Chrysin regulates NAD(P)H:quinoneoxidoreductase 1 and inflammatory mediators in rats exposed to smoking and thioacetamide

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A B S T R A C T

Tobacco smoking and hepatic damage causes oxidative stress in humans and underlay numerous chronic degenerative diseases. Heavy smoking yields toxins which induce necroinflammation and increase the severity of hepatic lesions. Accordingly, the main object of this study to investigate the effect of chrysin against thioacetamide (TAA)-induced liver injury in rats exposed to tobacco smoke in combination. The study was carried out by investigating the oxidative stress biomarkers (glutathione peroxidase, SOD, catalase, paroxynase-1 and NAD (P) H: quinoneoxidoreductase 1 activities), inflammatory markers (IL-6 and IL-8 levels and gene expressions of NF-κB and VEGF) as well as histopathogical examination. It was found a significant reduction in activities of antioxidant enzymes concomitant with cytokines elevation; (IL-6 and IL-8) in rats exposed to tobacco smoke in combination with (TAA) treatment compared with normal control. Additionally, the transcript levels of (NF-κB and VEGF) genes were significantly higher in the rats exposed to tobacco smoke in combination with (TAA) treatment compared with normal control. However, treatment with chrysin, the levels of these parameters were significantly decreased which demonstrates the ameliorative effect of chrysin against (TAA) induced liver injury in rats exposed to tobacco smoke in combination. Histological investigations revealed that chrysin gave more preventive effect against thioacetamide (TAA)-induced liver injury in rats exposed to tobacco smoke. In conclusion, chrysin modulated the damage induced by exposure to tobacco smoking and hepatic disease.

Keywords: Thioacetamide (TAA), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) , paroxynase -1, NAD P H oxidase , quinoneoxidoreductase 1 activities, interleukin 6 (IL-6),interleukin 8 (IL-8), nuclear factor-kappa B (NF-κB), vascular endothelial growth factor (VEGF)

1. INTRODUCTION

The use of tobacco products is the leading preventable cause of death in the world today, resulting in nearly six million deaths annually and assisting in the manifestation of many other diseases (World Health Organization, 2013). In addition, the economic damages caused by tobacco smoking in the US alone over the past four

decades could exceed $7 trillion USD (extrapolated from CDC reported data, unpublished).

Smoking considers being wide public health problem, which reached today to the level of global epidemical. It is a risk factor for variety of disease (cardiovascular disease, stroke, chronic pulmonary disease, Alzheimer's disease, Parkinson's disease) (Gboyega et al., 2013). About more than5 million people die from smoking related illnesses, as the report of World Health Organization [World Health Organization, 2002)], this number will be doubled by 2025.Smoking causes a variety of adverse effects on organs that have no direct contact with the smoke itself such as the liver. It induces three major adverse effects on the liver: direct or indirect toxic effects, immunological effects and oncogenic effects. Smoking yields chemical substances with cytotoxic potential which increase

Necroinflammation and fibrosis, In addition, smoking increases the production of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) that would be involved in liver cell injury. It contributes to the development of secondary polycythemia and in turn to increased red cell mass and turnover which might be a contributing factor to secondary iron overload disease promoting oxidative stress of hepatocytes. Increased red cell mass and turnover is associated with increased purine catabolism which promotes excessive production of uric acid. Smoking affects both cell-mediated and humeral immune responses by blocking lymphocyte proliferation and inducing apoptosis of lymphocytes. Smoking also increases serum and hepatic iron which induce oxidative stress and lipid peroxidation that lead to activation of stellate cells and development of fibrosis. Smoking yields chemicals with oncogenic potential that increase the risk of hepatocellular carcinoma

(HCC) in patients with viral hepatitis and are independent of viral infection as well. Tobacco smoking has been associated with suppression of p53 (tumor suppressor gene). In addition, smoking causes suppression of T-cell responses and is associated with decreased surveillance for tumor cells. Moreover, it has been reported that heavy smoking affects the sustained virological response to interferon (IFN) therapy in hepatitis C patients which can be improved by repeated phlebotomy. Smoker's syndrome is a clinico-pathological condition where patients complain of episodes of facial fleshing, warmth of the palms and soles of feet, throbbing headache, fullness in the head, dizziness, lethargy, prickling sensation, pruritus and arthralgia (El-Zayadi, 2006).

