Tropical Journal of Pharmaceutical Research November 2019; 18 (11): 2287-2292
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v18i11.8

Original Research Article

Osthole represses growth of multiple myeloma cells by regulating PI3K/AKT and ERK pathways

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Sent for review: 8 August 2019 *Revised accepted: 26 October 2019*

Abstract

Purpose: *To identify the effects of osthole on the growth of multiple myeloma cells and determine the probable molecular mechanism of action.*
Methods: *3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to measure multiple myeloma cell viability. Flow cytometry was used for the evaluation of cell cycle and apoptosis. For protein expression measurement, western blot analysis was employed.*
Results: *The results show that osthole dose- and time-dependently suppressed the viability of multiple myeloma cells ($p < 0.01$). Osthole also dose-dependently initiated multiple myeloma cell cycle arrest in G0/G1 phase ($p < 0.05$) and induced multiple myeloma cell apoptosis ($p < 0.01$). Moreover, western blotting revealed a significant reduction in cyclin D1 ($p < 0.01$) and induction in p21, cleaved caspase 3 and cleaved poly (ADP-ribose) polymerase ($p < 0.01$). Furthermore, osthole treatment significantly downregulated the phosphorylation levels of protein kinase B (AKTT308), AKTS473, and extracellular regulated protein kinase 1/2 (ERK1/2).*
Conclusion: *These findings demonstrated that osthole inhibited viability and induced cell cycle arrest and apoptosis of multiple myeloma cells by regulating PI3K/AKT and ERK pathways. Osthole may be considered as a potential anticancer agent for the therapy of multiple myeloma.*

Keywords: *Multiple myeloma, Osthole, Phosphatidylinositol 3 kinase, Protein kinase B, Extracellular regulated protein kinases, Cell growth*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Multiple myeloma is a hematological neoplasm caused by neoplastic clonal growth of plasma cells [1]. The clinical manifestations of patients with multiple myeloma are renal insufficiency, elevated blood calcium levels, bone damage, and anemia [2]. In recent years, the extensive uses of various therapies have greatly improved the quality of patients' life with multiple myeloma. However, the clinical treatment still has many difficulties [3]. Clinically, the treatment of multiple myeloma remains a major challenge.

Osthole, an active monomer isolated from *Cnidium monnieric (L.) Cuss*, has a wide range

of pharmacological effects. Many studies have shown that osthole has many effects, and particularly, anticancer [4]. For example, osthole exerts protective effects on various cancers such as nasopharyngeal carcinoma [5], and gastric cancer[6]. However, its role and the molecular mechanism have not been elucidated in multiple myeloma.

PI3K/AKT pathway is an important signal transduction pathway that plays a crucial role in proliferation, apoptosis, cell cycle, and autophagy. PI3K and AKT are abnormally expressed in cancers [7]. For instance, in multiple myeloma, knockdown of nuclear enriched abundant transcript 1 (NEAT1) inhibits cell proliferation and promotes cell apoptosis and cell cycle arrest through suppressing the PI3K/AKT pathway [8]. ERK1/2 is a subfamily of mitogen-activated protein kinase (MAPK) that regulates the differentiation of mammalian cells. Osthole inhibits migration and proliferation of panc02 cells *in vitro* and induces apoptosis by inhibiting phosphorylation of ERK1/2 [9]. Furthermore, ERK1/2 is abnormally phosphorylated in melphalan-resistant multiple myeloma cells and relapsed/refractory multiple myeloma [10]. These results indicated the important role of PI3K/AKT and ERK pathways in the progress of multiple myeloma. Based on these foundations, it raises the possibility that Osthole could repress multiple myeloma cell growth by activating ERK1/2. Therefore, this study investigated the role of Osthole in the growth of multiple myeloma cells and explored its potential molecular mechanism.

EXPERIMENTAL

Cell culture and treatment

RPMI-8226 cells purchased from ATCC (American Type Culture Collection; Manassas, VA, USA) were cultured in RPMI 1640 medium with fetal bovine serum (10% (v/v)) and Pen Strep (100 U/mL) at 37 °C with 5 % CO_2. The cells were exposed to osthole (0, 20, 50, and 100 μM) for 24, 48, and 72 h.

