The effect of MDMA and pentoxifylline drug on bad/bcl-xl gene dosage expression changes on rat liver

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ABSTRACT

MDMA generally known as ecstasy, have deleterious effects on the serotonergic neurotransmitter system. Recent findings suggest that the liver and brain are major target organs of MDMA-related toxicities. Although most research is being dynamically performed on brain, however, the molecular mechanisms by which MDMA elicits adverse effects in both organs are poorly undrestood.The present study was performed to obtain evidence for molecular mechanism of apoptosis involved in MDMA-induced hepatotoxicity in rat liver after MDMAadministration. Moreover, the antagonistic effect of pentoxifylline was assessed on hepatotoxicity after MDMA administration. In this experimental study, sample size and power in each group were calculated as 10 rats with 95% confidence level and 5% confidence interval. In the study, four experimental groups were selected including Control Normal, MDMA, MDMA+PTX and PTX+MDMA. MDMA was dissolved in PBS and intraperitoneally injected three doses of 7.5mg/kg with two hours gap between doses. Pentoxyfilline also was injected as 100mg/kg, simultaneously with third dose of MDMA. After treatment, total RNA was isolated from liver tissue (5mg). Absorbance at 260nm, 280nm and 230nm were measured and immediately reverse transcription was performed. Included target genes were *BAD* and *BCL*-XL as pro-apoptotic and anti-apoptotic gene, respectively. After set up and validation, Real-Time PCR were performed and obtaining data were statistically analyzed to determine significantly differences between groups. Using Real-Time quantitative PCR results, BCL-XL gene expression ratio significantly increased in MDMA+PTX group. Moreover, BAD gene expression ratio increased and up-regulated in PTX+MDMA group (*P*-value <0.001).Our study focused on molecular mechanism of MDMA in programmed cell death using gene expression quantification of a pro-apoptotic and anti-apoptoic gene in MDMA-induced hepatotoxocity. The results shown MDMA prompted apoptosis in liver and pentoxifylline protects hepatotoxicity after and befor taking MDMA.

Keywords: Anti-apoptotic gene; MDMA; Pentoxifylline; Pro-apoptotic gene; qReal-Time PCR

INTRODUCTION

Methylenedioxymethampetamine (MDMA) generally known as ecstasy, is an illicit drug used by individuals seeking mood enhancement [1]. neuroimaging researches have investigated the structural, chemical and functional differences of MDMA on the human brain and other organs. The effects of Ecstasy vary from almost universal minor symptoms to those that are rare but potentially life-threatening [2]. Increasing evidence suggests that one way of MDMA-induced toxicity involves abrupt death, rhabdomyolysis, hyperpyrexia and multi-organ failure, isolated liver failure, the serotonin syndrome, an acute panic disorder and hyponatraemia with cerebral oedema. The effects

of MDMA differ according to the dose, frequency and consumption period [3]. Researches and studies on MDMAin animals has been shown to have deleterious effects on the serotonergic neurotransmitter system [4]. Recent findings, moreover, suggest that the liver and brain are major target organs of MDMA-related toxicities [5]. Although most research is being dynamically performed on brain, however, the molecular mechanisms by which MDMA elicits adverse effects in both organs are poorly understood.

Apoptotic cell death is a genetically programmed mechanism(s) that allows the cell to commit suicide [6]. Methamphetamine and other amphetamine derivatives trigger the programmed cell death pathway by activation of pro-apoptotic and inhibition of anti-apoptotic genes [7]. The B-cell lymphoma-2 (Bcl-2) family is a group comprising *bcl2*, *Bcl-xL*, *Bcl-w* and *Mcl*-1 (anti-apoptotic), *bax*, *Bak*, *Bok*, *Bid*, *Bad*, *Bik*, *Bmf*, *Hrk*, *Noxa* and *Puma* (pro-apoptotic) genes which directly involved in regulating apoptosis.

Pentoxifylline (PTX), a non-specific phosphodiesterase inhibitor, is one of the main pharmacological treatment options for the treatment of severe alcoholic hepatitis [8] and routinely employed for circulatory diseases for >20 years. PTX is a ligand of adenosine receptors, a modulator of the ryanodine Ca^{2+}-release channel of the sarco(endo)plasmic reticulum, down-regulator of tumor necrosis factor-alpha (TNF-α) and the transcription factorNF-κB[9].