Tobacco smoking is the inhalation of smoke from burned dried or cured leavesof the tobacco plant, most often in the form of a cigarette. Cigarette smoke contains a toxic collection of more than 4000 chemicals including nicotine, which combined give rise to addictive stimulant and euphoriant properties. According to the World health Organization, about one third of the world's male population smokes tobacco and cigarette smoking (CS) killed 100 million people worldwide in the 20th century. Therefore, CS is considered a major cause of preventable morbidity and mortality worldwide (He*et al.* 2005; Pham *et al.* 2007). Hepatologists have traditionally paid scant attention to the deleterious effects of CS. (Bataller, 2006). This reflects the fact that smoking per se does not appear to cause liver injury and therefore is not considered a causative agent for chronic liver diseases (Whitehead *et al.* 1996). However, there is increasing evidence that CS may negatively impact the incidence, severity and clinical course of many types of chronic liver diseases including hepatocellular carcinoma (HCC) (El-Zayadi, 2006).

Chrysin, a natural flavonoid compound, has been reported to have antioxidant properties via free radical scavenging (Sathiavelu *et al.*, 2009) and upregulation of antioxidant defense mechanisms (Sultana *et al.*,2012). In addition, chrysin could decrease lipid peroxidation level induced by various oxidants (Anand *et al.*, 2012). Moreover, chrysin has been reported to have potent anti-inflammatory effects both *in vitro* and *in vivo* mainly via inhibiting several cytokines including Tumor Necrosis Factor-α (TNF-α),interlukin-6 (IL-6), interlukin-1β (IL-1β) and nitric oxide (NO) (Cho *et al.,* 2004; Harasstani *et al.,*2010). In this regard, inhibition of such inflammatory mediators could be attributed to suppression of (NF- B), a transcription factor regulating the expression of proinflammatory mediators. This occurs because chrysin was found to block its activation *in vitro* (Shin *et al.,* 2009; Ha *et al.,* 2010) and *in vivo*(Shin *et al.,* 2009; Rehman *et al.,* 2013) where it suppressed the phosphorylation of inhibitor of kappa B(I B) preventing its degradation and thus suspended the nuclear translocation of (NF- B). Finally, chrysin was reported to exert several biological properties such as antioxidant, anti-inflammatory, anti-apoptotic (Sultana *et al.,* 2012) and hepatoprotective effects (Ciftci*et al.,* 2012a). Based on the above facts and properties of the Chrysin, the study was designed to check the protective efficacy of Chrysin against nicotine-induced hepatotoxicity targeting oxidative stress and apoptosis.

2. Materials and methods

2.1. Drugs and Chemicals

Thioacetamide was purchased from Sigma Chemical Co. (St.Louis, MO, USA) in the form of powder. Chrysin was purchased from Sigma-Aldrich Chemical Company, (St. Louis, MO, USA). Chrysin was suspended in distilled water. Other chemicals were of the highest grade commercially available.

2.2. Study population

A total 35 adult male Wistar albino rats (8–10 weeks, 100–120 gm) were purchased from the animal house, Faculty of Medicine, Zagazig University, Egypt. Rats were kept in ventilated stainless steel cages in a clean room at a controlled temperature (20–25∘C) and illumination (12 h light/dark). They were fed a standard pellet diet and water ad-libitum. This diet was designed by the National Research Institute for Nutrition, Giza, according to the National Nutrition Database for Standard Reference. The animals were left for one week for acclimatization.

2.3. Animal treatments

Cleopatra cigarettes (king size, Eastern Company, Egypt) were obtained from Hyper One Mall (the 10th of Ramadan, El-Sharqia, Egypt). Each cigarette contains 1 mg of nicotine and 15 mg of tar.

