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Evaluation of Four Herbicides Cytotoxicity on Normal Liver THLE2 Cells

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THE widespread increase in pesticide use is a source of pollution worldwide. Although herbicides have various applications, they nonetheless represent a risk to human health due to their tendency to bioaccumulate in many different environments. The purpose of this research was to investigate the cytotoxic effects of pendimethalin (PEN), oxyfluorfen (OFN), fluazifop-p-butyl (FPB), and pyraflufen ethyl (PFE) herbicides on human normal liver (THLE2) cells through the evaluation of the cell viability (using the MTT assay), apoptosis (using qPCR), cell cycle (using flowcytometry), and oxidative stress status (using biochemical assays). The four herbicides induced cytotoxic effects on THLE2 cells as evidenced by suppressed cell viability with IC50 values as follows: PEN (60.50±3.19 µM), OFN (100.36±4.66 µM), FPB (174.90±6.54 µM) and PFE (186.72±6.82 µM). They also increased apoptosis (high *Bax* and caspase 3, and low *Bcl2* gene expression). However, only PEN and OFN induced cell cycle arrest in G0/G1 and both S and G2/M phases, respectively, with subsequent downregulated expression of cyclin D1, cyclin A2, and cyclin-dependent kinase 4 (*CDK4*). Among the four herbicides, only OFN, PFE, and FPB induced oxidative stress damage as revealed by higher levels of intracellular reactive oxygen species (ROS) and malondialdehyde (MDA), inhibited activities of antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx)], and downregulated expression of nuclear factor erythroid 2-related factor 2 (*Nrf2*) and heme oxygenase-1 (*HO-1*) genes. FPB and PFE had a greater safety margin on human normal THLE2 cells and might be used with less adverse consequences on human and animal health.

Keywords: Pendimethalin, Oxyfluorfen, Pyraflufen ethyl, Fluazifop-p-butyl, Cyclin-dependent kinase, Cytotoxicity.

Introduction

In many countries, the onion (*Allium cepa* L.) is a significant vegetable crop grown for export and domestic consumption [1,2]. It is grown across the world's tropics and subtropics when proper conditions exist [3,4]. Weeds significantly lower the crop's yield, quality, and value by competing with *A. cepa* for space, nutrients, light, and water [5,6], leading to substantial yield losses that have been reported to reach as high as 70-75% [7]. Several ways to get rid of weeds have been mentioned, such as using cultural, mechanical, herbicidal, and mulches [8]. Allium cepa weed population was greatly decreased by the use of pendimethalin (PEN), oxyfluorfen (OFN), fluazifop-p-butyl (FPB), and pyraflufen ethyl (PFE) herbicides, and *A. cepa* yield was enhanced [9,10]. The bad side of herbicides is their hazardous effects on not only plants but also humans and the environment. The Environmental Protection

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Agency of the United States (USEPA) has placed PEN in toxicity class 3, while the US Environmental Protection Agency's classified it as a category C (human potential carcinogen) substance. PEN can trigger DNA damage-dependent cell death in Chinese hamster ovary (CHO) cells [11,12], rat hepatocytes, and thyroid FRTL 5 cells [13,14], and mouse bone marrow cells [15]. PEN is also a pollutant and is very hazardous to fish and other aquatic invertebrates [16] and has cytotoxic effects on human umbilical vein endothelial cells (HUVECs) through induction of cell cycle arrest in the G0/G1 phase [17,18]. Inducing genetic damage by herbicides may raise the occurrence of hereditary illness in future generations while also contributing to somatic cell disorders such as cancer in the current generation [11]. Indeed, cancer rates have been shown to rise by PEN exposure [19].

OFN kills weeds by inhibiting the enzyme protoporphyrinogen oxidase (PPO), which oxidizes to protoporphyrin IX when exposed to light and oxygen, generating reactive oxygen species (ROS) that may cause cell death [20]. OFN main side effects include an increased incidence of liver cancer in mice, inhibition of the protoporphyrinogen IX oxidase enzyme in rats, and a possible carcinogenic influence in humans [21]. It also has ROS-dependent cyto-and geno-toxic effects on fish and other aquatic invertebrates [22-26]. In zebrafish, OFN disrupts hepatic lipid and glucose metabolism during embryonic development and causes hepatocyte mortality by increasing inflammatory factors [27]. OFN induces promotes cell cycle arrest through the regulation of MAPK, PI3K, and autophagy in bovine mammary epithelial cells [28]. PFE, like OFN, prevents weed growth by inactivating the PPO, but it has a much stronger, more immediate herbicidal impact and is hardly hazardous to animals at all [29]. FPB is readily absorbed via leaf surfaces and is mostly converted to fluazifop-p (FP), which inhibits lipid formation through the inactivation of acetyl coenzyme A carboxylase and induces ROS overproduction resulting in lipid peroxidation and damage of cell membrane and finally cell death of weeds [30]. FPB triggers a cytotoxic effect on mammalian cells by the same mechanism [31]. Rats exposed to FPB showed signs of liver, kidney, and testis damage caused by oxidative stress [32]. Based on the previously mentioned data, little is known regarding the comparison between the deleterious effects of the four herbicides on normal human cells. Therefore, this study aimed to evaluate the cytotoxic effects of PEN, OFN, FPB, and PFE on human normal liver (THLE2) cells through investigating their impacts on cell viability, apoptosis, cell cycle, and oxidative stress.

Material and Methods

Cytotoxicity by MTT Assay

The MTT assay was conducted to evaluate the cytotoxic potential of the four herbicides (PEN, OFN, FPB, and PFE) on the normal human liver cell line THLE2 as previously described [33,34]. The cells were purchased from VACSERA (Egypt). and were grown in Dulbecco's Modified Eagle's medium (DMEM, GIBCO, Grand Island, NY, USA, Cat. no.11995073) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Cat. no.10099133) at an initial number of 10000 cells per well. Cells were then treated by each herbicide with the following concentrations 0, 12.5, 25, 50, 100, 200, and 400 µM and were incubated for 24 h at 37°C. Cells were then treated with 12 mM (10 µl/well) MTT (Invitrogen, Waltham, MA, USA, Cat.no. M6494) and were re-incubated at 37°C for 4 h. Finally, we got rid of the MTT solution and added 100 µl dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA, Cat. no.673439) for 0.5 h. Using absorbance measurements taken at 570 nm, the IC_{50} was calculated using a sigmoidal curve plotted in GraphPad Prism 8 [35].

Experimental Design

THLE2 cells were allocated into 5 groups. In the control (Cnt) group, cells were treated with the vehicle (DMSO). In the PEN group, cells were treated with IC_{50} PEN (60.50±3.19 µM). In the OFN group, cells were treated with IC_{50} OFN (100.36±4.66 µM). In the FPB group, cells were treated with IC_{50} FPB (174.90±6.54 µM), and in the PFE group, cells were treated with IC_{50} PFE (186.72±6.82 µM). After all treatments, THLE2 cells were then incubated for one day until they reached suitable confluence for further analysis by qPCR, flow cytometry and spectrophotometer.

