THE PHTHALATE DBP-INDUCED CYTOTOXICITY AND APOPTOSIS *VIA* GENE EXPRESSION OF *p53*, *Bcl2* AND *Bax* IN TUMOR CELL LINES

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The phthalate compounds are widely used as plasticizers and softeners in plastic materials. There has been an increasing concern regarding their possible health hazard to humans (Kleinsasser *et al.*, 2000). Chemically, the phthalates are not bound to products, therefore, they can easily leak out to enter the environment and diffuse within the materials (Fujii *et al*., 2003). DBP is greatly used as plasticizer in food containers, furniture, toys, cosmetics, cellulose plastics, latex adhesives and dye solvents (Heudorf *et al*., 2007). Phthalates are quickly absorbed after oral administration or inhalation due to their lipophilic characteristics (Kavlock *et al*., 2006). In our previous study, Fahim *et al*. (2018) found that DBP has migratory effect from bottled natural drinking water stored under direct sunlight. The migration amount increased gradually by storage time.

Previous studies reported that DBP could reach high deleterious levels (Otake *et al*., 2004) and cause developmental toxicity in rat embryonic limb bud cells (Kim *et al*., 2002). It can cross through the placental and blood-brain barriers (Huang *et al*., 2014; Wo´jtowicz *et al*., 2017). High concentrations of DBP have been detected in milk, human cord blood and urine (Ho¨gberg *et al*., 2008; Huang *et al*., 2014). Moreover, Kawano (1980) found that accumulation of DBP in brain of rats was higher after chronic exposure than after a single inhalation, suggesting accumulation of phthalate in brain tissue. Xu *et al*. (2013) reported that DBP has the possibility to induce neurotoxicity in *zebra fish* embryos. In another study Chen *et al*. (2014) found that DBP enhanced-estrogenic activity and developmental toxicity in *zebra fish* embryo, and suggested that phthalates have the possibility to cause health hazards to human beings.

When, the male rats were exposed to high dose of DBP, inhibition of proliferation of fetal testicular somatic cells was observed (Boekelheide *et al*., 2009). Moreover, it disturbed the sex ratio of the offspring, delayed female sexual maturation, and deteriorated the sperm quality of F1 generation males at 500 mg/kg of DBP (Dobrzyńska *et al*., 2011). Li *et al*. (2009) reported that DBP also had adverse effects on cognitive abilities of male rats and their neurobehavioral aspects. In addition, exposures to DBP were reported to have positive correlation with developmental behavior disorders in eight-years-

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old children (Lien *et al*., 2015). Furthermore, exposure of DBP could induce neurotoxicity and apoptosis via activating caspase-3 in animal's neurons (Li *et al*., 2013; Li *et al*., 2014). Wellejus *et al*. (2002) found that di-*n*-butyl phthalate (DBP) induced ROS (reactive oxygen species) and oxidative DNA damage in the livers, kidneys and testes of rat. Also, Wo´jtowicz *et al*. (2017) observed that DBP stimulated apoptosis, neurotoxicity and ROS formation.

The hallmark of cancer is a cell death event that can be classified according to morphological variances (Wang *et al*., 2016). However, cytotoxic agents, radiation or drugs-induced cell death could result G2-M arrest. This phenomenon was associated with damage or incomplete mitosis (Bonelli *et al*., 1996). Wu *et al*. (2006) also mentioned that many investigators have shown that different cytotoxic agents can induce G2/M phase accumulation.

Apoptosis could by induced by *p53* gene through down regulation of *Bcl-2* gene, and activation of caspases (Haupt *et al*., 2004). It has been known that p53 contributed to the transcriptional activation of a great number of target genes, i.e. Bax (pro-apoptotic protein) (Mirzayans *et al*., 2012). Bcl-2 family genes play vital role in controlling the mitochondrial pathway of apoptosis (Dewson and Kluck, 2010; Wu *et al*., 2013), which consists of anti-apoptosis genes, such as *Bcl-2, Bcl-xl* and pro-apoptosis genes, such as *Bax, Noxa, Puma, Bim, Bid* (Chen and Lesnefsky, 2011).