CCK 8 assay

Cells were inoculated in 96-well plates and treated with osthole (0, 20, 50, and 100 μM) for 24, 48, and 72 h, respectively. Subsequently, the cells were exposed to CCK-8 reagent (10 μL) and incubated at 37°C for 2 h. Optical density of the cells was determined at 450 nm, and the cell viability was evaluated as previously reported [11].

Western blot analysis

RPMI cells treated with osthole were lysed with RIPA lysis buffer (Thermo Fisher, Guangzhou, China) containing phosphatase inhibitors. Proteins (20 μL) were separated by SDS-PAGE (10 × 10 cm) and transferred to a PVDF membrane, followed by treated with primary antibodies CyclinD1 (ab16663, 1:5,000), p21 (ab109520, 1:5,000), GAPDH (ab8245, 1:5,000), cleaved caspase 3 (ab2302, 1:5,000), cleaved PARP (ab205718, 1:5,000), p-AKT (T308, ab38449, 1:5,000), p-AKT (S473, ab81283, 1:5,000), AKT (ab8805, 1:5,000), p-ERK1/2 (ab223500, 1:5,000), and ERK1/2 (ab17942, 1:5,000). The primary antibodies were purchased from Abcam (Guangzhou, China). Subsequently, the samples were incubated with goat anti-rabbit IgG H/L (HRP, ab205718, Abcam, Guangzhou, China) and then visualized. GAPDH was used as an internal reference.

Determination of cell cycle and apoptosis

Cells were treated with osthole (0, 20, 50, and 100 μM) for 72 h. Then, the cells were inoculated into 6-well plates (2.3×10^6/well) and fixed with chilled ethanol (70%). The cells were washed with PBS buffer, resuspended in propidium iodide (PI) cold solution (RNase A), and incubated (37 °C; 30 min in the dark), and then determined using flow cytometry assay (BD, Franklin Lakes, NJ, USA). For apoptosis, the RPMI-8226 cells treated with osthole were exposed to CD34-FITC for 15 min. Then, cells were resuspended in PBS and determined using flow cytometry assay (BD, Franklin Lakes, NJ, USA).

Statistical analysis

Data were presented as mean ± SD. GraphPad Prism (California, USA) was employed to determine the significance of differences among groups via one-way ANOVA and post-hoc tests. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Osthole inhibited the viability of multiple myeloma cells

RPMI-8226 cells were exposed to osthole (0, 20, 50, and 100 μM) for 24, 48, and 72 h. As shown in Figure 1, osthole treatment dose- and time-dependently repressed the viability of multiple myeloma cells when compared to the control group.

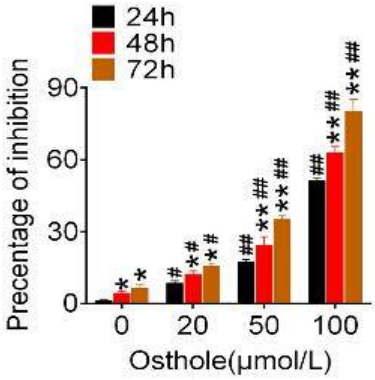

Figure 1: Osthole inhibited the viability of multiple myeloma cells**.** RPMI-8226 cells were exposed to osthole (0, 20, 50, and 100 μM) for 24, 48, and 72 h. Cell viability was assessed by CCK8 assay; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the 24h treatment group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the 0 μM group

Osthole induced multiple myeloma cell cycle arrest

The role of osthole in the cell cycle was studied using flow cytometry assay at 72 h. After osthole treatment, the cell population in the G1 phase was increased from 56.03% to 75.44, 79.54, and 89.87 %, respectively. However, the S phase cell population was decreased from 39.44% to 19.81, 14.09, and 4.45%, respectively. There were no significant changes in the G2/M phase (Figure 2A). Western blotting assay showed that Osthole dose-dependently increased p21 expression and increased CyclinD1 expression (Figure 2B). These investigations showed that osthole induced multiple myeloma cell cycle arrest.

