# Curcumin role during hepatic toxicity in Wistar rats: Preliminary Study

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## Abstract

In this study we examined the effect of curcumin on paracetamol overdose induced liver toxicity in rats. Forty male Wistar rats were allocated into 4 groups. The first group served as control and received corn oil; second group received curcumin (400 mg/kg BW daily) dissolved in corn oil; third group received a single dose of paracetamol (500 mg/kg BW); fourth group was served as protective group and received curcumin plus paracetamol.  Animals received the respective administrations orally for 7 successive days for first, second and third group, while paracetamol was administered on the 6$^{th}$ day for third and fourth group.  Animals were slaughtered and blood and liver tissues were collected for various biochemical and gene expression. Serum analysis revealed an alteration in GPT, GOT, Urea, albumin and lipid profiles that incluse LDL, TG, cholesterol and HDL. Moreover, a decrease in antioxidant activity of liver was reported and was normalized in curcumin protective group. Moreover, curcumin improved atherogenic index and Cholesterol ration that are increased during hepatic toxicity. Curcumin increased super oxide dismutase (SOD) and glutathione peroxidase expression while paracetamol decreased them. Co-administration of curcumin with paracetamol normalized SOD and GPx expression compared to paracetamol administered rats. Molecular gene expression revealed that paracetamol administered rats showed decrease in the expression of acute phase proteins that are represented by alpha-2 macroglobulin ($\alpha$-2M). Curcumin administration ameliorated the alteration in the expression of $\alpha$-2M. It can be concluded that curcumin has a protective effect on paracetamol induced hepatic toxicity in rats.

**Keywords:** Curcumin; Liver toxicity; Paracetamol; Antioxidants; gene expression; acute phase proteins; Wistar rats.

## Introduction

Curcumin (Turmeric) is widely used in therapeutic preparations against variable diseases (Chattopadhyay et al., 2004). Moreover it is used as food spice, additive, flavoring, preservative and as coloring agent in foods and textiles (Basnet and Skalko-Basnet, 2011). Curcumin has several activities among which is the antioxidant (Al-Jassabi et al., 2012), antimicrobial (Tajbakhsh et al., 2008), anti-inflammatory (Bereswill et al., 2010), antiviral (Kutluay et al., 2008), anti-carcinogenic activity (Das and Vinayak, 2012) and anti-diabetic activity (Aziz et al., 2013). Recently, it has been shown that curcumin and its constituents have hepatoprotective properties (Jobin et al., 1999; Somanawat et al., 2013). Curcumin has a protective effect against liver damage in animals induced by a variety of hepatotoxic substances such as carbon tetrachloride (Morsy et al., 2012). Moreover, curcumin has silymarin like actions (Girish et al., 2009). It has been shown that curcumin has antiapoptotic activity *in vitro* and *in vivo* through its action on hepatic injury (Li et al., 2013).

Liver functions include removal and inactivation of toxic substance and drugs to be excreted in urine. Hepatic toxicity is attributed primarily to the changes in oxidative stress and alteration in acute phase proteins (Flora et al., 2013). The imbalance between production of free radicals and reactive metabolites is named oxidative stress that alleviated by antioxidant systems (Evans et al., 2002). Oxidative stress leads to the damage of important biomolecules and organs (Durackova, 2010). This damage, is probably associated with DNA, protein, and lipid damage, and is the cause for liver diseases such as chronic liver injury, hepatic inflammation, fibrosis and hepatocellular carcinoma (Tanikawa and Torimura, 2006; Vera-Ramirez et al., 2013).

Paracetamol (P); acetaminophen or N-acetyl-p-aminophenol (APAP); is a widely analgesic medication in many countries. An overdose of paracetamol is the reason for liver and renal toxicity and probably death (Zhao et al., 1998). The exact mechanism of such toxicity is not clear but most studies focused on paracetamol effect on antioxidants levels in blood and tissue activity ( Li et al., 2013 ) together with liver and kidney function (Somanawat et al., 2013). High dose of paracetamol causes glutathione depletion, apoptosis and cell death (Sun et al., 2002).