The present study was performed to obtain evidence for molecular mechanism of apoptosis involved in MDMA-induced hepatotoxicity in rat liver after MDMAadministration. Moreover, the antagonistic effect of pentoxifylline was assessed on hepatotoxicity after MDMA administration.

MATERIALS AND METHODS
Animal drug administration
In this experimental study, sample size and power in each group were calculated as 10 rats with 95% confidence level and 5% confidence interval. All procedures were performed in accordance with Ethics Committee of Iran University of Medical Sciences and confirmed to European Communities Council Directive of November 1986 (86/609/EEC). All chemicals were purchased from Sigma except pentoxifylline powder that was gifted kindly by the Amin Pharmaceutical Co (Esfahan-Iran) and pure MDMA was gifted by Dr. Foroumadi, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences.

Adult male Wistar rats, in the study, were selected as weighting 250–300g (Cellular and molecular research center, Iran university of Medical Sciences, Tehran, Iran). The animals were housed under reversed 12:12 hours light and dark cycles at room temperature (22±2°C). In the study, four experimental groups (10 rats/group) were selected including Control Normal, MDMA, MDMA+PTX (MDMA followed by PTX) and PTX+MDMA (PTX followed by MDMA). MDMA was dissolved in Phosphate Buffer Saline (PBS) and intraperitoneally (IP) injected three doses of 7.5mg/kg with two hours gap between doses. In MDMA+PTX group, Pentoxyfilline was injected as 100mg/kg, simoultanously with third dose of MDMA. Furthermore, MDMA was received one week after pentoxyfilline in PTX+MDMA group.

Two weeks after first drug administration, animals were anaesthetized by Ketanamine and xylazine before being put into death. The liver tissue rapidly was removed, dissected and molecular procedures immediately performed.

Molecular procedures
Total RNA was isolated from liver tissue (5mg) through High Pure RNA isolation kit (Roche, Germany). According to the handbook kit, the samples were prepared using tissue lysis buffer. All samples, furthermore, were treated with DNase I enzyme (supported in the kit) to avoid DNA contamination. Finally, absorbance at 260nm (to calculate concentration), 280nm and 230nm (to determine purification) were measured using Nanophotometer 2000c (Thermo Science, USA). Immediately, Reverse transcription (RT) reaction was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania).

Primer design
Included target genes, in this experimental study, were *BAD* and *BCL-XL* as pro-apoptotic and anti-

apoptotic gene, respectively. In according to relative quantification analysis, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was selected as internal reference gene.

The sequences of interest genes were obtained from NCBI database and primer sets were designed using *PrimerExpress* v.3.0 (Applied Biosystems, Foster City, USA) and GeneRunner software. All designed oligonucleotids were analyzed in Basic Local Alignment Search Tool to avoid secondary structure and homology with other region of genome. Primer sequences are shown in Table 1.

Table 1. primer sequences, length, Tm and amplicons.

Oligo name	Sequence (5′→3′)	Tm
gapdh-F	AAGTTCAACGGCACAGTCAAGG	57.8
gapdh-R	CATACTCAGCACCAGCATCACC	56.7
bad-F	GGAGCATCGTTCAGCAGCAG	58
bad-R	CCATCCCTTCATCTTCCTCAGTC	57.6
Bcl-xl-F	GCTGGTGGTTGACTTTCTCTCC	56.4
bcl-xl-R	GGCTTCAGTCCTGTTCTCTTCG	56.7

Relative quantification analysis

In the assay, fluorescent dye and Real-Time PCR instrument were SYBR Green I and Rotor-Gene Q apparatus (Qiagen, Germeny), respectively. Total PCR reaction volume was 25μl including 12.5μl of SYBR Green I PCR Master Mix (TaKaRa, Japan), forward and reverse oligonucleotide (400nM), 5μl of cDNA template (60ng/μl) and ddH2O (5.5μl). Thermal cycling programme was performed including 30sec at 95°C, following 5sec at 95°C and 25sec at 60°C for 40 cycles and melting curve analysis carried out from 65°C to 95°C, rising 1°C each step. Melting curve analysis was used to confirm the specific amplification of fragments and no primer dimmers formation. Real-Time PCR reactions for each sample were performed in triplicate to confirm accuracy, reproducibility and intra-assay precision. Amplification efficiency for target and reference genes was validated using 10-fold dilution series of control cDNA template as 2000, 200, 20, 2ng. Then, standard curves were drawn using plotting the logarithmic input cDNA concentration versus mean CT and the slope was determined. PCR reaction efficiency was

calculated via formula; $E = (10^{-1/slope})$. Expression levels of target genes were calculated using comparative threshold cycle formula. The relative expression ratio (R) of target genes were calculated based on efficiency (*E*) and cross point deviation (CP) of unknown samples versus controls using formula;

$$\text{Ratio} = \frac{(E\,target)^{\Delta Cp\,target\,(control\,mean - sample\,mean)}}{(E\,ref.index)^{\Delta Cp\,ref.index(control\,mean - sample\,mean)}}$$