Assessment of cytotoxicity by mammalian cell lines to measure the activities of cytotoxic agents included either the MTT colorimetric cell viability assay (Borenfreund and Puerner, 1984) or neutral red cell viability assay (NR) (Fotakis and Timbrell, 2006).

The neutral red assay is more sensitive than the MTT assay (Aras *et al.*, 2008). The NR assay is based on the integration of the supravital dye inside the lysosomes of viable cells. If toxic agents injure the lysosomal membrane, damaged or dead cell cannot keep the dye. After NR dye has been extracted from lysosome, it is quantified spectrophotometrically (Fotakis and Timbrell, 2006).

The objective of this study was to assess the potential cytotoxic and apoptotic effects of DBP on human cell growth. Cell cycle arrest, apoptosis-related genes of the HepG2 cell line was also evaluated.

MATERIALS AND METHODS

In vitro cytotoxic activity using Neutral red cell viability/ cytotoxicity assay

Human cell lines and cultures

Two different types of human cancerous and normal cell lines were used: lung carcinoma (A549) and lung normal (Wi38). Moreover, liver cancer (HepG2) and liver normal cell line (THLE2) were

used. The plates with human cell lines were incubated at 37°C in 5% CO_2 for 24 hours to obtain monolayer confluence.

In vitro cytotoxicity by Neutral red assay (NR assay)

In vitro Neutral red cytotoxicity assay described by Fotakis and Timbrell (2006) was carried out. Culture medium containing different concentrations of DBP compound (10, 25, 50, 100 and 200 µg/ml) were added in triplicate and medium without DBP was considered as untreated control. The washed dye-medium was isolated and the plates with formol-calcium. 500 µl of acetic acid-ethanol (one ml glacial acetic acid in 100 ml 50% ethanol) was added and the plates were saved for fifteen min at room temperature to extract the dye. The plates were then shacked gently for few seconds, so that complete dissolution was achieved. The absorbance of the extracted dye was measured using spectrophotometric reading (Spectra max 190-Molecular devices) at 540 nm filter. The average of three measurements for each concentration was determined. The percent viability of the concentration of DBP which reflects half the maximum concentration of the cell proliferation (IC50) was estimated.

Statistical analysis

Cytotoxicity assay was measured as OD (optical density) at 540 nm. Dose-response curves were plotted, and the half maximum concentration 50% inhibitory concentrations of DBP (IC50) were calculated by Graph Pad Prism software program. For statistical analysis of data, multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by the LSD test for post hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. Data were analyzed using SPSS (version 11; Chicago, IL, USA).

Cell cycle analysis using flow cytometry

Cells were digested with warm Trypsin-EDTA + warm PBS-EDTA (0.25%) (500 µl + 500 µl), and incubated for ten min at 37°C. The mixture was centrifuged at 450 rpm for five min, and then the supernatant was removes carefully. The mixture was washed two times in warm PBS and the cell pellet was re-suspended in 500 µl warm PBS, centrifuged and supernatant was removed. A volume of 150 µl PBS + 350 µl ice-cold 70% ethanol was added and incubated at 4°C for one hour to fix the cells. The mixture was centrifuged at 350 rpm for 10 minutes and then ethanol was removed from supernatant carefully. The mixture was washed two times in warm PBS and the cells were re-suspended in 500 µl warm PBS, centrifuged and supernatant was removed. The cells were re-suspended in 100 µl PBS and stored at 4°C for up to 4 days. In darkness, the cells were stained with 100 µl of PI solution + 50 µl RNase A solution (100 µg/ml), and incubated in darkness for 30-60 min. The stained cells were read in Attune flow cytometer (Applied Bio-system, US).