Gene Expression by qPCR

The qPCR was applied to measure the changes in the fold changes of apoptosis-related genes (*Bax*, caspase3, and *Bcl2*) and cell cycle-associated genes

TABLE 1. Primer used for real-time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Bax	TGCTTCAGGGTTTCATCCAG	GGCGGCAATCATCCTCTG
Caspase3	GAAGCGAATCAATGGACTCTGG	GACCGAGATGTCATTCCAGTGC
Bcl2	AGGAAGTGAACATTTCGGTGAC	GCTCAGTTCCAGGACCAGGC
Cyclin D1	GCTGCTCCTGGTGAACAAG	ACAGAGGGCAACGAAGGTC
CDK4	CCATCAGCACAGTTCGTGAGGT	TCAGTTCGGGATGTGGCACAGA
Cyclin A2	TGGAAAGCAAACAGTAAACAGCC	GGGCATCTTCACGCTCTATTT
NrF2	CAGCGACGGAAAGAGTATG	TGGGCAACCTGGGAGTAG
HO-1	CGGGCCAGCAACAAAGTG	AGTGTAAGGACCCATCGGAGAA
B-actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG

[cyclin D1, cyclin A2, and cyclin-dependent kinase 4 (*CDK4*)] in THLE2 cells. We first extracted total RNA from THLE2 cells using Trizol (Invitrogen,Waltham, MA, USA, Cat# 15596026). We then used RevertAid H Minus Reverse (Thermo Scientific, #EP0451) to convert RNA into cDNA which was then quantified using a Nanodrop (Q5000, Quawell, San Jose, CA, USA). The qPCR test and data analysis were conducted using a Piko qPCR thermal cycler (Thermo Scientific) and its corresponding software. The PCR mixture included cDNA, primers (Table 1), and 2X SYBR Green Mix (Thermo Scientific). We maintained the temperature range as specified by the manufacturer and as previously detailed [36,37]. Gene expression in the form of fold change was quantified using the $2^{-\Delta\Delta Ct}$ method against the internal control *B-actin* [38,39].

Cell Cycle by Flow Cytometry

Flow cytometry was used to quantify the number of THLE2 in each phase of the cell cycle as previously reported [40,41]. One day after trypsinization, THLE2 cells were fixed at -20°C in 70% ethanol, underwent 3 centrifugations (10,000 rpm/5 min) with interval twice washes with 50% ethanol/PBS, and stained with 100 µl propidium iodide (PI) in PBS (640932, Biolegend, CA) for 30 min. Cells stained positive for PI were counted using flow cytometry (FACScan, BD Biosciences, USA), and the percentage of cells in each cell cycle phase was evaluated using FlowJo V.10 (BD Biosciences).

Oxidant and Antioxidant Status

By measuring the production of the fluorescent metabolite dichlorofluorescein (DCF) from the non-fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA), we were able to undertake a fluorometric analysis of intracellular reactive oxygen species (ROS) as previously described [42,43]. THLE2 cells (86% confluence) were treated with each of the herbicides at IC_{50} value and were incubated for one day before the addition of 10 µM DCF-DA for 1 h. The fluorescence was measured (at 485 nm for excitation and 535 nm for emission) using a fluorescent microplate reader. The level of lipid peroxide malondialdehyde (MDA) marker and the activities of the antioxidant enzymes [superoxide dismutase activity (SOD) and catalase (CAT), and glutathione peroxidase (GPx)] were measured in THLE2 cells using commercially available kits (Biodiagnostic, Cairo, Egypt) and as previously described [44-47]. Determining the amount of thiobarbituric acid reactive substances (TBARS) is necessary for measuring MDA. TBA combined with MDA in an acidic media forming TBARS. SOD inhibits epinephrine's conversion to adrenochrome at a pH of 10.2, making measuring epinephrine concentrations possible. The CAT activity was determined by calculating the H_2O_2 dissociation rate at 240 nm.

Statistical Analysis

Data was analyzed statistically using GraphPad Prism 8.0. (San Diego, CA). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to identify significant differences between groups. The data was shown as a mean ± standard error of mean (SEM), and a value of $P<0.05$ was used to indicate statistical significance.

Results

Effect of Herbicides on THLE2 Cell Viability

The cytotoxic potential of the four herbicides on the human normal liver THLE2 cells was determined by the MTT assay (Fig. 1). PEN, OFN, FPB, and PFE exerted cytotoxic effects on THLE2 with IC_{50} values of 60.50±3.19, 100.36±4.66, 174.90±6.54, and 186.72±6.82 µM, respectively. This infers that the greater IC_{50} values of FPB, and PFE resulted in less cytotoxicity being generated in THLE2 cells. In contrast, the IC_{50} values for PEN and OFN were much lower, leading to greater THLE2 cytotoxicity.

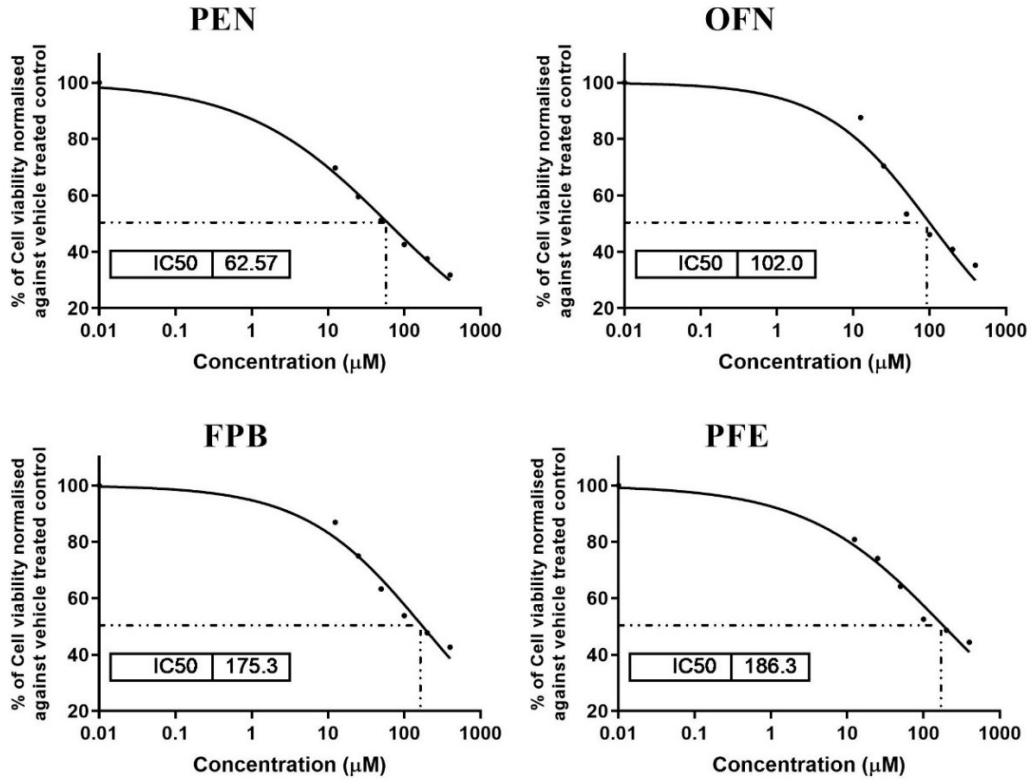

Fig.1. Cytotoxic effects of PEN, OFN, FPB, and PFE herbicides on the human normal liver THLE2 cells as detected by the MTT assay. The representative sigmoidal curves exhibited the IC_{50} values of each herbicide as displayed within the rectangles. The values shown are the averages of three separate studies (n = 3).