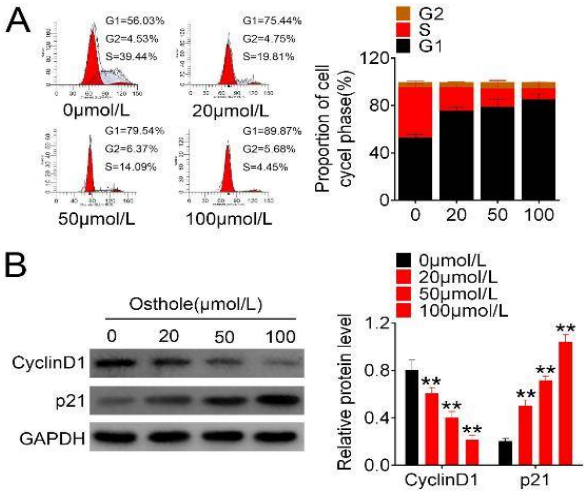

Figure 2: Osthole induced multiple myeloma cell cycle arrest. RPMI-8226 cells were exposed to osthole (0, 20, 50, and 100 μM) for 72 h. A. Cell cycle was determined using flow cytometry. B. The protein level was measured using western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with 0-μM group

Osthole promoted s apoptosis of multiple myeloma cells

Apoptosis was measured using flow cytometry. The number of apoptotic cells was significantly increased after osthole treatment (Figure 3A). In addition, western blot analysis indicated that osthole treatment promoted the expressions of cleaved caspase 3 and cleaved PARP in a dose-dependent manner (Figure 3B).

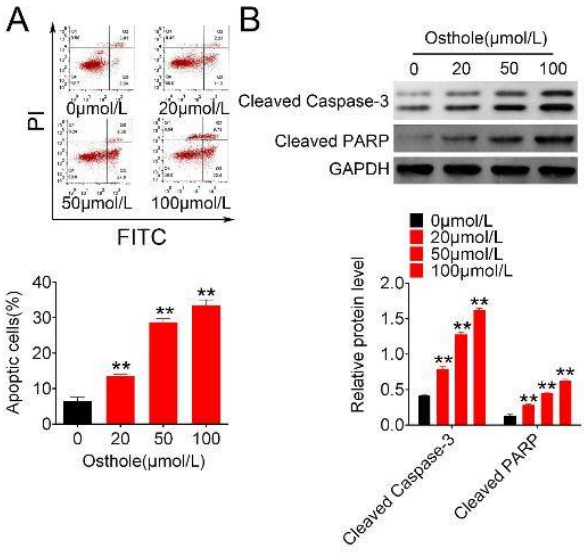

Figure 3: Osthole induced apoptosis of multiple myeloma cells. RPMI-8226 cells were exposed to osthole (0, 20, 50, and 100 μM) for 72 h. A. The number of apoptotic cells was measured using flow cytometry. B. The protein level was measured using western blotting assay; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with 0-μM group

Osthole regulated PI3K/AKT and ERK pathway activity in multiple myeloma cells

The underlying mechanism was also investigated. The phosphorylation levels of the AKT^{T308} and AKT^{S473} in multiple myeloma cells were significantly reduced after osthole treatment. Similarly, the phosphorylation level of ERK1/2 was dose-dependently reduced. However, there were no significant change in AKT and ERK1/2 (Figure 4). These results demonstrate that osthole regulated PI3K/AKT and ERK pathway in multiple myeloma cells.

DISCUSSION

Clonal proliferation of plasma cells causes multiple myeloma, a malignant hematological tumor that is difficult to cure. The treatment of multiple myeloma mainly includes conventional chemotherapy and autologous hematopoietic stem cell transplantation [12,13].