Data processing and statistical analysis

All mathematical procedures were calculated using Statistical Package for the Social Sciences software (*SPSS* Inc v. 22). The statistical operations were included Mean ratio (M), Standard Deviation (SD), Determination coefficient (R^2), Confidence Intervals (95% CI) and Standard Error of Mean (SEM). One-way ANOVA, non-parametric (independent sample Kruskal-Wallis test) and Tukey analysis (Post hoc test) were performed to determine significantly differences between gene expression of interest groups. *P*-value<0.05 was considered statistically significant.

RESULTS

The slope of standard curves for *BCL*-XL, *BAD* and *GAPDH* genes were obtained -3.46, -3.31 and -3.39, respectively (Figure 1).
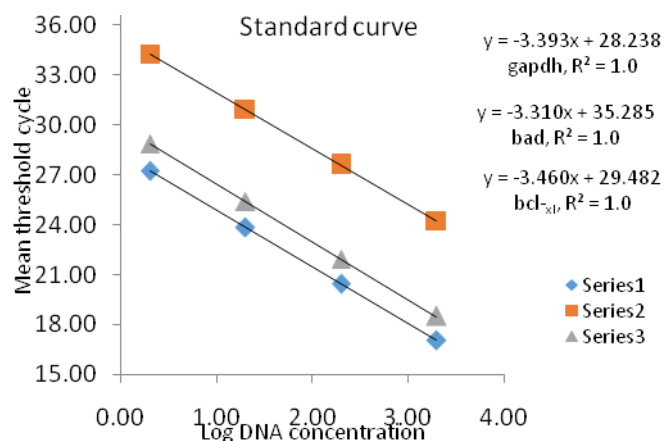

Figure 1. Standard curves: Mean threshold cycle versus log cDNA concentration. Y-intercept and Determination coefficient for interest genes; bad (y = -3.310x + 35.285 and R² = 1.00), bcl-xl

(y = -3.460x + 29.482 and R² = 1.00) and gapdh (y = -3.393x + 28.238 and R² = 1.00).

PCR efficiency (*E*), therefore, for target and reference genes were calculated 1.94 (*BCL*-$_{XL}$), 2.00 (*BAD*) and 1.97 (*GAPDH*). Melting curve analysis shown the specific amplicon for *BCL*-$_{XL}$, *BAD* and *GAPDH* melted at 82.5ºC, 89.0ºC and 85.5ºC, respectively (Figure 2). The results of melting curves were confirmed by gel electrophoresis of PCR product. To determine whether the gene expression of pro-apoptotic and anti-apoptotic changed in rat liver after MDMAexposure, the level of mRNA for *BCL*-$_{XL}$ and *BAD* were measured.
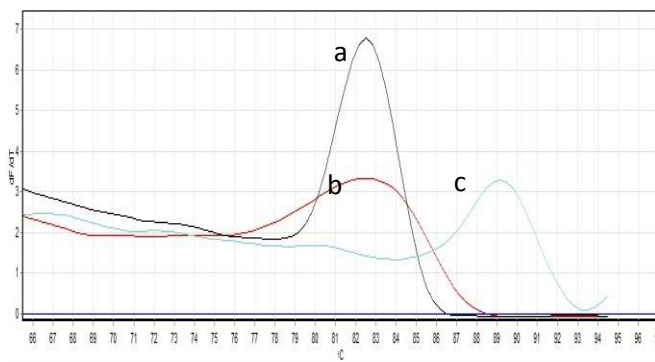

Figure 2. Melting curve analysis: derevative fluorescent versus temperature.a: gapdh (85.5ºC), b: bcl-$_{xl}$ (82.5ºC), c: bad (89.0ºC).