The aim of the current study is to examine the protective effect of curcumin against hepatic toxicity induced by paracetamol in Wistar rats based on biochemical and molecular study. The expression of antioxidant genes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and acute phase protein α-2 macroglobulin, α-2M) were examined using semi-quantitative RT-PCR analysis.

## Materials and methods
### *Reagents and Kits*

Acetaminophen, ethidium bromide and agarose were purchased from Sigma-Aldrich Co., MO, USA. The Wistar albino rats were purchased from King Fahd center for Scientific Research, King Abdel-Aziz University, Jeddah, Saudi Arabia. Serologic kits for GPT, GOT, albumin and urea were purchased from Bio-diagnostic

Co., Dokki, Giza, Egypt. The deoxyribonucleic acid (DNA) ladder was purchased from MBI, Fermentas, USA. Qiazol for RNA extraction was purchased from QIAGEN Inc., Valencia, CA.

## *Animals and Experimental Design*

All animal procedures were approved by the Ethical Committee Office of the dean of scientific affairs of Taif University, Saudi Arabia. Forty male Sprague Dawley rats, weighing 200–280 g were used for this study. The animals were kept a 12-h light-dark cycle and gained access to food and water ad libitum. Rats were randomly divided into 4 groups (10 rats per group and 5 rats per cage) as follows:

**Group1**, Control (C) served as negative control. **Group2**, curcumin (CUR) received curcumin dissolved in corn oil in a dose of 400 mg/kg bw daily for 7 days. **Group 3**, Paracetamol received single intra-gastric dose of paracetamol (500 mg /kg B W), 24 h before sampling. **Group 4**, protective group received curcumin dissolved in corn oil (400 mg/kg BW) daily for 7 days and on the 6$^{th}$ day after curcumin administration, paracetamol (500 mg /kg BW intra-gastric) was administered. The dose of paracetamol used in this study, was determined based on study of Li et al. (2013).

## *Sampling*

Twenty four hours after administration of tested chemicals, all animals were anesthetized; blood and tissues were collected after rats sacrificing by diethylether inhalation. Serum was extracted after blood centrifugation for 10 min at 4000 $xg$. For gene expression, liver tissues were kept in TriZol reagent for RNA extraction and in 10% neutral formalin for histopathological and immunohistochemistry.

## *Determination of liver antioxidant activity*

For Catalase activity, one gram of liver tissues was homogenized in 5ml of cold buffer of 50mM potassium phosphate buffer (PBS, pH 7.4), containing 1mM EDTA and 1mL/l Triton X-100. After centrifugation at 4000 $xg$ for15 minutes at 4ºC, the supernatant was removed and stored frozen at -80ºC until the time of analysis of catalase (U/g tissue). For malondialdehyde (MDA) measurements, one gram of liver tissues was homogenized in 5ml of cold buffer of 50mM potassium phosphate buffer (pH 7.4). After centrifugation at 4000 $xg$ for15 minutes at 4ºC, the supernatant was removed and stored frozen at -80ºC until the time of analysis of MDA (nmol/g tissue) The activities of catalase and MDA were determined by ELISA reader (Absorbance Microplate Reader ELx 800TM BioTek®, USA). Results were calculated according to the manufacture instructions.

## *Gene expression analysis*
## *RNA Extraction*

Total RNA was extracted from liver tissue samples (approximately 100 mg per sample) of experimental rats. Liver samples were flash frozen in liquid nitrogen and

subsequently stored at -70°C in 1 ml Qiazol (QIAGEN Inc., Valencia, CA). Frozen samples were homogenized using a Polytron 300 D homogenizer (Brinkman Instruments, Westbury, NY). Then, 0.3 ml chloroform was added to the homogenate. The mixtures were shaken for 30 seconds followed by centrifugation at 4°C and 16,400 $x g$ for 15 min. The supernatant was transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shacked for 15 seconds and centrifuged at 4°C and 16,400 $x g$ for 15 min. The RNA pellets were washed with 70% ethanol, briefly dries up, and then dissolved in Diethylpyrocarbonate (DEPC) water. RNA concentration and purity were determined spectrophotometrically at 260 nm. The RNA integrity was confirmed in 1.5% agarose stained with ethedium bromide. The ratio of the 260/280 optical density of all RNA samples was 1.7-1.9.