Determination of the expression levels of apoptosis-regulatory genes

Total RNA was isolated from HepG2 cells using Gene JET RNA Purification Kit (Thermo Scientific, # K0731, USA) according to the manufacturer's protocol. The reverse transcribed of 5 µg from total RNA by using RevertAid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, USA) to produce cDNA. cDNA was used as a template to determine the relative expression of the apoptosis-related genes using StepOnePlus real time PCR system (Applied Biosystem, USA).

The primers were designed by Primer 5.0 software, Table (1). The housekeeping gene β-actin was used as a reference to calculate fold change in target gene expression. A 25-µL PCR mix was prepared by adding 12.5 µL of 2X Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 2 µL of cDNA template, 1 µL forward primer, 1 µL reverse primer, and 8.5 µL of nuclease free water. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40-45 cycles of amplification of DNA denaturation at 95°C for 15 s, annealing at 60°C for 30s, extension at 72°C for 30 s. At the end of the last cycle, the temperature was increased from 63 to 95°C for melting curve analysis. The cycle threshold (Ct) values were calculated for target genes and the housekeeping gene, and relative gene expression was determined using 2-ΔΔCt method .

Statistical analysis

All data were expressed as means ± standard error (SE). The statistical significance was evaluated by one way ANOVA using SPSS 18.0 software. Values were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

In vitro cytotoxic effect on human cancer and normal cells

Cytotoxicity of DBP was screened on multiple cell lines and examined by NR assay (Neutral red assay). In this study, Doxorubicin was used as a positive control, which exhibited cytotoxic activity against human hepatocellular carcinoma cells (HepG2). The DBP exhibited marked cytotoxic activity against liver normal cell line (THLE2), the IC50 of Doxorubicin at 500.9 µg/ml and 254 µg/ml in DBP (Fig. 1A). IC50 levels were measured for Doxorubicin and DBP which showed 26.82 µg/ml and 43.77, respectively, as shown in Fig. (1B). Induced growth inhibitory effect on normal lung fibroblast cell line (Wi38) was examined at IC50 of Doxorubicin and DBP which were observed at 530.3 µg/ml and 445.3 µg/ml, respectively (Fig. 2A). The cytotoxic activity was noticed also against lung cancer cell line (A549), at IC50 of 93.54 µg/ml or Doxorubicinis and 106.3 µg/ml for (DBP (Fig. 2B). These data indicated that DBP decreased cell viability in malignant and non-malignant cells as well and confirmed the occurrence of cytotoxic effect.

Our results agreed with those of Kim *et al*. (2002), who suggested that DBP and MBuP induced developmental toxicity in rat embryonic limb bud cells due to oxidative stress. The IC50 values of DBP for cytotoxicity were 25.54 µg/ml and cell differentiation was 21.21 µg/ml were observed in neutral red assay.

Boekelheide *et al*. (2009) reported that DBP inhibited proliferation and increased apoptosis in somatic cells in the fetal rat testis. Moreover, DBP might cause genetic defects in male gametes, which could deteriorate sperm quality of male offspring, and delayed sexual maturation of female offspring (Dobrzyńska *et al*., 2011).

Abdel-Ghani *et al*. (2014) observed that DBP decreased in fertility of male and female rats and increased mortality rate with DNA damage. DBP has hepatotoxic effect as increased GOT and GPT activities were measured. Moreover, embryonic, developmental and reproductive toxicity were observed.

The high concentrations of DBP stimulated cytotoxicity in mesencephalic neurospheres of rat embryo via decreased number of proliferating cells (Ishido and Suzuki, 2014).

Wo´jtowicz *et al*. (2017) observed that DBP-induced cytotoxic effects (decreased cell viability) at high concentrations. Fahim *et al*. (2018) reported that DBP has cytotoxic effect on human cell line and potential carcinogenic activity in SMART protocol (Somatic Mutation and Recombination Test) of *Drosophila melanogaster*.

