Tramadol-induced hepato- and nephrotoxicity in rats: Role of Curcumin and Gallic acid as antioxidants

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Abstract

Tramadol is an analgesic used to treat moderate to severe pain caused by cancer, osteoarthritis, and other musculoskeletal diseases. Cytochrome P450 system metabolizes tramadol and induces oxidative stress in different organs. Therefore, the present study aims at investigating the changes in the activities and the protein expressions of CYPs isozymes (2E1, 3A4, 2B1/2), antioxidants status, free radicals levels after pretreatment of rats with Curcumin and/or Gallic acid as single- and/or repeated-doses before administration of tramadol. In repeated-dose treatments of rats with tramadol, the activities of cytochrome P450, cytochrome b5, and NADPH-cytochrome-c-reductase, and the antioxidant enzymes including glutathione reductase, glutathione peroxidase, glutathione S-transferase, catalase, superoxide dismutase, and levels of glutathione were inhibited in the liver and the kidney of rats. Interestingly, such changes caused by tramadol restored to their normal levels after pretreatment of rats with either Curcumin and/or Gallic acid. On the other hand, repeated-dose treatment of rats with tramadol increased the activities of both dimethylnitrosamine N-demethylase I (DMN-dI), and aryl hydrocarbon hydroxylase (AHH) compared to the control group. However, pretreatment of rats with Curcumin and/or Gallic acid prior to administration of tramadol restored the inhibited DMN-dI activity and its protein expression (CYP 2E1) to their normal levels. In conclusion, Curcumin and/or Gallic acid alleviated the adverse effects caused by tramadol. In addition, patients should be advice to take Curcumin and/or Gallic acid prior to tramadol treatment to alleviate the hepatic and renal toxicities caused by tramadol.

1. Introduction

Tramadol is a potent analgesic medication prescribed worldwide for treatment of acute and chronic pains [1–3]. The mechanisms of action of the tramadol are mainly due to binding to...
the μ-opioid receptor and inhibition of the neuronal uptake of norepinephrine and serotonin [1,2]

CYP450s play a significant role in the activation and inactivation of many exogenous and endogenous compounds. For example, CYP2E1 metabolizes N-nitrosamines to genotoxic products that methylate DNA and other macromolecules [4]. Furthermore, CYP2E1 is able to produce reactive oxygen species (ROS), leading to oxidative stress which consequently induces different cytotoxic effects [5]. CYP3A4 and CYP2D6 metabolize tramadol into more potent opioid analgesic metabolite M1 [3,6]. In addition, CYP2D6 gene polymorphisms increased the hepatotoxicity through the accumulation of tramadol bioactive metabolite (M1), and consequently induced oxidative stress [6,7]. The opioid analgesic potency of tramadol was influenced by an individual’s CYP genetics since poor metabolizers have experienced little conversion to the more active M1 opioid metabolite, whereas individuals with a high metabolic rates have experienced greatest analgesic effects. Detoxification of M1 opioid metabolite is mainly carried out through phase II reactions with glucuronic acid and/or sulphate [3,8]. The toxic effects of tramadol could be due to the generation of more than one metabolites that were associated with the hepatic- and the nephrotoxicity especially after long-term therapy [9].

Imbalance in the production of reactive oxygen species (ROS) and the deficiency to detoxify ROS caused induction of the oxidative stress. Herbal medicines detoxify these ROS via their antioxidant capacities [10,11]. Moreover, it has been found a linear association between ROS absorbance capacity and the total phenolic, flavonoid and flavone contents in the medicinal plants [10,12,13]. Curcumin, a yellow pigment from Curcuma longa, is a major component of turmeric and commonly used as a spice and food coloring agent [12]. It also used in cosmetic manufacturers, and in some medical preparations. The desirable preventive or putative therapeutic properties of Curcumin are mainly due to its antioxidant efficiency [14,15]. Gallic acid, known as 3, 4, 5-trihydroxybenzoic acid, is present widely in different plant species including gallnuts, grapes, sumac, witch hazel, tea leaves, hops, and oak bark [12]. Gallic acid is existed as a free molecule and/or as part of tannins [16]. Recently, Curcumin showed higher antioxidant activity than ascorbic acid and xanthone but less than Gallic acid on the free radical scavenging action [17]. These findings suggested that Curcumin-Gallic acid combination was the potential antioxidant mixture that could be used in place of the individual substance, whereas using of Curcumin in combination with ascorbic acid or xanthone should be avoid [17].

In recent years, consumption of fresh fruits and vegetables had found to reduce the incidence of some diseases due to the presence of flavonoids, anthocyanin and phenolic compounds [18]. In addition, antioxidant enzymes (CAT, SOD, GPx, GR and GST) are playing a critical role in the protection of myocardium against oxidative stress caused by different toxic compounds [19]. Oxidative stress increased ROS levels that consequently inhibited the activities of antioxidant enzymes [10]. In addition, inhibition of SOD activity was associated with the accumulation of superoxide ions that consequently inactivated GPx and increased H$_2$O$_2$ level [10,11,20,21]. Following cell membrane structural damage due to the presence of excessive amounts of ROS, the membrane permeability increased and some of the intracellular enzymes like Ck-MB, CPK, and LDH leaked from the cell [22,23].