2.4. Emphysematous model

Our smoking inhalation system device was based on the work of Cendon *et al.* (1997) and Kozma *et al.* (2014). The rats were exposed to the smoke of 10 cigarettes/three times/day for 4 weeks. Each exposure period was one hour (from 9:00 a.m. to 10:00 a.m.; from12:00 p.m. to 1:00 p.m.; from 7:00 p.m. to 8:00 p.m.). During each exposure period, the animals were exposed to smoke for 10 min, followed by 5 min of exposure to fresh room air to avoid hypoxia.

2.5. Experimental design

The rats were randomly divided into the following groups; Group 1 (Control): Healthy rats were kept as control. Group 2 (Chry): Rats received chrysin orally at the dose of 25 mg/kg b.wt. once daily for one month (Pushpavalli *et al.,* 2010). Group 3 (Cig. + TAA): Rats were received 250 mg/kg, i.p. three times a week for 6 weeks then exposed tobacco smoke for 2 months .Group 4 (Cig. + TAA +Chry): Rats were received

250 mg/kg, i.p. three times a week for 6 weeks then exposed tobacco smoke for 2 months, after the exposure to tobacco smoke, rats received chrysin as in group 2.

2.6. *Tissue preparation for histological liver evaluation and homogenate*

The rats were euthanized with an intraperitoneal injection of0.2 ml of 260 mg/ml sodium pentobarbital after 4 weeks post-last treatment. Then, instillation of fixative (10% neutral buffered formalin, pH 7.4) was carried out under a constant pressure of 20 cmH$_2$O. 1 gm of the liver were dissected, immersed in fixative and processed for paraffin block. Then, 5 µm thick sections were cut, deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) (Bancroft and Layton, 2012). The rest of the liver was cut into small pieces and subsequently homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mMEthyleneDiamine Tetra Acetic acid (Sigma, St. Louis MO), 0.1 mM phenyl-methanesulfonyl fluoride (NacalaiTesque, Kyoto, Japan), 1µMpepstatin A (Peptide Institute, Osaka, Japan), and 2 µM leupeptin(Peptide Institute). The homogenates were then centrifuged at105,000 × g for 1 h at 4∘C. Thereafter, the supernatants were stored at−80∘C until used.

2.7. *Assay of antioxidant biomarkers*

To demonstrate systemic liver redox, activities of antioxidant enzymes; superoxide dismutase (SOD),glutathione peroxidase (GPx),Paraoxonase (PON-1) and catalase (CAT) were determined. SOD (U/g protein, EC 1.15.1.1) activity was determined based on the method of Sun *et al.* (1988).CAT (U/g protein; EC 1.11.1.6) activity was determined with respect to Aebi (1984) method. Glutathione peroxidase (GPx) activity was determined based on the method of Rotruck *et al.* (1973) method [E.C. 1.11.1.9]. PON-1 (U/mg tissue) activity was determined

according to the method of Beltowski *et al.* 2005.

2.7. *Real-time PCR*

The liver was disrupted using a motorized pestle and mortar, and total RNA was extracted using an RNeasy mini kit (Qiagen, cat. number. 74104, Germany). RNA concentration was determined using a Spectrophotometer (Thermo scientific, USA). RNA (500 ng) was reverse transcribed to cDNA using Quantitect Reverse Transcription Kit (Qiagen, cat. number. 205310, Germany). The cDNA was used as template for real-time PCR using Quanti Fast SYBR Green PCR Kit (Qiagen, cat. number. 204052, Germany). The sequences of the primers for NF-κ B were F: 5′-ACAACCCCTTCCAAGTTCCCT-3′ (sense) and R: 5′-TGGTCCCGTGAAATACACCT-3′ (anti-sense). The sequences of the primers for VEGF were F: 5′-TGCACCCACGACAGAAGGGGA-3′ (sense) and R: 5′-TCACCGCCTTGGCTTGTCACAT-3′ (anti-sense). The sequences of the primers for NADPH F: 5′-CAGCGGCTCCATGTACT-3′ (sense) and R: 5′-GACCTGGAAGCCACAGAAG-3′ (anti-sense). β -actin (housekeeping gene) was used as an internal control to ensure equal loading. All the experiments were performed according to the manufacturer's protocols. All samples were loaded into the qRT-PCR system (Light Cycler 480 II, Roche, USA) and run in triplicate using the following cycling parameters: 95 ℃ for 5 min for PCR initial heat activation; 40 repetitions of cycling: 95 ℃ for 10 s for denaturation and combined annealing/extension at 60 ℃ for 30 s. Data analysis was carried out using the 2-dCT method.