*cDNA Synthesis and Semi-quantitative PCR Analysis*

For cDNA synthesis, mixture of 3 μg total RNA and 0.5 ng oligo dT primer (Qiagen Valencia,CA, USA) in a total volume of 11 μl sterilized DEPC water was incubated in the Bio-Rad T100$^{TM}$ Thermal cycle at 65°C for 10 min for denaturation. Then, 2 μl of 10X RT-buffer, 2 μl of 10 mM dNTPs and 100 U Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (SibEnzyme Ltd. Ak, Novosibirsk, Russia) were added and the total volume was completed up to 20 μl by DEPC water. The mixture was then re-incubated in the thermal Cycler at 37°C for one hour, then at 90°C for 10 min to inactivate the enzyme. For semi-quantitative RT-PCR analysis, specific primers for examined genes (table 1) were designed using Oligo-4 computer program and synthesized by Macrogen (Macrogen Company, GAsa-dong, Geumcheon-gu. Korea). PCR was conducted in a final volume of 25 μl consisting of 1 μl cDNA, 1 μl of 10 pM of each primer (forward and reverse), and 12.5 μl PCR master mix (Promega Corporation, Madison, WI), the volume was brought up to 25 μl using sterilized, deionized water. PCR was carried out using Bio-Rad T100$^{TM}$ Thermal Cycle machine with the cycle sequence at 94 °C for 5 minutes one cycle, followed by variable cycles (stated in table 1) each of which consists of denaturation at 94 °C for one minute, annealing at the specific temperature corresponding to each primer (table 1) and extension at 72 °C for one minute with additional final extension at 72 °C for 7 minutes. As a reference, expression of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was examined (table 1). PCR products were electrophorized on 1.5% agarose (Bio Basic INC. Konrad Cres, Markham Ontario) gel stained with ethidium bromide in TBE (Tris-Borate-EDTA) buffer. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were quantified densitometrically using Image J software version 1.47 (http://imagej.en.softonic.com/).

*Table 1. PCR conditions for rat antioxidants, cytokines and acute phase proteins genes*

| Gene | Forward primer (5'-3') | Reverse primer (5'-3') | PCR cycles and conditions |
|---|---|---|---|
| GPx (406 bp) | AAGGTGCTGCTCATTGAGAATG | CGTCTGGACCTACCAGGAACTT | 40 cycles 57°C 1 min |
| SOD (410 bp) | AGGATTAACTGAAGGCGAGCAT | TCTACAGTTAGCAGGCCAGCAG | 35 cycles, 55°C 1 min |
| α2 –macroglobulin (325 bp) | GCTCCTGTCTGTTTCCTTAGTT | ATTGGCCTTTCGTGGTTTAG | 30 cycles, 56°C 1 min |
| GAPDH (309 bp) | AGATCCACAACGGATACATT | TCCCTCAAGATTGTCAGCAA | 25 cycles, 52 °C 1 min |

## Statistical Analysis

Results were shown as means ± standard error of means (SEM). Data were analyzed using analysis of variance (ANOVA) and post-hoc descriptive tests by SPSS software version 11.5 for Windows with $p < 0.05$ regarded as statistically significant. Regression analysis was performed using the same software.

## Results

### Serum renal and hepatic biochemical measurements

Renal and hepatic changes after paracetamol administration were tested. Paracetamol overdose increased serum levels of urea, albumin, GOT and GPT (table 2). Administration of curcumin together with paracetamol inhibited the increase in serum parameters of kidney and liver (table 2). Serum GOT values in paracetamol group were 156±9.4 *Vs.* 83 ± 2 for control group, while GPT values were 136±27.9 for paracetamol group *Vs.* 58.3 ± 6 for control group.