The gene expression ratio of *BCL*-$_{XL}$ increased in MDMA+PTX group compared to MDMA and PTX+MDMA groups (Table 2, Figure 3-A). The statistical analysis shown significant difference for *BCL*-$_{XL}$ expression between experimental groups   (*P*-value<

0.001). *BAD* gene expression ratio, furthermore, increased approximately 5-fold in PTX-MDMA group compared to other groups (Table 2, Figure 3-B) in statistical analysis, outlier data were excluded to achieve pure results.

Table 2. Quantitative results of *BCL*-$_{XL}$ and *BAD* gene expression ratio in excremental groups

	gene expression ratio (Mean±IQR)			
	MDMA	MDMA+PTX	PTX+MDMA	*P*-value
BCL-$_{XL}$	6.85±0.37	8.48±1.33	4.45±0.84	<0.001
BAD	135.33±8.76	89.92±6.82	49.26±6.85	<0.001
BAD/BCL-$_{XL}$	19.75	10.61	11.06	<0.001

The ratio of pro-apoptotic to anti-apoptotic gene (*BAD/BCL*-$_{XL}$) for experimental groups were calculated as 19.75 (MDMA), 10.61 (MDMA+PTX) and 11.06 (PTX+MDMA) (Figure 3-C).
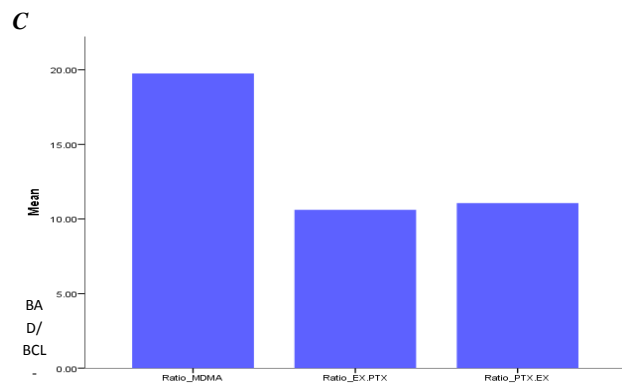
A

B

C
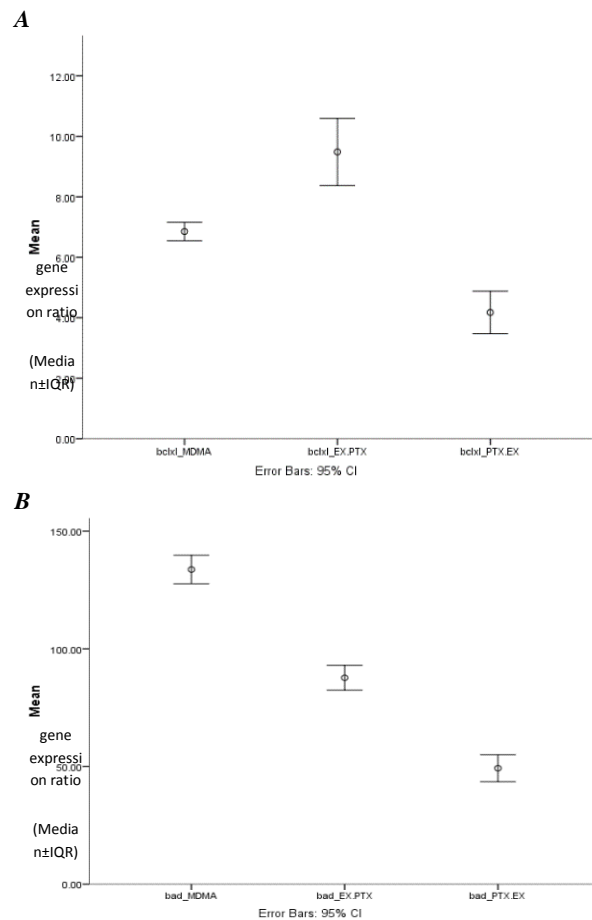
Figure 3. Gene expression (Median±IQR) and pro-apoptotic to anti-apoptotic gene ratio. A) BCL-$_{XL}$. B) BAD. C) BAD/BCL-$_{XL}$ ratio

DISCUSSION

Although MDMA alone or in combination with other abused substances can damage various organs (such as liver, heart, brain, and kidney), recent epidemiological study are shown that MDMA abuse has increased almost in all parts of the world. Most research is being dynamically performed to undrestand the mechanism of MDMA-mediated neurotoxicity [10-12] and hepatotoxicity [13]. recent studies have shown that co-administration of MDMA with ethanol significantly enhances neurotoxicity and hepatotoxicity [14]. Although many investigations have reported MDMA-induced liver damage [13, 15, 16], the fundamental mechanism accounting for hepatic toxicity is poorly understood.