The Four Herbicides Triggered Apoptosis in THLE2 Cells

To check whether the four herbicides could inhibit THLE2 proliferation through apoptosis, we conducted qPCR to evaluate the relative expression of the apoptotic genes (*Bax* and Caspase3) and the anti-apoptotic *Bcl2* gene following treatment with the herbicides. As shown in Fig.2, the four herbicides induced apoptosis in THLE2 cells as evidenced by significant ($P<0.05$) upregulation of *Bax* and caspase 3 and significant ($P<0.05$) downregulation of *Bcl2* in cells treated with the herbicides compared to the control (vehicle-treated) cells. Comparing the apoptotic effects of the four herbicides, PFE showed the lowest apoptosis followed by FPB, while PEN exhibited the highest apoptosis. These results agree with the results of the MTT assay and both assays confirmed that the four herbicides inhibited THLE2 proliferation through induction of apoptosis with lowest cytotoxic effect for PFE followed by FPB.

Effect of the Herbicides on the THLE2 Cell Cycle

To determine whether any of the four herbicides would affect the number of THLE2 in each phase of the cell cycle, PI staining was executed after treatments with the IC_{50} value of each herbicide. As displayed in Fig. 3, the number of THLE2 cells treated with PEN significantly ($P<0.05$) increased in the G0/G1 phase, indicating cell cycle arrest in this phase, relative to the control cells. On the other hand, OFN induced arrest in both S and G2/M phases as revealed by a significantly ($P<0.05$) higher number of THLE2 cells in the two phases compared to the control group. Moreover, cells treated with PEN and OFN showed a significant ($P<0.05$) reduction in G2/M and G1 phases, respectively, relative to the control group. On the other hand, there were no significant differences in cell number in any phase of the cell cycle after treatment with FPB or PFE as compared to the control groups. Cell cycle arrest in different phases was further confirmed at a molecular level via the detection of relative expression of cell cycle-associated genes (cyclin D1, cyclin A2, and *CDK4*) by

qPCR and the results were presented in Fig.4. Interestingly, cells treated with PEN showed significantly ($P<0.05$) lower expression of cyclin D1 and its partner *CDK4* and significantly ($P<0.05$) higher cyclin A2 expression compared to the control groups. Moreover, OFN-treated cells exhibited significantly ($P<0.05$) lower expression of the cyclin A2 gene and significantly higher expression of cyclin D1 and its partner *CDK4* than the control group. However, no significant changes were observed in the expression of cyclin D1, cyclin A2, and *CDK4* genes following treatment with FPB or PFE relative to the control group. These results confirm cell cycle arrest in the G0/G1 phase (via PEN) and both S and G2/M phases (via OFN).

Effect of the Herbicides on the Oxidant and Antioxidant Status of THLE2 Cells

Treatment of THLE2 cells with OFN, FPB, or PFE led to a significant ($P<0.05$) increase in intracellular ROS as compared to control and PEN-treated cells, as determined by ELISA (Fig.5). In contrast, no significant ($P>0.05$) difference was observed in the intracellular ROS levels between PEN-treated cells and the control cells. For further confirmation, we determined MDA levels and found similar results. Among the OFN, FPB, and PFE groups, PFE exhibited the lowest ROS and MDA levels. We also detected the activities of the antioxidant enzymes (SOD, CAT, and GPx) and the expression of antioxidant-related genes (*NrF2* and *HO-1*) and found significantly ($P<0.05$) lowered levels of these antioxidant markers in THLE2 cells treated with OFN, FPB, or PFE, with lowest levels in OFN-treated cells, as compared to the control and PEN-treated cells (Fig.5). On the other hand, these antioxidant indicators were not significantly ($P>0.05$) different in PEN-treated cells compared to controls.

Discussion

The use of herbicides to protect crops and household plants has been an integral component of farming and gardening for decades. The global rise in the usage of pesticides that has resulted from the creation of pesticide-resistant crops is a major contributor to environmental pollution [48,49]. Despite pesticides' many uses, they pose a threat to human health since they bioaccumulate in vegetation, soil, water, and even the air. The use of pesticides has been linked to the onset of potentially fatal illnesses. Farmworkers' exposure to pesticides causes DNA degradation and immunological alterations [50].

PEN treatment resulted in the maximum cytotoxicity, indicating that this herbicide may pose a threat to human cells. In agreement, several other studies reported cytotoxic potential for PEN in normal human and rodent cells. PEN induced cytotoxic effects on human umbilical vein endothelial cells (HUVECs) [17,18], Chinese hamster ovary (CHO) cells [11,12], rat hepatocytes and thyroid FRTL 5 cells [13,14], and mouse bone marrow cells [15]. The IC_{50} of PEN (60.50±3.19 µM) on THLE2 cells was quite similar to that reported by Lee, et al. [18] on HUVECs (nearly 50 µM) after 24 h incubation. However, Saquib, et al. [17] found a very high IC_{50} for PEN on HUVECs (500 - 1000 µM) after 4 h incubation. Another very high IC_{50} (1-10 mM) for PEN was recorded on CHO cells following 4 h incubation [11]. Therefore, PEN could induce cytotoxicity but at different concentrations based on the type of the cells and the incubation time. OFN and FPB also induced cytotoxic effects on THLE2 cells but at IC_{50} higher than that of PEN. In consistency, OFN inhibited cell viability in fish and other aquatic invertebrates [22-26] and FPB triggers a cytotoxic effect on mammalian cells [31]. On the other hand, to the best of our knowledge, no available study in the literature addressed a cytotoxic potential for PFE on mammalian cells, so far. Hence, our study could be the first to detect the cytotoxic effect of this herbicide on the THLE2 cells.

Real-time PCR results revealed induction of THLE2 apoptosis by the four herbicides as evidenced by higher expression of the apoptotic genes (*Bax* and Caspase3) and lower expression of the anti-apoptotic *Bcl2* gene. These results of the MTT assay and qPCR confirmed that the four herbicides inhibited THLE2 proliferation through induction of apoptosis with lowest cytotoxic effect for PFE followed by FPB. Consistent with our findings, a prior investigation utilizing a comparable dosage of OFN also observed elevated expression of apoptotic genes (*Bax*, caspase 8, and caspase 9) in bovine mammary epithelial cells [28]. Similarly, other studies reported apoptotic potential for PEN on HUVECs (at a higher dose, 500 and 1000 µM) [17], and mouse testicular cells (at a lower dose 20 µM) [51] as revealed by cell cycle arrest in the sub-G0/G1 phase. Unlike FPB or PFE which did not affect cellular distribution, both PEN and OFN induced cell cycle arrest in the G0/G1 phase (via PEN) and both S and G2/M phases (via OFN) in human normal liver THLE2 cells. These data were further confirmed by qPCR which revealed a significant downregulated expression of the G1 phase-associated genes (cyclin D1 and its partner *CDK4*) in cells treated with PEN and a significant lowered expression of S+G2/M phases-associated cyclin A2 gene in cells treated with OFN. Consistent with our data, a previous study also reported G1 arrest and downregulated expression of cyclin D1, cyclin D3, and CDK4 in HUVECs treated with 50 and 100 µM PEN [18]. In contrast, Ansari*, et al.* [52] found an arrest in both

sub-G0/G1 and G2/M phases in human lymphocytes treated with similar doses of PEN. On the other hand, our results are in agreement with Jang, et al. [28] who reported cell cycle arrest in the S, and G2/M phases with lower expression of *CDK2*, a marker for S/G2/M transition, in bovine mammary epithelial cells treated with OFN.