2.8. *Cytokine Analysis.*

Cytokines, IL-6 and IL-8 that are prominent cytokines involved in inflammation were measured in serum using

Rat interleukin-6 ELISA kit (Bender Medsystem, Burlingham, CA, USA (Cat no BMS625) and Rat interleukin-8 ELISA kit (My BioSource, Southern California, San Diego (USA). (Cat no MBS025179).

2.9. Statistical analysis:

Statistical analysis of differences between means was carried out using one-way analysis of variance (ANOVA). In case of a significant F-ratio, post hoc least significant difference (LSD) test for multiple comparisons was used to evaluate the statistical significance between treatment groups at $P < 0.05$ level of significance. All the statistical analysis was done using Statistical Package for Social Science (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

3.RESULTS

3.1. Effect of Chrysin on endogenous antioxidant status

The changes in the activities of SOD and CAT in liver of control and experimental rats are given in Fig. 1. The hepatic activitiesofGPx and PON-1 are shown in Fig. 2. The activities of SOD, CAT, GPx and PON-1 were significantly decreased in liver of nicotine-treated group when compared with control. Chrysin co-treatments were significantly increased the activities of SOD, CAT and GPx and PON-1 in liver.

3.2. Effect of Chrysin on Inflammatory markers

The changes in the levels of IL-6 and IL-8 in liver of control and experimental rats are given in Fig. 3. The hepatic levels of IL-6 and IL-8 were significantly increased in liver of nicotine-treated group when compared with control. Chrysin co-treatments were significantly decreased the levels of IL-6 and IL-8 in liver.

3.2. Effect of Chrysin on gene expression of NQO1, NF-κB and VEGF.

The changes in the levels of *NQO1, NF-κB and VEGF* in liver of control and experimental rats are given in Fig. 4. The hepatic levels of IL-6 and IL-8 were significantly increased in liver of nicotine-treated group when compared with control. Chrysin co-treatments were significantly decreased the levels of IL-6 and IL-8 in liver.
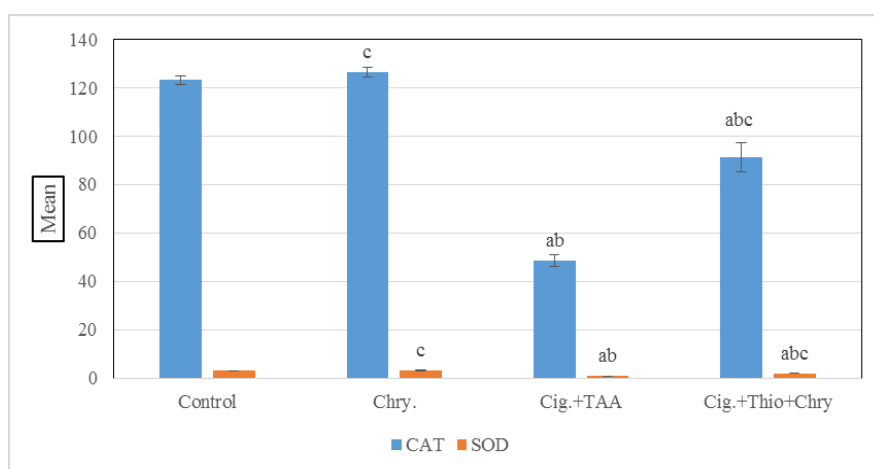