*Table 2. Protective effect of curcumin on paracetamol induced changes in serum levels of renal and hepatic parameters and hepatic antioxidant activity in Wistar rats.*

|  | Control | Curcumin | Paracetamol | Curcumin + Paracetamol |
|---|---|---|---|---|
| Urea (mg/dl) | 35.6±3.8 | 35.3±2.3 | 42.0 ±1.5* | 31.0 ±1.0[#] |
| Albumin (g/dl) | 3.2±0.1 | 3.1±0.1 | 4.8±0.4* | 3.5±0.1[#] |
| GOT (U/L) | 83 ± 2 | 72.7± 9.6 | 156±9.4* | 90.3±3.7[#] |
| GPT(U/L) | 58.3± 6 | 64.7±6.01 | 136±27.9* | 98±4.9[#] |
| MDA (nmol/g tissue) | 9.78±1.9 | 10.7±1.0 | 18.2±0.5* | 12.6±0.4[#] |
| Catalase (U/g tissue) | 33±6.1 | 37±1.1* | 21±0.4* | 33.9±2.6[#] |

*Values are means ± standard error (SE) for 5 different rats. Values are statistically significant at *p<0.05 Vs. control and #p<0.05 Vs. paracetamol group.*

### Serum lipids measurements

As shown in table 3, paracetamol induced significant increase in cholesterol, TG, LDL and a decrease in HDL levels. Curcumin co-Administration induced normalization in all parameters measured. Moreover. Curcumin coadministration

increase HDL levels and normalized the changes induced in atherogenic index and cholesterol ratio.

Table (3). Protective effect of curcumin on paracetamol induced serum changes of lipid profiles in Wistar rats.

| | Control | Curcumin | Paracetamol | Curcumin + Paracetamol |
|---|---|---|---|---|
| Cholesterol | 96.3±5.8 | 97.7±6.1 | 137.7±8.1* | 103.7±3.7# |
| Triglycerides | 55.7±7.9 | 72±4.2 | 100.3±1.5* | 86.3±6.7# |
| LDL | 66.3±4.1 | 62.2±7 | 107±7.8* | 65.7±2.2# |
| VLDL | 11.1±1.6 | 14.5±0.8 | 18.1±0.2* | 19±1 |
| HDL | 19.1±0.5 | 22.1±0.5 | 12.7±0.6* | 19.7±0.5 |
| Cholesterol ratio | 5±0.2 | 4.4±0.2 | 10.9±0.3* | 5.3±0.1 |
| Atherogenic Index | 0.5±0.1 | 0.5±0.0 | 0.8±0.0* | 0.6±0.0 |

*Values are means ± standard error (SE) for 5 different rats. Values are statistically significant at *p<0.05 Vs. control and #p<0.05 Vs. paracetamol group.*

### Hepatic antioxidant activity

The results for the protective effect of curcumin on MDA as oxidative stress marker, and catalase as antioxidant enzyme are illustrated in table 2. The current results revealed that, MDA increased significantly ($P<0.05$) in paracetamol administered rats compared to control (18.2±0.5 for paracetamol group Vs. 9.78 ±1.9 for control). Administration of curcumin to paracetamol group normalized MDA activity (12.6±0.4 for paracetamol plus curcumin group Vs. 9.78 ±1.9 for control group). In paracetamol group, the activity of catalase enzyme was decreased significantly compared to control rats ($P<0.05$) (21±0.4 for paracetamol Vs. 33± 6.1 for control). There is a significant increase in catalase activity (U/g/protein) in curcumin administered group (37±1.1). Administration of curcumin plus paracetamol inhibited significantly the decrease in catalase activity and returned to normal levels (33.9±2.6 for curcumin plus paracetamol Vs. 33± 6.1 for control).

### Molecular findings
### Semi-quantitative RT-PCR analysis of hepatic antioxidant enzymes

RT-PCR analysis for antioxidants expression is illustrated in figure 1. Parallel to tissue catalase antioxidant activity (table 2), mRNA expressions of SOD, and GPx were significantly decreased in paracetamol administered rats and increased in curcumin administered rats (Fig. 2). Curcumin administration plus paracetamol reversed the decrease in antioxidants expression reported in paracetamol administered rats.

### Semi-quantitative RT-PCR analysis of hepatic alpa-2 macroglobulin expression

To explore the possible involvement of acute phase proteins in curcumin protective effect, we examined the expression of α-2M in liver of treated groups. The expression of α-2M was down-regulated in paracetamol group and returned to control expression in curcumin plus paracetamol administered rats (Fig. 3).