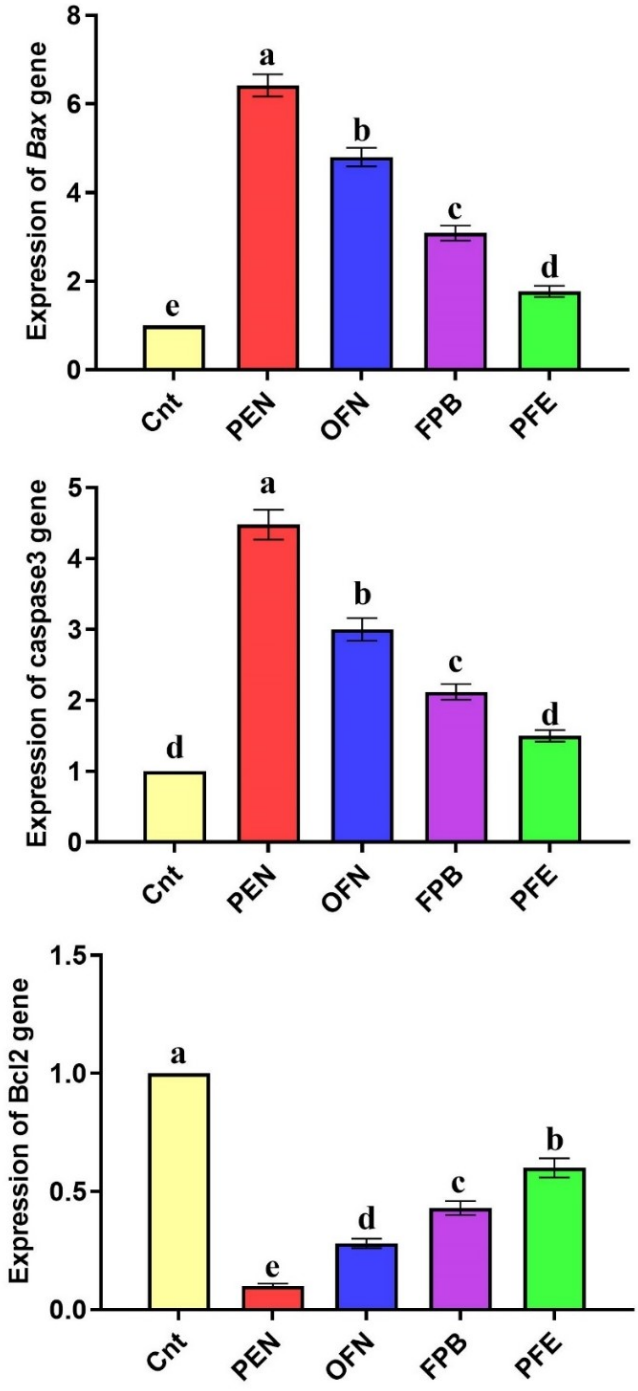

Fig.2. Analysis of qPCR data of apoptosis-related genes (*Bax*, caspase3, and *Bcl2*) in the human normal liver THLE2 cells. Columns and error bars represented fold change mean ± SEM (n = 5/group) and the different letters [a (highest) – e (lowest)] indicate significant difference between groups at P < 0.05. Each group was measured against every other group. Cnt, control (DMSO-treated) cells, PEN, pendimethalin-treated cells, OFN, oxyfluorfen- treated cells, FPB, fluazifop-p-butyl-treated cells, and PFE, pyraflufen ethyl-treated cells.

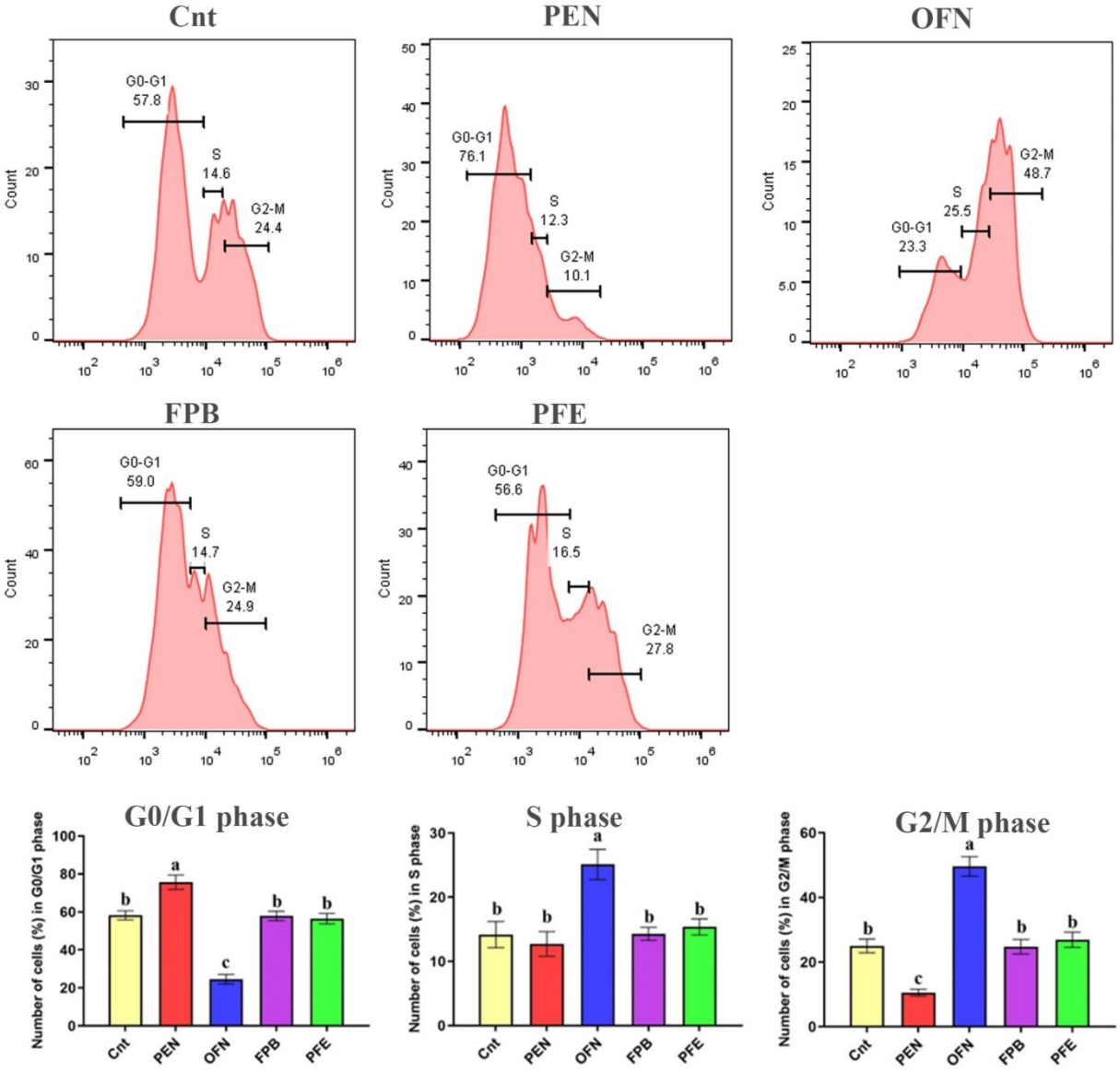
Cnt
G0-G1
57.8
S
14.6
G2-M
24.4
PEN
G0-G1
76.1
S
12.3
G2-M
10.1
OFN
G0-G1
23.3
S
25.5
G2-M
48.7
FPB
G0-G1
59.0
S
14.7
G2-M
24.9
PFE
G0-G1
56.6
S
16.5
G2-M
27.8
Count
G0/G1 phase
Number of cells (%) in G0/G1 phase
S phase
Number of cells (%) in S phase
G2/M phase
Number of cells (%) in G2/M phase
Cnt
PEN
OFN
FPB
PFE

