis when DNA synthesis is blocked. *Oncogene*, **18**, 283-95.
- Zare L, Esmaili-Mahani S, Abbasnejad M, et al (2012). Oleuropein, chief constituent of olive leaf extract, prevents the development of morphine antinociceptive tolerance through inhibition of morphine-induced L-type calcium channel overexpression. *Phytother Res*, **26**, 1731-7.