Effect of DBP on cell cycle arrest utilizing flow cytometry assay

According to the inhibition rate of liver cancer cell line (HepG2) viability, it was necessary to assess cytotoxic effect of DBP on cell cycle arrest using flow cytometry based on cell cycle distribution. Figure (3) showed that compared with the control group, DBP at 43.77 µg/ml affected the cell cycle distribution on HepG2 cells. The G0/G1 phase showed a decrease from 58% to 33%. Similarly, the S-phase percentage also exhibited a decrease from 30% to 18% in the control and DBP, respectively. While, the percentage of HepG2 cells at the G2/M phase was highly increased after incubation with DBP (49%) as compared to the control (12%).These results showed significant accumulation of HepG2 cells in the G2/M phase, and confirmed that DBP has marked cytotoxic effect via induction of G2/M phase arrest of the cell cycle.

Sleiman and Stewart (2000) suggested that many cytotoxic molecules induce mitotic cell death (apoptosis) which occurs in parallel with G2/M arrest. In addition, the sinularin induced G2/M arrest by increasing the expressions of genes related to G2/M such as p53, and p21 was reported. Furthermore, sinularin stimulated apoptosis was observed via decreased anti-apoptotic Bcl-2 expression whereas; it increased the expressions of Bax (Chung *et al*., 2017).

Determination of the expression levels of apoptosis-regulatory genes

The role of apoptosis in DBP induced cytotoxicity on liver cancer cell lines (HepG2) was studied. The expression levels of apoptosis-related genes such as *p53*, *Bcl*-2 and *Bax* in HepG2 cells were estimated by real time PCR (qRT-PCR). Figure (4) showed that, compared to the untreated group, the expression levels of p53 and Bax were increased, whereas that of *Bcl*-2 gene was decreased. These results indicated that the DBP killed HepG2cells through apoptosis mechanism mainly via over expression of *p53* and *Bax* genes, while Bcl-2 was down regulated.

Several investigators pointed to an essential role for p53 in the balance between proliferation and apoptosis (Polager and Ginsberg, 2009), as, the *p53* gene plays a key role in G2 checkpoint, by halting G2/M transition. In addition, p53 regulates the balance between the proapoptotic gene Bax and the antiapoptotic gene Bcl-2 through its transcriptional activities (Leu *et al*., 2004).

Li *et al*. (2013) indicated that DBP induced caspase-3 activity in the hippocampi of rats. Wo´jtowicz *et al*. (2017) demonstrated that DBP-induced neurotoxicity and apoptosis via stimulation of caspase-3 and enhancement of AhR mRNA levels at lower concentrations. Moreover, DBP-increased ROS production and LDH activities in concentration from 10 nM to 100 μM were observed.

Finally, cytotoxicity and programmed cell death (apoptosis) of phathalate di-n-butyl phthalate (DBP) was tested on *in vitro* human cell growth. Cell cycle arrest and apoptosis-related genes of the HepG2 cell line were also evaluated. The results revealed that DBP stimulated cytotoxicity by decreased cell viability in cancer and normal cell lines. Cell cycle blocking at the G2/M phase was evident. Moreover, induction of apoptosis via increased p53 and Bax mRNA expression levels accompanied with reduced *Bcl2* gene expression correlated with apoptosis pathway was observed. These studies provided evidence that DBP can inhibit cell proliferation in the human cell lines and also shed light on dose-dependent cellular cytotoxicity consequences of exposure to this phthalate. Our findings detected the toxic and apoptotic effects of DBP in human cell lines. Thus, the use of phthalates could presents negative health biohazards to human beings. This calls for strict control of the threshold levels that should be permitted for the industrial products containing DBP as it could reflect biosafety hazards to human and environment.

SUMMARY

The components of phthalate are utilized as softeners and plasticizers in a wide range of plastic materials. There has been a high concern of potential health risks to humans. DBP is commonly used as plasticizer in food containers, furniture, toys, cosmetics, cellulose plastics, latex adhesives and dye solvents.