Fig. 3. Flow cytometry histograms and representative graphs showing the impact of the four herbicides on the number of THLE2 cells in the G0/G1, S, and G2/M phases of the cell cycle. The cell count in each phase was expressed as %. Columns and error bars represented fold change mean ± SEM (n = 5/group) and the different letters [a (highest) – e (lowest)] indicate significant difference between groups at P < 0.05. Each group was measured against every other group. Cnt, control (DMSO-treated) cells, PEN, pendimethalin-treated cells, OFN, oxyfluorfen- treated cells, FPB, fluazifop-p-butyl-treated cells, and PFE, pyraflufen ethyl-treated cells.

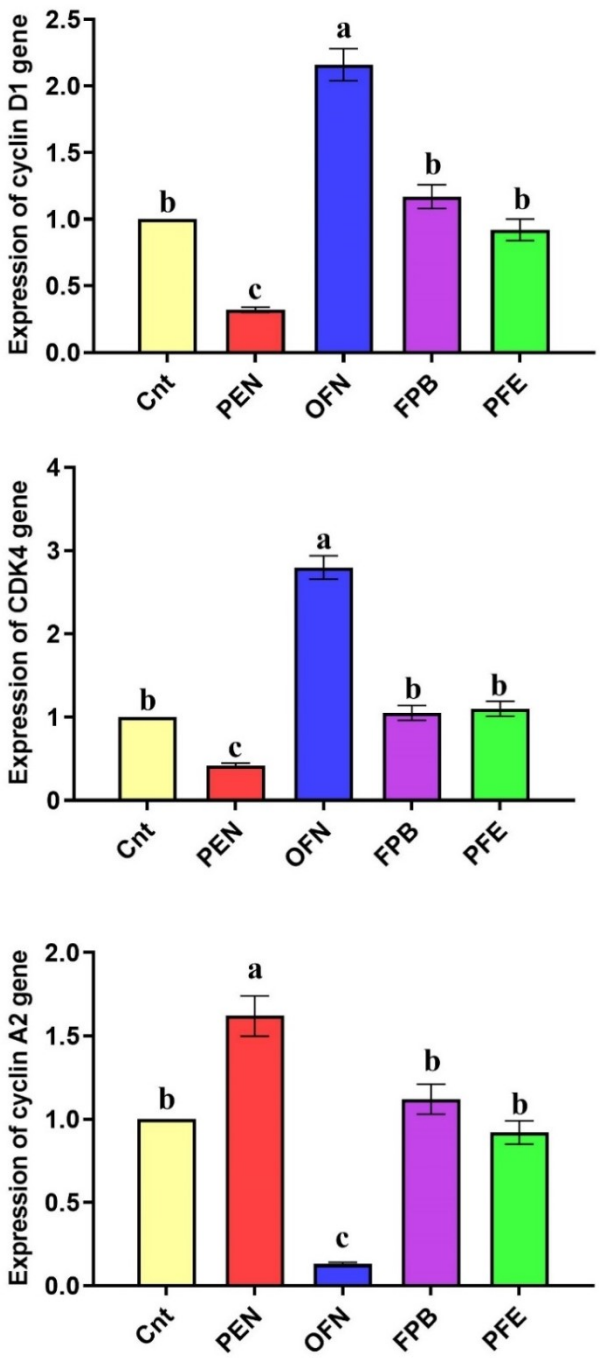

Fig.4. Analysis of qPCR data showing the effect of the four herbicides on the relative expression of cell cycle associated genes (cyclin D1, *CDK4*, and cyclin A2) in the human normal liver THLE2 cells. Columns and error bars represented fold change mean ± SEM (n = 5/group) and the different letters [a (highest) – e (lowest)] indicate significant difference between groups at P < 0.05. Each group was measured against every other group.