**Discussion**

        This study demonstrated that paracetamol overdose induced hepatotocity in rats and curcumin administration attenuated hepatic toxicity through re-impairment of antioxidants capacity of hepatic cells together with a decrease in the expression of some cytokines that initiate the inflammatory cascade in the body. Previous observations studied in mice and rats (Yousef et al., 2010) focused on the biochemical alterations in levels of liver and kidney parameters. Here, we focused on the molecular regulation of hepatic toxicity and possible attenuation by curcumin. During inflammatory conditions like hepatitis, curcumin shows beneficial effects through its antioxidants activity (Samuhasaneeto et al., 2009).

        Cellular antioxidant defenses are classified into primary antioxidant enzymes which include SOD, catalase and GPx, and secondary non-enzymatic antioxidants such as ubiquinol, vitamin E, vitamin C and β carotene. SOD converts superoxide radical to hydrogen peroxide, catalase enzyme catalyzes the decomposition of hydrogen peroxide to water ($H_2O$) and oxygen molecule ($O_2$) where as GPx catalyzes the reduction of hydrogen peroxide and organic hydroperoxide into water and corresponding alcohol at the expense of glutathione (Evans et al., 2002).

        Lipid peroxidation represented by MDA as an oxidative stress marker and reduced GST, SOD and catalase as indicators of antioxidant potency for cells were used to assess the degree of hepatic cell stability and integrity. Our results showed that oxidative damage was caused by overdose of paracetamol significantly attenuated by curcumin administration. Therefore, we can postulate that curcumin could protect against free radical mediated oxidative stress by scavenging for free radicals that limits lipid peroxidation involved in membrane damage through attenuation of antioxidants depletion (Del Rio et al., 2005). The protective effect of curcumin can be explained by induction of gene expression for SOD, GPx, and catalase. Curcumin is known to protect cell membrane against oxidative damage. Most of the antioxidants have either a phenolic functional group or a β-diketone group. Curcumin is a super H-atom donor by donating the H-atom from the central methylenic group rather than from the phenolic group in acidic and neutral aqueous and acetonitrile solutions (Priyadarsini et al., 2003). It has been suggested that curcumin was not able to prevent MDA production (Reyes-Gordillo et al., 2007). The most likely explanation is that about 75% of oral consumed curcumin is excreted in the feces and only traces appeared in the urine, suggesting poor absorption of curcumin. However, it is confirmed that curcumin is bio-transformed to dihydrocurcumin, tetrahidrocurcumin, and hexahidrocurcumin; subsequently, these products are converted to glucuronide conjugates, which are more polar and have better absorption than curcumin. Therefore, the pharmacological actions of curcumin are mostly due to its hydrosoluble derivatives (Maheswari et al., 2006).

        Alterations in serum levels of hepatic transaminases (GOT and GPT) are used as markers and their increase indicates liver damage. In our study, there is a significant increase in GPT and GOT levels in paracetamol overdose administered

rats. Such increase was reduced by the administration of curcumin coinciding with the changes in urea and albumin levels as their increase was decreased by curcumin administration confirming curcumin role in protecting liver and kidney from paracetamol toxicity.

Acute-phase proteins are proteins that increase in response to inflammation. The variability in protein plasma levels, and following impact on drug binding extent, cause modifications in the mode of drug action, distribution, disposition and elimination. One of the most important acute phase proteins is alpha-2 macroglobulin. Our findings confirmed that the expression of $\alpha$-2M expression was increased after curcumin administration and decreased in paracetamol overdose administered rats. Co-administration of curcumin with paracetamol normalized $\alpha$-2M expression. $\alpha$-2M is a large plasma protein found in the blood. It is produced mainly by the liver and locally by macrophages, fibroblasts, and adrenocortical cells. $\alpha$-2M acts as an antiprotease and is able to inactivate an enormous variety of proteinases. $\alpha$-2M functions as an inhibitor of fibrinolysis by inhibiting plasmin and acts as a carrier protein because it binds to numerous growth factors and cytokines such as IL-1$\beta$ (Lyoumi et al., 1998). It has been shown that $\alpha$-2M secretion is decreased during acute liver inflammation induced by turpentine oil and the possible cause for the reduction in $\alpha$-2M expression is presumed to be hepatocyte dysfunction irrespective of cytokines profiles (Kuribayashi et al., 2012).