Fig. 1 Changes the CAT and SOD activities in liver after co-administration of Chry. in nicotine treated rats. Data represents mean ± SE, N = 10. a p<0.05 compared to control, b p<0.05 compared to chrysin and c p<0.05 compared to Cig. + TAA groups.
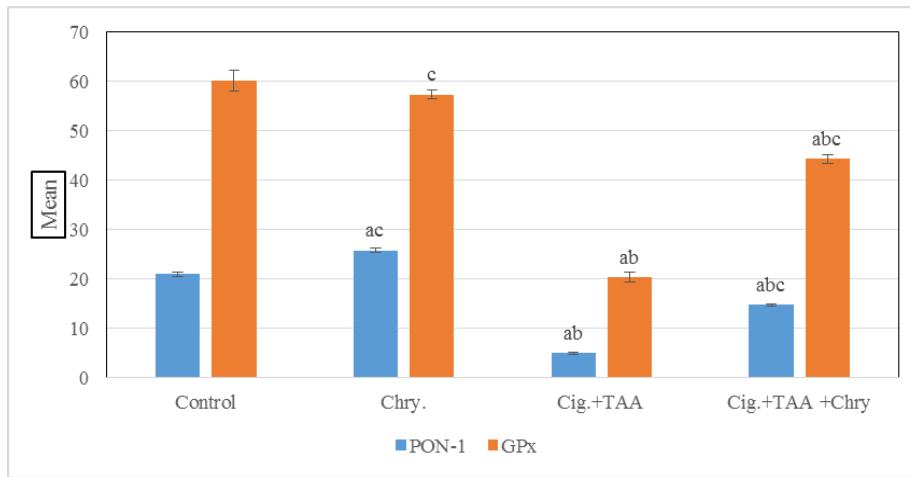
Fig. 2 Changes the GPx activity and PON-1 level in liver after co-administration of Chry. in nicotine treated rats. Data represents mean ± SE, N = 10. a $p<0.05$ compared to control, b $p<0.05$ compared to chrysin and c $p<0.05$ compared to Cig. + TAA groups.
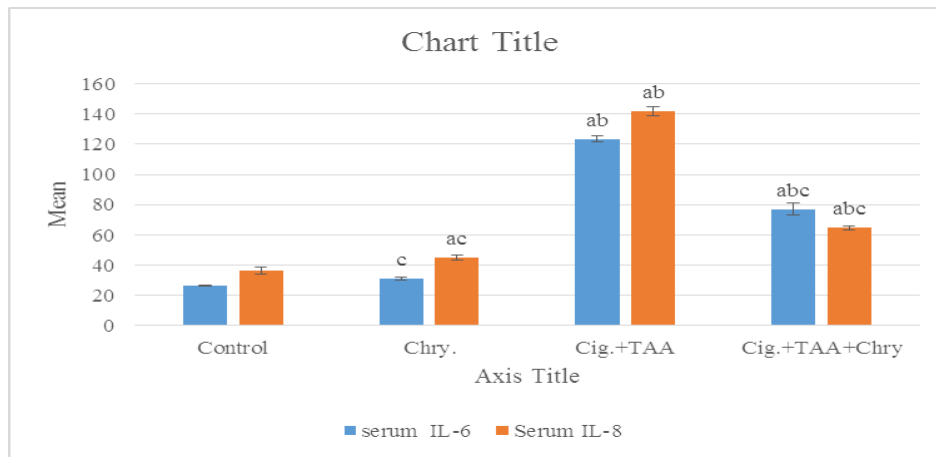