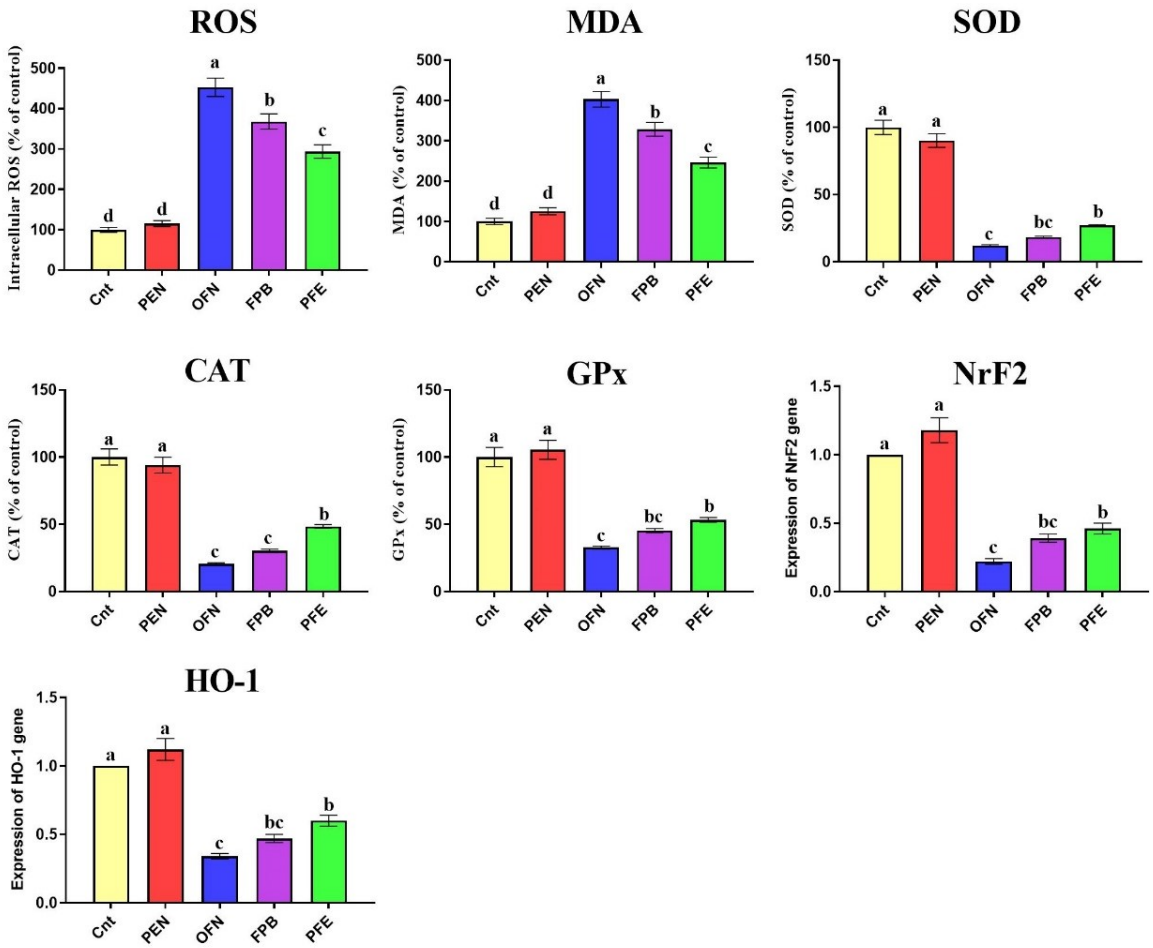

Fig.5. Effect of the four herbicides on the levels of oxidative stress related markers (intracellular ROS, and MDA) and activities on antioxidant enzymes (SOD, CAT, GPx) and expression of antioxidant *NrF2* and *HO-1* genes in THLE2 cells as measured with ELISA, spectrophotometer and qPCR, respectively. Values were presented as mean ± SEM (n = 5/group). Columns with different letters [a (highest) – e (lowest)] indicate significant difference between groups at P < 0.05. Each group was measured against every other group. Cnt, control (DMSO-treated) cells, PEN, pendimethalin-treated cells, OFN, oxyfluorfen- treated cells, FPB, fluazifop-p-butyl-treated cells, and PFE, pyraflufen ethyl-treated cells.

We and Jang, *et al*. [28] also found a decreased number of cells in the G0/G1. In contrast, treatment with FPB or PFE did not alter cell distribution, indicating that cell cycle arrest is not required for the cytotoxic potential of these herbicides.

In the present study, THLE2 cells treated with OFN, FPB, or PFE showed higher oxidative stress (as indicated by high levels of intracellular ROS and MDA, lower levels of the antioxidant enzyme activities (SOD, CAT, and GPx), and down regulated expression of *NrF2* and *HO-1* genes as compared to control and PEN-treated cells. In contrast, these oxidant/antioxidant indicators were not significantly different in PEN-treated cells compared to controls. In agreement, OFN, FPB, and PFE kill weeds through the overproduction of ROS which causes lipid peroxidation and damage to the cell membrane and finally cell death [20,29,30]. Similar oxidative stress dependant cytotoxic effects were also reported in fish for OFN [22-27], and in rat liver, kidney and testis for FPB [32]. However, no cytotoxic potential has been reported for PFE on animals or humans so far [29]. Thus, to the best of our knowledge, this could be the first study to report the cytotoxic effect of PFE on human normal liver THLE2 cells. However, compared to other herbicides used in the present study, PFE has the lowest cytotoxic effects.

Our findings highlighted the cytotoxic mechanism of four regularly used herbicides (PEN, ORF, FPB, and PFE) by assessing their effects on the survival of human normal liver (THLE2) cells as a non-target organism. Among these herbicides, PFE had the lowest cytotoxic effects against the human normal liver THLE2 cells and therefore could be used to control weeds with less adverse consequences on human health. The lack of

investigation of the cytotoxic effects of the four herbicides on another normal cell line and in vivo and their related signaling pathways is a significant shortcoming of our work. Another limitation of this study is the doses of four different herbicides employed in THLE2 that did not accurately represent actual environmental conditions. The cytotoxic effects of these herbicides on the occupational populations of workers and farmers have to be assessed as well. To properly assess the underlying mechanisms of these herbicides' cytotoxicity, further research is needed on animals and humans.

Conclusions

To the best of our knowledge, this is the first study that reported the cytotoxic potential of four commonly used herbicides (PEN, ORF, FPB, and PFE) on the human normal liver THLE2 cells with little toxicity for PFE followed by FPB. All four herbicides promoted THLE2 apoptosis, although they did so in different ways: ORF, FPB, and PFE increased ROS levels, whereas PEN and ORF caused cell cycle arrest. Although these findings provide insight into a potential threat to human health, it will be important to test these herbicides in vivo on non-targeted species at ecologically relevant amounts in the future.

Conflicts of interest

"There are no conflicts to declare".

Funding statement

"There is no funding statement to declare".

Author`s contributions

"Authors contribute equally in this work"

References

1. Etana, M.B., Aga, M.C. and Fufa, B.O. Major onion (Allium cepa L.) production challenges in Ethiopia: A review. *Journal of Biology, Agriculture and Healthcare*, **9**, 42-47 (2019).
2. Amalfitano, C., Golubkina, N.A., Del Vacchio, L., Russo, G., Cannoniero, M., Somma, S., Morano, G., Cuciniello, A. and Caruso, G. Yield, antioxidant components, oil content, and composition of onion seeds are influenced by planting time and density. *Plants*, **8**, 293 (2019).
3. Brewster, J.L. Onions and other vegetable alliums. *CABI*: Vol. 15. 2008.
4. Hanci, F. A comprehensive overview of onion production: worldwide and Turkey. *Journal of Agriculture and Veterinary Science*, **11**, 17-27 (2018).
5. Uygur, S., Gürbüz, R. and Uygur, F.N. Weeds of onion fields and effects of some herbicides on weeds in Cukurova region, Turkey. *African Journal of Biotechnology*, **9**, 7037-7042 (2010).
6. Ganesh, M., Pandey, B., Kewat, M. and Ankita, S. Effects of various weed management practices on growth, yield and economics of Rabi onion (Allium cepa L.) cv. Bhima Shakti. *The Pharma Innovation Journal*, **11**(4), 611-615 (2022).
7. Arakeri, M.P., Kumar, B.V., Barsaiya, S. and Sairam, H. In *Computer vision based robotic weed control system for precision agriculture*, 2017 International Conference on Advances in Computing, Communications and Informatics (ICACCI), IEEE: pp 1201-1205(2017). DOI:10.1109/ICACCI.2017. 8126005
8. Pushpa, U. and Choudhary, M. A review on efficacy of weed management methods in onion. *International Journal of Current Microbiology and Applied Sciences*, **8**, 895-905 (2019).
9. Sanjeev, A., Sandhu, K. and Ahuja, S. Efficiency of weed control in cabbage onion relay cropping system. *Annals of Biology*, **19**, 1-31 (2003).
10. El-Sagheer, S., Hilal, T., Temerak, S. and Abouelkassem, S. Performance of different herbicides and their combinations on weed control, growth and yield parameters of onion (Allium cepa L.). *Journal of Research in Weed Science*, **3**, 355 (2020).
11. Demir, N., Aydin, S. and Ündeğer Bucurgat, Ü. Assessment of Genotoxic Effects of Pendimethalin in Chinese Hamster Over Cells by the Single Cell Gel Electrophoresis (Comet) Assay. *Turk. J. Pharm. Sci.*, **14**, 185-190 (2017).
12. Patel, S., Bajpayee, M., Pandey, A.K., Parmar, D. and Dhawan, A. In vitro induction of cytotoxicity and DNA strand breaks in CHO cells exposed to cypermethrin, pendimethalin and dichlorvos. *Toxicology in vitro*, **21**, 1409-1418 (2007).
13. Yamano, T. and Morita, S. Effects of pesticides on isolated rat hepatocytes, mitochondria, and microsomes II. *Archives of Environmental Contamination and Toxicology*, **28**, 1-7 (1995).
14. Pan, H., Sun, D., Wang, J. and Wu, D. Using the FRTL-5 cell to screen the thyroxine disrupting effects of the two pesticides-ethylenethiourea and pendimethalin. *Wei Sheng yan JIU. Journal of Hygiene Research*, **33**, 267-269 (2004).
15. Dimitrov, B.D., Gadeva, P.G., Benova, D.K. and Bineva, M.V. Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems. *Mutagenesis*, **21**, 375-382 (2006).
16. Singh, B. and Singh, K. Microbial degradation of herbicides. *Critical Reviews in Microbiology*, **42**, 245-261 (2016).