In summary, the data from biochemical and molecular findings reveal that curcumin is a natural antioxidant and anti-inflammatory polyphenol food supplement that attenuates paracetamol overdose induced hepatic toxicity in Wistar rats.
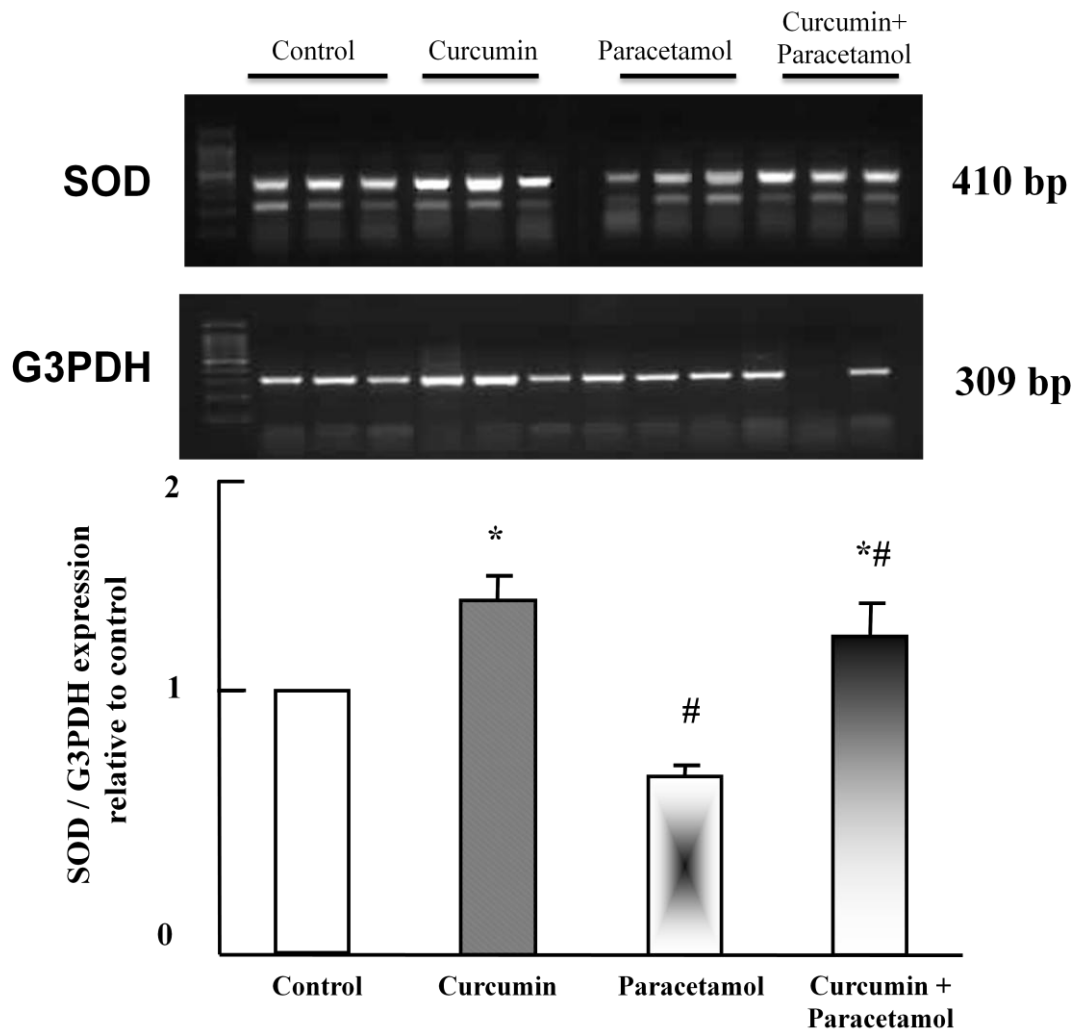
**Figure 1.**