Fig. 3 Changes the IL-6 and IL-8 levels in liver after co-administration of Chry. in nicotine treated rats. Data represents mean ± SE, N = 10. a $p<0.05$ compared to control, b $p<0.05$ compared to chrysin and c $p<0.05$ compared to Cig. + TAA groups.
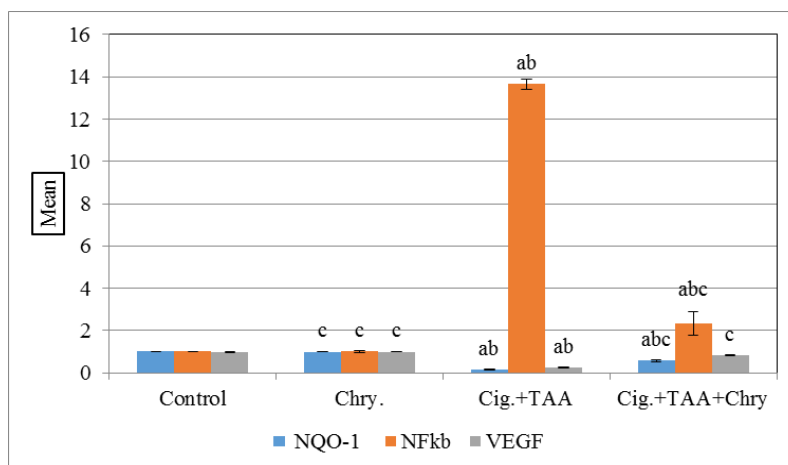

Fig. 4 Changes the NQO1, NF-κB and VEGF gene expressions in liver after co-administration of Chry. in nicotine treated rats. Data represents mean ± SE, N = 10. a $p<0.05$ compared to control, b $p<0.05$ compared to chrysin and c $p<0.05$ compared to Cig. + TAA groups.

4. DISCUSSION

Cigarette smoking induces three major adverse effects on the liver including immunological, oncogenic and other toxics effects (El-Zayadi, 2006). Nicotine-induced oxidative stress in the liver that was prevented by chrysin in this study. Our study findings showed significantly decreasing in all of CAT, SOD and GXp in cigarette group comparable with control group. We suggested that the decreased in CAT level in smokers group due to accumulation of hydrogen peroxide, which inhibit this enzyme or alter enzymatic conformation. Also this decreased CAT activity could be due to inactivation of CAT by the cross linking or the impairment of nitric oxide (NO) synthase, which can bind reversible to ferric iron and inhibiting afterwards CAT activity (Bogdansk *et al*. 2003-Yosri*et al*. 2013).

Our findings about CAT activity were disagreeing with previous results (Omar *et al*. 2012), which observed increased level of CAT significantly in smokers. Our observation about CAT, SOD and GPx confirm the results of many studies (Anshu and Flora, 2012). Our result disagrees with other studies (Syed Anzar and Alvina, 2016), which observed that about SOD and GXp, which decreased in group of smokers as compared to nonsmokers. The significantly differences of oxidative enzymes levels CAT, SOD and GXp may be due to nicotine increased generation of superoxide anion and hydrogen peroxide, which in turn results in generation of hydroxyl free radicals. Generation of these free radicals have been shown to participate in many toxic reaction and increased production of superoxide and hydrogen peroxide may cause deleterious injury to alveolar macrophages by causing release of proteolytic enzymes (Yildiz, 2004).

Cigarette smoking is a potent inhibitor of PON1 enzymatic activity, making the effectiveness of PON1 in smoker's particularly important (Senti*et al.*2003; Nishio and Watanabe, 1997). It was shown that cigarette smoke extract decreases PON1 activity by modifying the free thiol groups of the enzyme. It can be explained by the effects of several of the hundreds of chemical components of tobacco smoke including various reactive aldehydes as well as aromatic hydrocarbons (Nishio and Watanabe, 1997). Moreover, it is important that smoking may increase neutrophil myeloperoxidase activity (Loke*et al.* 2012), which has been shown to interact with HDL and to modulate paraoxonase 1 function (Huang *et al.* 2013).Nicotine increases the cytokines production that could be involved in liver injury (El-Zayadi, 2006).In the present study, proinflammatory cytokines secreted from activatedneutrophils, monocytes and macrophages of nicotine-treatedanimals to remove dead cells and promote tissue repair, as IL-8,and IL-6 have been elevated.Our observation is in coincident with Cromwell *et al.*(1992) who found increasing the production of α-TNF, IL- 1 and IL-6 within the lung of rats is similar to the effect nicotine. This increase in the levels of cytokines and adhesion molecules results in the activation of neutrophils and macrophages to the lung that can lead to tissue destruction (Wang *et al.* 2004).