17. Saquib, Q., Siddiqui, M.A., Ansari, S.M., Alwathnani, H.A., Musarrat, J. and Al-Khedhairy, A.A. Cytotoxicity and genotoxicity of methomyl, carbaryl, metalaxyl, and pendimethalin in human umbilical vein endothelial cells. *J. Appl. Toxicol*., **41**, 832-846(2021).

18. Lee, H.-S., Amarakoon, D., Tamia, G., Park, Y., Smolensky, D. and Lee, S.-H. Pendimethalin induces apoptotic cell death through activating ER stress-mediated mitochondrial dysfunction in human umbilical vein endothelial cells. *Food and Chemical Toxicology*, **168**, 113370 (2022).

19. Alavanja, M.C., Dosemeci, M., Samanic, C., Lubin, J., Lynch, C.F., Knott, C., Barker, J., Hoppin, J.A., Sandler, D.P. and Coble, J. Pesticides and lung cancer risk in the agricultural health study cohort. *American Journal of Epidemiology*, **160**, 876-885 (2004).

20. Bianchi, J., Fernandes, T.C. and Marin-Morales, M.A. Induction of mitotic and chromosomal abnormalities on Allium cepa cells by pesticides imidacloprid and sulfentrazone and the mixture of them. *Chemosphere*, **144**, 475-483 (2016).

21. Narayanan, K.B., Ali, M., Barclay, B.J., Cheng, Q.S., D'Abronzo, L., Dornetshuber-Fleiss, R., Ghosh, P.M., Gonzalez Guzman, M.J., Lee, T.J. and Leung, P.S. Disruptive environmental chemicals and cellular mechanisms that confer resistance to cell death. *Carcinogenesis*, **36 (**Suppl 1), S89-110 (2015).

22. de Vasconcelos Lima, M., de Siqueira, W.N., Silva, H., de Melo Lima Filho, J., de França, E.J. and de Albuquerque Melo, A.M.M. Cytotoxic and genotoxic effect of oxyfluorfen on hemocytes of Biomphalaria glabrata. *Environ. Sci. Pollut. Res Int*., **26**, 3350-3356(2019).

23. Hassanein, H.M.A. Toxicological effects of the herbicide oxyfluorfen on acetylcholinesterase in two fish species: Oreochromis niloticus and Gambusia affinis. *Journal of Environmental Science and Health, Part A*, **37**, 521-527 (2002).

24. Abd El-Rahman, G.I., Ahmed, S.A.A., Khalil, A.A. and Abd-Elhakim, Y.M. Assessment of hematological, hepato-renal, antioxidant, and hormonal responses of Clarias gariepinus exposed to sub-lethal concentrations of oxyfluorfen. *Aquat. Toxicol*., **217**, 105329 (2019).

25. Mansour, A.T., Amen, R.M., Mahboub, H.H., Shawky, S.M., Orabi, S.H., Ramah, A. and Hamed, H.S. Exposure to oxyfluorfen-induced hematobiochemical alterations, oxidative stress, genotoxicity, and disruption of sex hormones in male African catfish and the potential to confront by Chlorella vulgaris. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **267**, 109583 (2023).

26. Huang, L., Jia, K., Xiong, H., Tian, G., Xu, J., Yuan, W., Lu, C., Xiao, X. and Lu, H. Oxyfluorfen exposure can cause acute kidney injury by promoting ROS-induced oxidative stress and inflammation in zebrafish. *Journal of Hazardous Materials*, **440**, 129823(2022).

27. Li, Z., Guo, J., Jia, K., Zheng, Z., Chen, X., Bai, Z., Yang, Y., Chen, B., Yuan, W. and Chen, W. Oxyfluorfen induces hepatotoxicity through lipo-sugar accumulation and inflammation in zebrafish (Danio rerio). *Ecotoxicology and Environmental Safety*, **230**, 113140 (2022).

28. Jang, H., Song, J., Ham, J., An, G., Lee, H., Song, G. and Lim, W. Oxyfluorfen induces cell cycle arrest by regulating MAPK, PI3K and autophagy in ruminant immortalized mammary epithelial cells. *Pestic. Biochem. Physiol*., **193**, 105461 (2023).

29. Miura, Y., Mabuchi, T., Higashimura, M. and Amanuma, T. Development of a New Herbicide, Pyraflufen-ethyl. *Journal of Pesticide Science*, **28**, 219-220 (2003).

30. Luo, X.-Y., Sunohara, Y. and Matsumoto, H. Fluazifop-butyl causes membrane peroxidation in the herbicide-susceptible broad leaf weed bristly starbur (Acanthospermum hispidum). *Pesticide Biochemistry and Physiology*, **78**, 93-102(2004).

31. Tong, L. Acetyl-coenzyme A carboxylase: crucial metabolic enzyme and attractive target for drug discovery. *Cellular and Molecular Life Sciences CMLS*, **62**, 1784-1803(2005).

32. Ore, A. and Olayinka, E.T. Fluazifop- p-butyl, an aryloxyphenoxypropionate herbicide, diminishes renal and hepatic functions and triggers testicular oxidative stress in orally exposed rats. *Toxicol. Ind. Health*, **33**, 406-415 (2017).

33. Zedan, A.M.G., Sakran, M.I., Bahattab, O., Hawsawi, Y.M., Al-Amer, O., Oyouni, A.A.A., Nasr Eldeen, S.K. and El-Magd, M.A. Oriental Hornet (Vespa orientalis) Larval Extracts Induce Antiproliferative, Antioxidant, Anti-Inflammatory, and Anti-Migratory Effects on MCF7 Cells. *Molecules*, **26**, 3303(2021). https://doi.org/3310.3390/molecules26113303,

34. Elgazar, A.A., Selim, N.M., Abdel-Hamid, N.M., El-Magd, M.A. and El Hefnawy, H.M. Isolates from Alpinia officinarum Hance attenuate LPS induced inflammation in HepG2: Evidence from In Silico and In Vitro Studies. *Phytotherapy Research,* **32**, 1273-1288(2018).

35. El-kattawy, A.M., Algezawy, O., Alfaifi, M.Y., Noseer, E.A., Hawsawi, Y.M., Alzahrani, O.R., Algarni, A., Kahilo, K.A. and El-Magd, M.A. Therapeutic potential of camel milk exosomes against HepaRG cells with potent apoptotic, anti-inflammatory, and anti-angiogenesis effects for colostrum exosomes. *Biomedicine & Pharmacotherapy*, **143**, 112220(2021).

36. Saleh, A.A., Amber, K., El-Magd, M.A., Atta, M.S., Mohammed, A.A., Ragab, M.M., Abd El-Kader, H. Integrative effects of feeding Aspergillus awamori and fructooligosaccharide on growth performance and digestibility in broilers: promotion muscle protein metabolism. *BioMed. Research International*, **2014**, 946859 (2014).

37. El-Adawy, M., El-Aziz, M.A., El-Shazly, K., Ali, N.G. and El-Magd, M.A. Dietary propionic acid enhances antibacterial and immunomodulatory effects of oxytetracycline on Nile tilapia, Oreochromis

niloticus. *Environmental Science and Pollution Research*, **25**, 34200-34211 (2018).