The present study focused on the cytotoxic activity of the DBP and its possible underlying mechanisms were also investigated. The results showed that phathalate di-*n*-butyl phthalate (DBP) induced profound cytotoxicity in cancer cells, i. e. Human hepatocellular carcinoma (HepG2) (IC50 = 43.77 µg/ml), against human lung cancer cell line (A549) (IC50 = 106.3 µg/ml). Moreover, DBP exhibited cytotoxic activity on normal cell lines; the IC50 of DBP is 254 µg/ml and 445.3 µg/ml on THLE2 and Wi38, respectively. Flow cytometric analysis demonstrated that treatment of HepG2 cells with DBP increased G2/M phase cell cycle arrest. The quantitative real time-PCR was used to measure the mRNA levels of *p53*, *Bax*, and *Bcl-2* genes. The data showed that DBP changed transcriptional levels of these apoptosis-related genes. The mRNA expression of p53 and Bax were up-regulated, but, the transcription of Bcl2 was significantly down-regulated compared to the control.

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Table (1): Primers used for each gene.

Primer	Sequence
p53	F- 5'-CCCAGGTCCAGATGAAG-3' R- 5'-CAGACGGAAACCGTAGC-3'
Bcl-2	F-5'-GGATGCCTTTGTGGAACTGT-3' R-5'-AGCCTGCAGCTTTGTTTCAT-3'
Bax	F-5'-TTTGCTTCAGGGTTTCATC-3' R-5'-CAGTTGAAGTTGCCGTCAGA-3'.

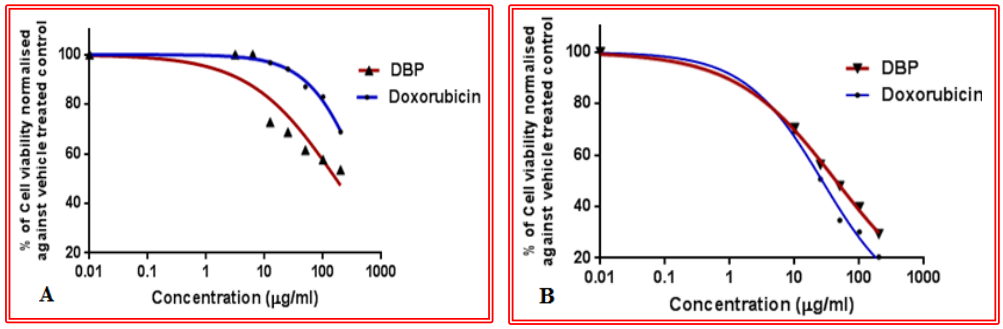

Fig. (1): Dose-dependent growth inhibition by Doxorubicin as a positive control and DBP on, A) non-malignant human liver cells *(THLE2)* and B) malignant liver cell line *(HepG2cells).* Cell viability was quantified by NR assay. Results are mean ± (n = 3). *p < 0.05 to compared to control.

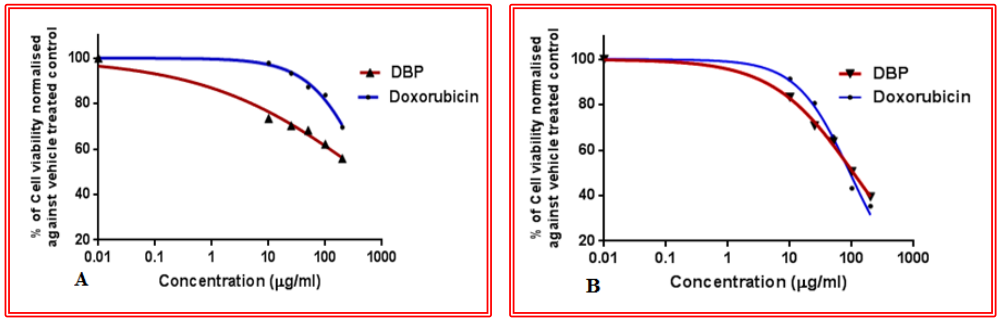

Fig. (2): Dose-dependent growth inhibition by the Doxorubicin as a positive control and DBP on, A) non-malignant human lung cells (Wi38) and B) malignant human lung cells(A549cells). Cell viability was quantified by NR assay. Results are mean ± (n = 3). *p < 0.05 to compared to control.