The present study focused on molecular mechanism of MDMA in apoptosis using gene expression quantification of a pro-apoptotic and an anti-apoptotic gene in hepatotoxocity. Our findings shown that BCL-$_{XL}$ gene expression increased in MDMA+PTX group compared to MDMA and PTX+MDMA groups. BCL-$_{XL}$ is an anti-apoptotic gene that its expression up-regulated after PTX administration.

This result may confirmed the role of PTX as an anti-apoptotic-inducer. BAD, the another target, is a pro-apoptic gene that its expression up-regulated in MDMA group, and decreased in MDMA+PTX and PTX+MDMA groups, respectively. This pattern could be shown the effect of MDMA triggering pro-apoptotic gene

expression and the antagonistic effect of pentoxifylline on hepatotoxicity before and after MDMA administration. According to the data, furthermore, the ratio of pro-apoptotic to anti-apoptotic gene (BAD/BCL-$_{XL}$) revealed this ratio increased in MDMA group compared to other excremental groups (Table 2, Figure 3-C). This result confirmed that MDMA activated pro-apoptotic gene expression and induced apoptosis.

Upreti and his team assessed on the levels of Bcl-2 and phospho-Bcl-2 (or Bcl-XL) protein in cytosol and mitochondria [5]. They were reported that anti-apoptotic Bcl-2 was phosphorylated (inactivated), although its protein level was unchanged in MDMA-exposed tissues. In addition, the level of Bcl-XL, another member of the anti-apoptotic Bcl-2 family, was decreased in the cytoplasm, however, this protein increased in the mitochondria, suggesting its translocation after MDMA exposure.

Histopathological examination revealed that treatment with PTX significantly suppressed early inflammation and the apoptosis of hepatocytes induced by ConA [17]. Moreover, some reports suggest

that PTX administration might increase the effectiveness of antitumor chemotherapy [18]. Pentoxifylline effects depends on its dose and time duration. Ji Q and colleagues reported that single dose of PTX dramatically reduced brain inflammation and apoptosis for up to 16 h post-injury [19].

Hernandez-Flores and his coleagues investigated on cytotoxic effect of PTX and CIS either alone or in combination on HeLa, SiHa and HaCaT cells [9]. They reported that PTX is able to markedly down-regulate the expression of Bcl-2 and BCL-$_{XL}$ proteins in both HeLa and SiHa cells as compared with untreated cells.

In some types of cancers pentoxifylline sensitizes these cells to the toxic action of chemotherapeutics drugs such as adriamycin and inducing apoptosis. Recent investigation have shown that PTX is toxic for cancer cells affecting cell viability and inducing apoptosis. PTX also has antimetastatic activity and arrests

the cell cycle in which the tumors are more sensitive to the toxic effects of some chemotherapeutic and radiotherapeutic agents.

In the parallel analysis, Movassaghi et al. performed chromosome staining in Rat liver to investigating of apoptotic bodies using TUNEL (Terminal deoxynuclotidyl transfrased UTP nickend labeling) assay [20]. They shown that the number of TUNEL-positive cells in rat liver was significantly decreased in MDMA followed PTX group compared to MDMA and vehicle groups. In addition, they published the apoptotic bodies were significantly decreased by PTX in the liver and there was no significant difference between the number of apoptotic bodies in MDMA followed PTX group and control groups.

Our study focused on molecular mechanism of MDMA in programmed cell death using gene expression quantification of a pro-apoptotic and anti-apoptoic gene in MDMA-induced hepatotoxocity. The results shown MDMA prompted apoptosis in liver and pentoxifylline protects hepatotoxicity after and before taking MDMA.