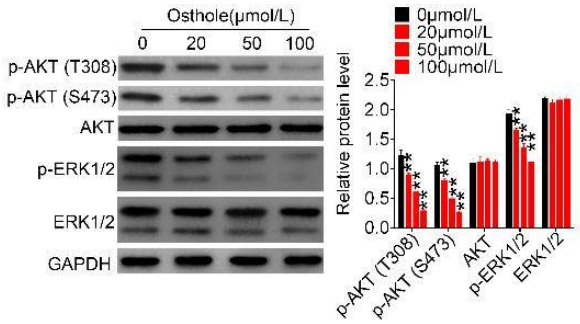

Figure 4: Osthole regulated the PI3K/AKT and ERK pathway in multiple myeloma cells. RPMI-8226 cells were exposed to osthole (0, 20, 50, and 100 μM) for 72 h. The protein level was measured by western blot; **p* < 0.05 and ***p* < 0.01 compared with 0-μM group

However, conventional chemotherapy produces strong drug resistance and serious side effects. The high cost of treatment limits the clinical application of autologous hematopoietic stem cell transplantation [13]. Therefore, the exploration of non-toxic and efficient new drugs is the key to the treatment. Results in this study indicated that osthole repressed the multiple myeloma cell growth and regulated PI3K/AKT and ERK1/2 signaling pathways. Therefore, osthole may be a new active component for multiple myeloma therapy.

Osthole is a biologically active monomer extracted from the species of the family *Umbelliferae* that has a variety of biological activities. This study found that osthole inhibited multiple myeloma cell growth and induced apoptosis and caused G1 phase cell cycle arrest.

As an active monomer, osthole has an inhibitory effect on the occurrence of various tumors [14]. Liu *et al* revealed that osthole represses the proliferation of renal cancer cells, induces apoptosis, and weakens the motor ability of renal cancer cells [15]. Lin *et al* also reported that osthole restrains the proliferation, migration, and epithelial–mesenchymal transition process of hepatocellular carcinoma cells. In addition, osthole induces G2/M phase arrest and DNA damage by down-regulating the expression of Cdc2 and cyclinB1 and increasing the expression of ERCC1 [16].

Jiang *et al* suggested that osthole hinders ovarian cancer cell growth by inhibiting cell viability, thereby promoting G2/M phase arrest and inducing apoptosis. Furthermore, osthole impedes ovarian cancer cell migration through inhibiting MMP-2 and MMP-9 [17]. Similarly, this study found that osthole suppressed multiple myeloma cell viability Osthole also induced G1 phase arrest by inhibiting the expression of CyclinD1 and inducing the expression of p21. In addition, osthole induced multiple myeloma cell apoptosis by upregulating cleaved caspase-3 and cleaved PARP.

It is worth noting that the PI3K/AKT pathway plays a regulatory role in cell survival and regulated by osthole in various tumors, such as lung cancer [4]. Su *et al* believed that osthole is a potential sensitizer to reverse the chemical resistance of cisplatin-resistant cervical cancer to cisplatin by inhibiting partial NRF2 expression correlated with PI3K/AKT blockade [18]. Xu *et al* reported that osthole treatment hinders gastric cancer cell proliferation by inhibiting the activation of the PI3K/p-AKT pathway and causing cell cycle arrest in the G2/M phase [6]. Zhu *et al* suggested that osthole could inhibit esophageal squamous cell carcinoma proliferation by inducing cell cycle arrest and apoptosis by adjusting the PTEN/PI3K/AKT axis [19].

In multiple myeloma, PI3K/AKT pathway is aberrantly activated, and miR-30d inactivated the downstream PI3K/AKT pathway by targeting metadherin [20]. Besides, co-administration of metformin and FTY720 inhibited the proliferation of multiple myeloma cells and induced apoptosis by inhibiting the activation of the PI3K/AKT/mTOR signaling pathway [21]. The present study is the first evidence demonstrating that osthole inactivated the PI3K/AKT pathway in multiple myeloma cells, and thus inhibited multiple myeloma cell growth.

Studies have shown that ERK1/2 is associated with multifarious human cancers. Hu *et al.* found that dihydrocelastrol down-regulated the phosphorylation of ERK1/2 and STAT3 and suppressed cell proliferation and inspired apoptosis and cell cycle arrest in G0/G1 phase in multiple myeloma cells through an *in vitro* caspase-dependent pathway [22].