**Figure 1.** Semi-quantitative RT-PCR analysis of SOD mRNA expressions and its corresponding G3PDH in liver. Experimental groups were administered corn oil as a control (**C**), curcumin (**CUR**), paracetamol (**P**), or curcumin plus paracetamol (**CUR+P**) as described in materials and methods. Values are means ± SEM obtained from 10 different rats per group. P*< 0.05 vs. control group and P#< 0.05 vs. paracetamol administered group.
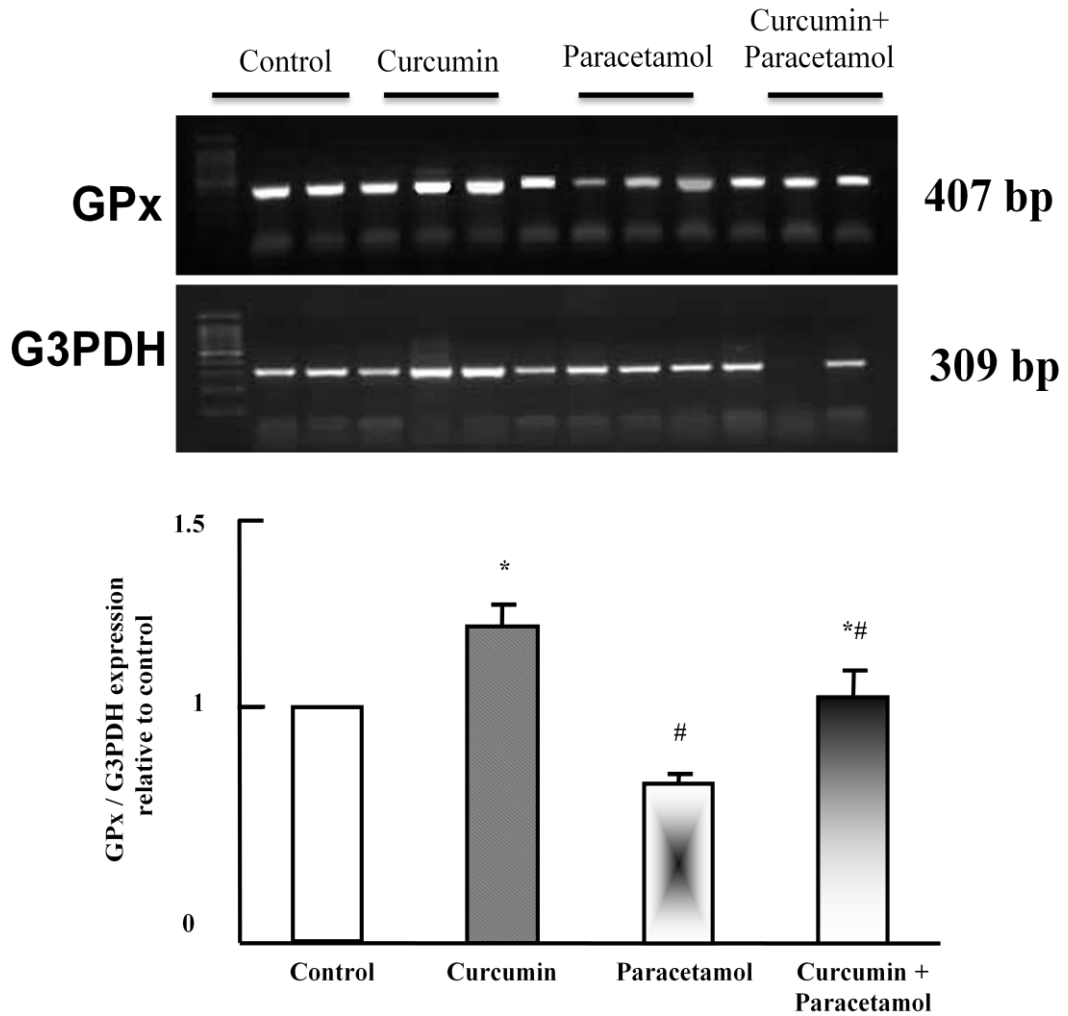
**Figure 2**