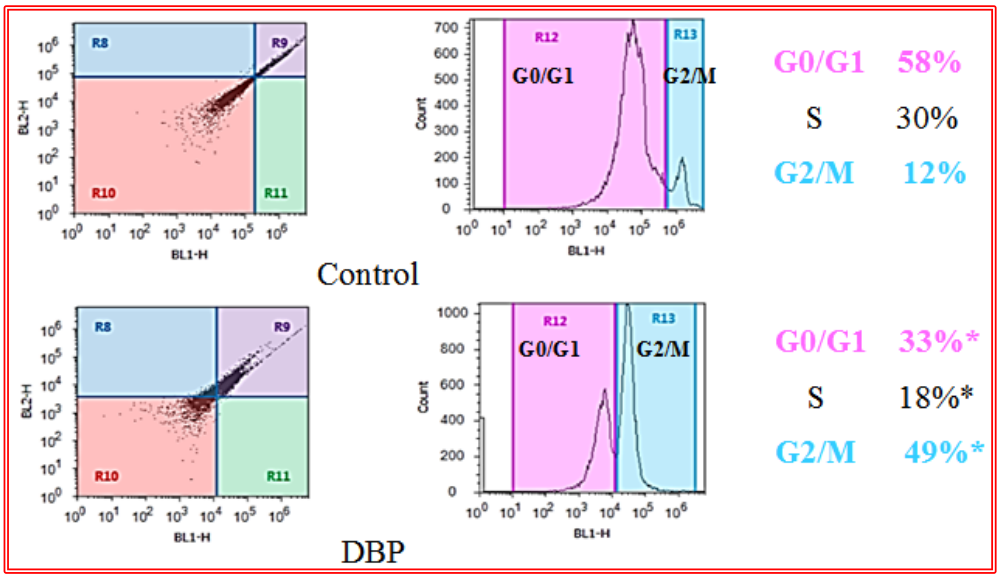

Fig. (3): Effect of DBP on the G2/M cell cycle arrest. Liver cancer cells (HepG2) were treated with DBP at the concentration of 43.77 μg/mL in order to check the cell cycle distribution and then were analyzed by *flow cytometry.*

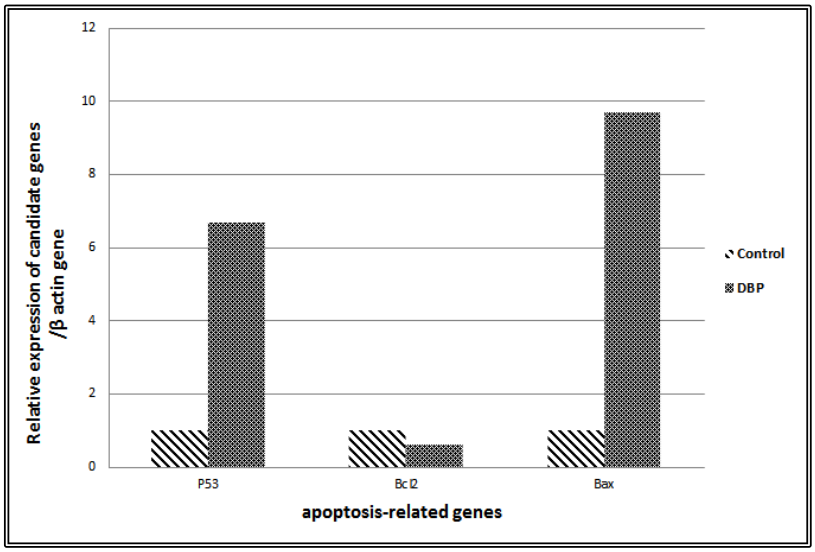

Fig. (4): Effects of DBP on apoptosis-related genes after exposure to 54.86 μg/mL, mRNA expression of p53, Bcl-2, and Bax was assessed by quantitative RT-PCR *P < 0.05, compared to the control group.