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REFERENCES

1. Maloney WJ, Raymond G. The significance of ecstasy use to dental practice. *N Y State Dent J* 2014,80:24-27.
2. Hall AP, Henry JA. Acute toxic effects of 'Ecstasy' (MDMA) and related compounds: overview of pathophysiology and clinical management. *Br J Anaesth* 2006,96:678-685.
3. Karimi S, Jahanshahi M, Golalipour MJ. The effect of MDMA-induced anxiety on neuronal apoptosis in adult male rats' hippocampus. *Folia Biol (Praha)* 2014,60:187-191.
4. Garg A, Kapoor S, Goel M, Chopra S, Chopra M, Kapoor A*, et al.* Functional Magnetic Resonance Imaging in Abstinent MDMA Users: A Review. *Curr Drug Abuse Rev* 2015.
5. Upreti VV, Moon KH, Yu LR, Lee IJ, Eddington ND, Ye X*, et al.* Increased oxidative-modifications of cytosolic proteins in 3,4-methylenedioxymethamphetamine (MDMA, ecstasy)-exposed rat liver. *Proteomics* 2011,11:202-211.
6. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. *Biochim Biophys Acta* 2011,1813:238-259.

7. Cerretani D, Bello S, Cantatore S, Fiaschi AI, Montefrancesco G, Neri M*, et al.* Acute administration of 3,4-methylenedioxymethamphetamine (MDMA) induces oxidative stress, lipoperoxidation and TNFalpha-mediated apoptosis in rat liver. *Pharmacol Res* 2011,64:517-527.
8. Chayanupatkul M, Liangpunsakul S. Alcoholic hepatitis: a comprehensive review of pathogenesis and treatment. *World J Gastroenterol* 2014,20:6279-6286.
9. Hernandez-Flores G, Ortiz-Lazareno PC, Lerma-Diaz JM, Dominguez-Rodriguez JR, Jave-Suarez LF, Aguilar-Lemarroy Adel C*, et al.* Pentoxifylline sensitizes human cervical tumor cells to cisplatin-induced apoptosis by suppressing NF-kappa B and decreased cell senescence. *BMC Cancer* 2011,11:483.
10. Roseman L, Leech R, Feilding A, Nutt DJ, Carhart-Harris RL. The effects of psilocybin and MDMA on between-network resting state functional connectivity in healthy volunteers. *Front Hum Neurosci* 2014,8:204.
11. Capela JP, Carmo H, Remiao F, Bastos ML, Meisel A, Carvalho F. Molecular and cellular mechanisms of ecstasy-induced neurotoxicity: an overview. *Mol Neurobiol* 2009,39:210-271.

12.   Sarkar S, Schmued L. Neurotoxicity of ecstasy (MDMA): an overview. *Curr Pharm Biotechnol* 2010,11:460-469.

13.   Carvalho M, Pontes H, Remiao F, Bastos ML, Carvalho F. Mechanisms underlying the hepatotoxic effects of ecstasy. *Curr Pharm Biotechnol* 2010,11:476-495.

14.   Upreti VV, Eddington ND, Moon KH, Song BJ, Lee IJ. Drug interaction between ethanol and 3,4-methylenedioxymethamphetamine ("ecstasy"). *Toxicol Lett* 2009,188:167-172.

15.   Downey C, Daly F, O'Boyle KM. An in vitro approach to assessing a potential drug interaction between MDMA (ecstasy) and caffeine. *Toxicol In Vitro* 2014,28:231-239.

16.   Payance A, Scotto B, Perarnau JM, de Muret A, Bacq Y. Severe chronic hepatitis secondary to prolonged use of ecstasy and cocaine. *Clin Res Hepatol Gastroenterol* 2013,37:e109-113.

17.   Mohamed DI, Elmelegy AA, El-Aziz LF, Abdel Kawy HS, AbdEl-Samad AA, El-Kharashi OA. Hepatoprotective effects of early pentoxifylline administration on hepatic injury induced by concanavalin A in rat. *Can J Physiol Pharmacol* 2014,92:490-497.

18.   Barancik M, Bohacova V, Gibalova L, Sedlak J, Sulova Z, Breier A. Potentiation of anticancer drugs: effects of pentoxifylline on neoplastic cells. *Int J Mol Sci* 2012,13:369-382.

19.   Ji Q, Jia H, Dai H, Li W, Zhang L. Protective effects of pentoxifylline on the brain following remote burn injury. *Burns* 2010,36:1300-1308.

20.   Movassaghi S, Nadia Sharifi Z, Mohammadzadeh F, Soleimani M. Pentoxifylline Protects the Rat Liver Against Fibrosis and Apoptosis Induced by Acute Administration of 3,4-Methylenedioxymethamphetamine (MDMA or Ecstasy). *Iran J Basic Med Sci* 2013,16:922-927.