Cigarette constituents (particularly ROS) activate epithelial cell intracellular signaling cascades that lead to inflammatory gene activation [*e.g*., interleukin-8 or IL-8 and tumor necrosis factor-alpha (TNFα)] (Churg *et al*., 2002; Chung, 2005). The secretion of these inflammatory mediators promotes chronic immune cell recruitment and inflammation.

We have shown in the present study that cigarette smoke reduces both NQO1 and VEGF expression. This result is in coincidence with the researchers who showed that VEGF and VEGFR-2 expression are decreased in emphysematous lungs concomitant with increased endothelial cell apoptosis (Kasahara *et al.* 2001). VEGF levels are also reduced in the bronchoalveolar lavage fluid of smokers (26). Interestingly, VEGF receptor inhibition leads to the enlargement of the air spaces, increased endothelial cell death, and decreased capillary density, characteristic of emphysema (Jakkula *et al.* 2000, Kasahara *et al.* 2000).

Living tissues are endowed with innate antioxidant defense mechanisms, namely CAT, SOD and Gpx. A reduction in the activities of CAT, SOD and Gpx enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes (Reedy and Lokesh, 1992).Rats that received chrysin along with nicotine in combination with thioacetamide (Group IV), the activities of CAT, SOD and Gpx were significantly higher than in rats that received nicotine in combination with thioacetamide(Group III) rats. These results corroborate previous observations that chrysin treatment increases the activities of antioxidants, such as CAT, SOD and Gpx, in ethanol-induced toxicity in female albino rats (Sathiavelu *et al.,* 2009). Chrysin possibly confers this protective effect by dampening the generation of free radicals that are induced by CCl4.Paraoxonase-1 (PON-1), an enzyme with lactonase and esterase activities, is synthesized mainly by the liver [Leviev *et al.* 1997, Rodrigo *et al.* 1997] and it plays a role in the regulation of oxidative stress, fibrosis and hepatic cell apoptosis in chronic liver diseases. [Ferre *et al.* 2006]. Our result showed that administration of chrysin increased the level of PON-1 and that was

agreed with Zhang et al., who proposed that PON-1 over expression provides strong protection against the development of experimental liver disease [Zhang *et al.* 2008].

The pro-inflammatory cytokines IFN-γ, IL-17A, IL-6, IL-1β and TNF-α contribute to the recruitment, activation and activity of M1 macrophages, leading to ocular inflammation, thus, we evaluated the expression of these cytokines at both the mRNA and protein levels. We observed that chrysin suppressed the levels of IFN-γ, IL-17A, IL-6, IL-1β and TNF-α. Thus, chrysin suppresses intraocular inflammation (Meng X *et al.* 2017).In the present study, NF-κB expression was markedly elevated in the Cig+TAA-intoxicated animals while chrysin supplementation effectively counteracted this effect. Our results are in accordance with pervious one reporting that downregulating NF-κB protein expression could be a promising target for protection against DOX cardiotoxicity (Saeed *et al.* 2015).. Tumor angiogenesis is stimulated by angiogenic growth factors, such as VEGF, basic Fibroblast Growth Factor (bFGF), Transforming Growth Factor (TGF) and Interleukin-8 (IL-8). VEGF and its receptors have been described as the fundamental regulators of angiogenesis and play an important role in tumor progression (Fang *et al.,* 2005). Generally, the important effects of chrysin in cellular processes can be concluded to be (1) the inhibition of HIF-1α/VEGF-regulated angiogenesis via the AKT signaling pathway and (2) the sensitization of TNFα-induced cell death via downregulation of NFκB and activation of caspase-8. In addition, (3) chrysin induces also apoptosis via activation of caspase-3, which involves the inactivation of AKT signaling pathway and downregulation of the X-linked Inhibitor of Apoptosis Protein (XIAP) (Samarghandian *et al.,* 2011).

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