**Figure 2.** Semi-quantitative RT-PCR analysis of GPx and its corresponding G3PDH in liver. Experimental groups were administered corn oil as a control (**C**), curcumin (**CUR**), paracetamol (**P**), or curcumin plus paracetamol (**CUR+P**) as described in materials and methods.. Values are means ± SEM obtained from 10 different rats per group. P*< 0.05 *vs.* control group and P#< 0.05 *vs.* paracetamol administered group.
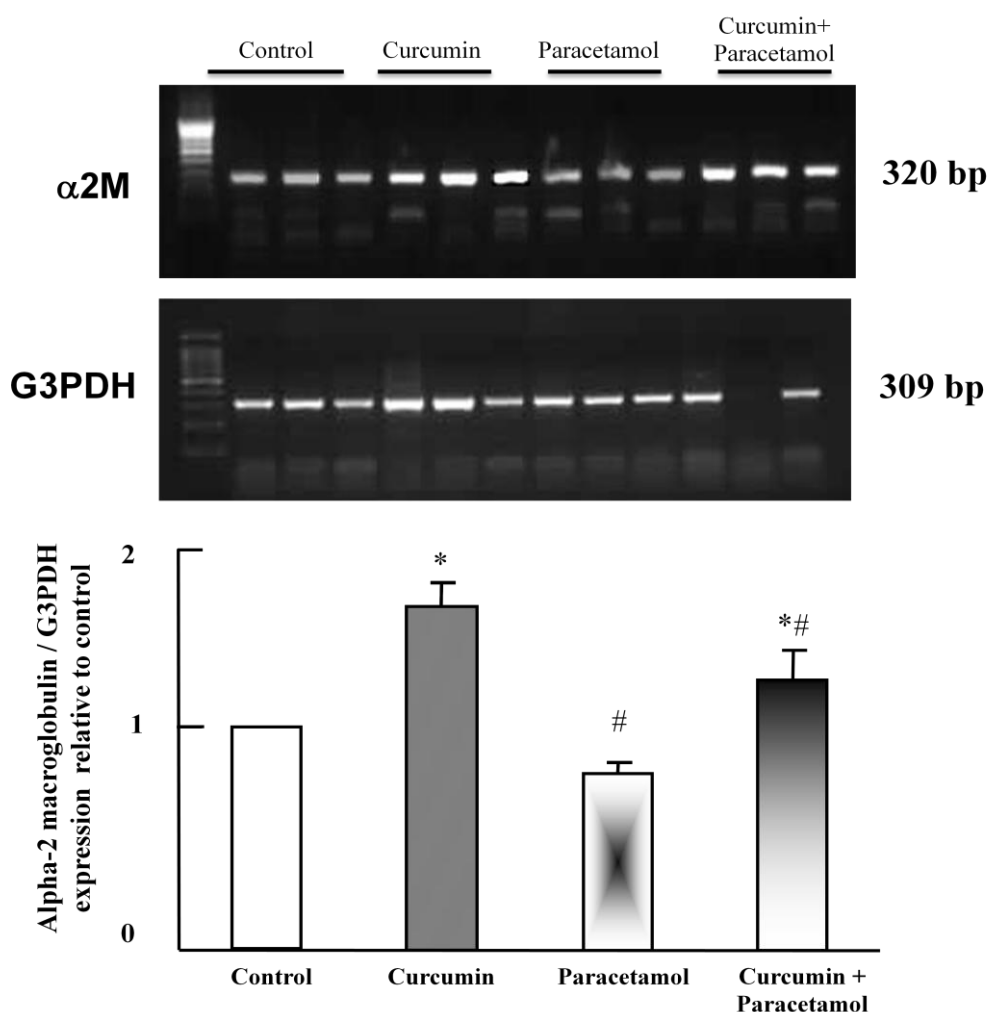
**Figure 2**



**Figure 3.** Semi-quantitative RT-PCR analysis of acute phase proteins α-2M mRNA expressions and its corresponding G3PDH in liver. Experimental groups were administered corn oil as a control (**C**), curcumin (**CUR**), paracetamol (**P**), or curcumin plus paracetamol (**CUR+P**) as described in materials and methods. Values are means ± SEM obtained from 10 different rats per group. P*< 0.05 *vs.* control group and P#< 0.05 *vs.* paracetamol administered group.

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