38. Abd-Allah, S.H., Shalaby, S.M., Abd-Elbary, E., Saleh, A.A. and El-Magd, M.A. Human peripheral blood CD34+ cells attenuate oleic acid-induced acute lung injury in rats. *Cytotherapy*, **17**, 443-453(2015).

39. Awad, M.G., Ali, R.A., Abd El-Monem, D.D. and El-Magd, M.A. Graviola leaves extract enhances the anticancer effect of cisplatin on various cancer cell lines. *Molecular & Cellular Toxicology,* **16**, 385–399(2020) https://doi.org/10.1007/s13273-020-00092-8,

40. Elsied, M.A., Sharawi, Z.W., Al-Amrah, H., Hegazy, R.A., Mohamed, A.E., Saleh, R.M., El-kholy, S.S., Farrag, F.A., Fayed, M.H. and El-Magd, M.A. Walnut Kernel Oil and Defatted Extracts Enhance Mesenchymal Stem Cell Stemness and Delay Senescence. *Molecules*, **28**, 6281 (2023).

41. Abdelwahab, M.A., El-Barbary, A.A., El-Said, K.S., Betiha, M., Elkholy, H.M., Chiellini, E. and El-Magd, M.A. Functionalization of poly(3-hydroxybutyrate) with different thiol compounds inhibits MDM2–p53 interactions in MCF7 cells. *Journal of Applied Polymer Science*, **136**, 46924 (2019).

42. El-Magd, M.A., Khalifa, S.F., A. Alzahrani, F.A., Badawy, A.A., El-Shetry, E.S., Dawood, L.M., Alruwaili, M.M., Alrawaili, H.A., Risha, E.F. and El-Taweel, F.M. Incensole acetate prevents beta-amyloid-induced neurotoxicity in human olfactory bulb neural stem cells. *Biomedicine & Pharmacotherapy*, **105**, 813-823 (2018).

43. Mansour, G.H., El-Magd, M.A., Mahfouz, D.H., Abdelhamid, I.A., Mohamed, M.F., Ibrahim, N.S., Hady A. Abdel Wahab, A. and Elzayat, E.M. Bee venom and its active component Melittin synergistically potentiate the anticancer effect of Sorafenib against HepG2 cells. *Bioorganic Chemistry,* 105329 (2021). https://doi.org/10.1016/j.bioorg.2021.105329,

44. El-Magd, M.A., Kahilo, K.A., Nasr, N.E., Kamal, T., Shukry, M. and Saleh, A.A. A potential mechanism associated with lead-induced testicular toxicity in rats. *Andrologia*, **49**, e12750, (2016).

45. Sallam, A.A., Ahmed, M.M., El-Magd, M.A., Magdy, A., Ghamry, H.I., Alshahrani, M.Y. and Abou El-Fotoh, M.F. Quercetin Ameliorated Multi-Walled Carbon Nanotubes-Induced Immunotoxic, Inflammatory, and Oxidative Effects in Mice. *Molecules*, **27**(7),2117(2022). doi: 10.3390/molecules 27072117

46. Mohamed, A.E., El-Magd, M.A., El-Said, K.S., El-Sharnouby, M., Tousson, E.M. and Salama, A.F. Potential therapeutic effect of thymoquinone and/or bee pollen on fluvastatin-induced hepatitis in rats. *Scientific Reports*, **11**, 15688(2021).

47. Attia, A.A., Salama, A.F., Eldiasty, J.G., Mosallam, S.A.E.-R., El-Naggar, S.A., El-Magd, M.A., Nasser, H.M. and Elmetwalli, A. Amygdalin potentiates the anti-cancer effect of Sorafenib on Ehrlich ascites carcinoma and ameliorates the associated liver damage. *Scientific Reports*, **12**, 6494 (2022).

48. Park, H., Song, G., Hong, T., An, G., Park, S. and Lim, W. Exposure to the herbicide fluridone induces cardiovascular toxicity in early developmental stages of zebrafish. *Science of The Total Environment*, **867**, 161535 (2023).

49. Abdelhady, D.H., El-Magd, M.A., Elbialy, Z.I. and Saleh, A.A. Bromuconazole-induced hepatotoxicity is accompanied by upregulation of PXR/CYP3A1 and downregulation of CAR/CYP2B1 gene expression. *Toxicol. Mech. Methods*, **27**, 544-550 (2017).

50. Ramos, J.S.A., Pedroso, T.M.A., Godoy, F.R., Batista, R.E., de Almeida, F.B., Francelin, C., Ribeiro, F.L., Parise, M.R. and de Melo, E.S.D. Multi-biomarker responses to pesticides in an agricultural population from Central Brazil. *Sci. Total Environ*., **754**, 141893 (2021).

51. Ham, J., Lim, W. and Song, G. Pendimethalin induces apoptosis in testicular cells via hampering ER-mitochondrial function and autophagy. *Environmental Pollution*, **278**, 116835 (2021).

52. Ansari, S.M., Saquib, Q., Attia, S.M., Abdel-Salam, E.M., Alwathnani, H.A., Faisal, M., Alatar, A.A., Al-Khedhairy, A.A. and Musarrat, J. Pendimethalin induces oxidative stress, DNA damage, and mitochondrial dysfunction to trigger apoptosis in human lymphocytes and rat bone-marrow cells. *Histochemistry and Cell Biology*, **149**, 127-141 (2018).

تقييم سمية أربعة مبيدات أعشاب على خلايا الكبد الطبيعية THLE2

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الغرض من هذا البحث هو دراسة التأثيرات السمية لمبيدات الأعشاب بنديميثالين (PEN) وأوكسيفلورفين (OFN) وفلوازيفوب-ب-بوتيل (FPB) وبيرافلوفين إيثيل (PFE) على خلايا الكبد الطبيعية للإنسان (THLE2) من خلال تقييم حيوية الخلايا والموت الخلوي المبرمج ودورة الخلية وحالة التوتر التأكسدي باستخدام التحاليل الكيميائية والتفاعل البولميراز المتسلسل وتحاليل التدفق الخلوي. أدت المبيدات الأربعة إلى تأثيرات سمية على خلايا THLE2 كما يتضح من انخفاض حيوية الخلايا وزيادة الموت الخلوي المبرمج (ارتفاع تعبير جين Bax و3 caspase ، وانخفاض تعبير جين Bcl2) ومع ذلك ، فقط PEN و OFN أدت إلى توقف دورة الخلية في مرحلة G0 / G1 وكلا المرحلتين S و G2 / Mعلى التوالي ، مع انخفاض التعبيرالجينى ل cyclin D1 و cyclin A2 و cyclin-dependent kinase 4 (CDK4). من بين المبيدات الأربعة ، فقط OFN و PFE و FPB أدت إلى تلف التوتر التأكسدي كما يظهر من مستويات أعلى من ROS و MDA داخل الخلايا ، وأنشطة مثبطة لإنزيمات المضادة للأكسدة (CAT و SOD و GPx)، وانخفاض تعبير جين NrF2 و HO-1 كان لـ FPB و PFE هامش أمان أكبر على خلايا THLE2 الطبيعية للإنسان ، وقد يستخدمان بعواقب سلبية أقل على صحة الإنسان.

الكلمات الدالة: بنديميثالين (PEN) ،أوكسيفلورفين (OFN) ،فلوازيفوب-ب-بوتيل (FPB) ،بيرافلوفين إيثيل، السمية الخلوية.