Fan *et al* found that high expression of phosphorylated ERK1/2 is implicated in the poor prognosis in patients with multiple myeloma [23]. In nasopharyngeal carcinoma cells, osthole treatment significantly increased the expression levels of phosphorylated ERK1/2 and JNK1/2[24]. Similarly, this study determined the phosphorylation level of ERK1/2 in multiple myeloma cells after osthole treatment and found that osthole treatment dose-dependently inhibited the phosphorylation level of ERK1/2, suggesting that osthole may have a positive therapeutic effect on poor prognosis of multiple myeloma.

CONCLUSION

The findings indicate that osthole represses cell viability and induces G0/G1 phase arrest of multiple myeloma cells. Osthole also induces multiple myeloma cell apoptosis by upregulating the levels of cleaved caspase 3 and cleaved PARP. The underlying mechanism of action is at least partially via inactivation of PI3K/AKT and ERK1/2 signaling pathways. Therefore, osthole is a potential therapeutic agent for the treatment of multiple myeloma.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. LH and LG designed all the experiments and revised the paper. XZ and ZH formed the experiments. LH and LG wrote the paper.

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REFERENCES

1. *Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA: a cancer journal for clinicians 2018; 68(1): 7-30.*
2. *Liu X, Zhao Y, Zhang E, Yan H, Lv N, Cai Z. The synergistic effect of PFK15 with metformin exerts anti-myeloma activity via PFKFB3. Biochem Biophys Res Commun 2019.*
3. *Child JA, Morgan GJ, Davies FE, Owen RG, Bell SE, Hawkins K, Brown J, Drayson MT, Selby PJ. High-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. N Engl J Med 2003; 348(19): 1875-1883.*
4. *Xu X, Zhang Y, Qu D, Jiang T, Li S. Osthole induces G2/M arrest and apoptosis in lung cancer A549 cells by modulating PI3K/Akt pathway. J Exp Clin Cancer Res 2011; 30(1):33.*
5. *Peng L, Huang Y-T, Chen J, Zhuang Y-X, Zhang F, Chen J-Y, Zhou L, Zhang D-H. Osthole sensitizes with radiotherapy to suppress tumorigenesis of human nasopharyngeal carcinoma in vitro and in vivo. Cancer Manag Res 2018; 10: 5471-5477.*
6. *Xu X, Liu X, Zhang Y. Osthole inhibits gastric cancer cell proliferation through regulation of PI3K/AKT. PLoS One 2018; 13(3): e0193449.*
7. *Ham J, Lim W, Kim K, Heo YM, Ryu SM, Lee D, Kim JJ, Song G. Gentisyl Alcohol Inhibits Proliferation and Induces Apoptosis via Mitochondrial Dysfunction and Regulation of MAPK and PI3K/AKT Pathways in Epithelial Ovarian Cancer Cells. Mar Drugs 2019; 17(6):331.*
8. *Xu H, Li J, Zhou ZG. NEAT1 promotes cell proliferation in multiple myeloma by activating PI3K/AKT pathway. Eur Rev Med Pharmacol Sci 2018; 22(19): 6403-6411.*
9. *Wang B, Zheng X, Liu J, Zhang Z, Qiu C, Yang L, Zhang L, Zhang Q, Gao H, Wang X. Osthole inhibits pancreatic cancer progression by directly exerting negative effects on cancer cells and attenuating tumor-infiltrating M2 macrophages. J Pharmacol Sci 2018; 137(3): 290-298.*
10. *Tsubaki M, Takeda T, Tomonari Y, Koumoto YI, Imano M, Satou T, Nishida S. Overexpression of HIF-1alpha contributes to melphalan resistance in multiple myeloma cells by activation of ERK1/2, Akt, and NF-kappaB. Lab Invest 2019; 99(1): 72-84.*
11. *Prabhu KS, Siveen KS, Kuttikrishnan S, Iskandarani AN, Khan AQ, Merhi M, Omri HE, Dermime S, El-Elimat T, Oberlies NH et al. Greensporone C, a Freshwater Fungal Secondary Metabolite Induces Mitochondrial-Mediated Apoptotic Cell Death in Leukemic Cell Lines. Front Pharmacol 2018; 9: 720.*
12. *Schilling G, Schulz-Kindermann F. Psychosocial Impact of Personalized Therapies in Oncology. Recent Results Cancer Res 2018; 210: 181-190.*
13. *Hussein M. Role of high-dose chemotherapy with hematopoietic stem-cell rescue for multiple myeloma. Leukemia 2004; 18(4): 893.*
14. *Che Y, Li J, Li Z, Li J, Wang S, Yan Y, Zou K, Zou L. Osthole enhances antitumor activity and irradiation sensitivity of cervical cancer cells by suppressing ATM/NFkappaB signaling. Oncol Rep 2018; 40(2): 737-747.*
15. *Liu L, Mao J, Wang Q, Zhang Z, Wu G, Tang Q, Zhao B, Li L, Li Q. In vitro anticancer activities of osthole against renal cell carcinoma cells. Biomed Pharmacother 2017; 94: 1020-1027.*
16. *Lin ZK, Liu J, Jiang GQ, Tan G, Gong P, Luo HF, Li HM, Du J, Ning Z, Xin Y et al. Osthole inhibits the tumorigenesis of hepatocellular carcinoma cells. Oncol Rep 2017; 37(3): 1611-1618.*
17. *Jiang G, Liu J, Ren B, Tang Y, Owusu L, Li M, Zhang J, Liu L, Li W. Anti-tumor effects of osthole on ovarian cancer cells in vitro. J Ethnopharmacol 2016; 193: 368-376.*

18. Su J, Zhang F, Li X, Liu Z. Osthole promotes the suppressive effects of cisplatin on NRF2 expression to prevent drug-resistant cervical cancer progression. Biochem Biophys Res Commun 2019; 514(2): 510-517.
19. Zhu X, Li Z, Li T, Long F, Lv Y, Liu L, Liu X, Zhan Q. Osthole inhibits the PI3K/AKT signaling pathway via activation of PTEN and induces cell cycle arrest and apoptosis in esophageal squamous cell carcinoma. Biomed Pharmacother 2018; 102: 502-509.
20. Zhu B, Chen H, Zhang X, Pan Y, Jing R, Shen L, Wang X, Ju S, Jin C, Cong H. Serum miR-30d as a novel biomarker for multiple myeloma and its antitumor role in U266 cells through the targeting of the MTDH/PI3K/Akt signaling pathway. Int J Oncol 2018; 53(5): 2131-2144.
21. Zhao Y, Zhang E, Lv N, Ma L, Yao S, Yan M, Zi F, Deng G, Liu X, He J et al. Metformin and FTY720 Synergistically Induce Apoptosis in Multiple Myeloma Cells. Cell Physiol Biochem 2018; 48(2): 785-800.
22. Hu L, Wu H, Li B, Song D, Yang G, Chen G, Xie B, Xu Z, Zhang Y, Yu D et al. Dihydrocelastrol inhibits multiple myeloma cell proliferation and promotes apoptosis through ERK1/2 and IL-6/STAT3 pathways in vitro and in vivo. Acta Biochim Biophys Sin (Shanghai) 2017; 49(5): 420-427.
23. Fan L, Hong J, Huang H, Fu D, Wu S, Wang Q, Ye Y, Liu Y. High Expression of Phosphorylated Extracellular Signal-Regulated Kinase (ERK1/2) is Associated with Poor Prognosis in Newly Diagnosed Patients with Multiple Myeloma. Med Sci Monit 2017; 23: 2636-2643.
24. Liu PY, Chang DC, Lo YS, Hsi YT, Lin CC, Chuang YC, Lin SH, Hsieh MJ, Chen MK. Osthole induces human nasopharyngeal cancer cells apoptosis through Fas-Fas ligand and mitochondrial pathway. Environ Toxicol 2018; 33(4): 446-453.