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Tramadol causes oxidative stress via different mechanisms [24]. Therefore, the present study showed the changes in antioxidant enzymes activities, protein expression of different CYP isoymes (2E1, 3A4 and 2B1/2), as well as the biomarkers of liver and kidney functions after pretreatment of rats with Curcumin, Gallic acid and/or their combination before administration of tramadol.
2. Materials and methods

2.1 Chemicals

Tramadol hydrochloride purchased from Medis Company (Tunisie). Gallic acid and Curcumin crystalline manufactured by Euromedex (France), Lobachemie (India), respectively. All other chemicals purchased from Sigma Chemical Company (Saint Louis, USA). Western blotting detection kit, primary anti-rabbit antibodies for CYP 2E1, 3A4, 2B1/2 and secondary antibody anti-rabbit-HRP were obtained from ABCAM® pharmaceuticals, UK.

2.2 Experimental design

One hundred male Sprague–Dawely rats were obtained from the Animal House, Faculty of Medicine, Alexandria University, Egypt. The committee of Postgraduate Studies & Research, IGSR, Alexandria University, approved the design of the experiments, and the protocol conforms to the guidelines of the National Institutes of Health (NIH). The weights of rats were 220±30 g with an average age of 75±5 days. Rats housed in a stainless steel wire bottom cages and placed in a well-ventilated animal house, maintained for two weeks for acclimatization period on food and water ad libitum, and subjected to the natural photoperiod of 12 hours light: dark cycle. After the period of acclimation, Table 1 summarized the protocol of oral administration of tramadol and antioxidants to rats. The control animals received normal saline as a vehicle. The number of animals in each treatment was ten rats. In the single-dose experiment, tramadol administered as a single dose after administration of Curcumin and/or Gallic acid by two hours (Table 1). In the repeated-dose experiment, tramadol administered after two hours of Curcumin and/or Gallic acid administration, and such treatments have continued for 30 consecutive days. Tramadol (1.5 mg/kg), Curcumin (50 mg/kg), and Gallic acid (30 mg/kg) doses selected and administered to rats according to the previous reports [25–27]. At the end of the experimental period, rats anesthetized with diethyl ether and sacrificed by cervical dislocation. The liver and kidney tissues removed, washed, and cleaned. In addition, fasting blood samples collected in heparinized tubes, and plasma obtained after centrifugation at 1,000 xg for 20 min was stored at −80˚C until used.

2.3 Enzyme assays

Activities of both alanine transaminase (ALT) and aspartate transaminase (AST) were assayed in the plasma according to the method of Reitman and Frankel (1957) [28], gamma-glutamyl-transferase (GGT) was assayed according to the method of Szasz and Persijn (1974) [29].

Table 1. Protocol of antioxidants administration as single- and repeated-doses treatments before administration of tramadol to male rats.

| Treatments | Single-dose Treatments * | Repeated-dose Treatments (30 days)b |
|------------|--------------------------|----------------------------------|
|            | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| Saline     | 0.2 ml | 0.2 ml | 0.2 ml | 0.2 ml | 0.2 ml | 0.2 ml | 0.2 ml |
| Tramadol   | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg | 1.5 mg/kg |
| Curcumin   | 50 mg/kg | 50 mg/kg | 50 mg/kg | 50 mg/kg | 50 mg/kg | 50 mg/kg | 50 mg/kg | 50 mg/kg | 50 mg/kg |
| Gallic acid| 30 mg/kg | 30 mg/kg | 30 mg/kg | 30 mg/kg | 30 mg/kg | 30 mg/kg | 30 mg/kg | 30 mg/kg | 30 mg/kg |

a- In single-dose treatment, groups 1, 2 received normal saline and tramadol, respectively, whereas 3, 4, 5 received Curcumin, Gallic acid and/or their mixture for two hours before administration of tramadol as a single dose.  
b- In repeated dose treatment, groups 1,2 received normal saline and tramadol, respectively, whereas 3,4,5 received Curcumin, Gallic acid and/or their mixture for two hours before administration of tramadol as a repeated doses for 30 consecutive days.  
c- The number of animals in each treatment was 10 rats.

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concentration was determined according to the method of Batton and Coruch (1977) [30], and creatinine level was determined according to the method of Bowers and Wong (1980) [31]. Creatine kinase-MB (CK-MB) was assayed according to the method of IFCC (1989) [32]. Total cholesterol concentration was determined according to the method of Allain et al. (1974) [33]. High-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) concentration were determined according to the methods of Assmann et al., 1984 [34].

Livers and kidney tissues homogenized in three volumes of 0.1 M phosphate buffer, pH 7.4, and centrifuged at 11,000 xg for 20 min at 4˚C. The supernatant fractions of livers divided into two unequal volumes. The small proportion used for assaying of superoxide dismutase (SOD) activity (EC 1.15.1.1) according to the method of Misra and Fridovich, 1972 [35]. The assay of SOD based on the inhibition of epinephrine autoxidation in an alkaline medium to adrenochrome, which markedly inhibited in the presence of SOD. Two tubes for each sample were used. The first tube contained 20 μl of S9 fraction and 960 μl sodium carbonate buffer (0.05 M sodium carbonate, PH 10.2, 0.1 mM EDTA). The second tube contained all reagents of the first tube except 20 μl dH2O was added instead of the S9 fraction. The reaction initiated by addition of 20 μl of 30 mM epinephrine to both tubes. The increase in absorbance of adrenochrome measured spectrophotometrically at 480 nm every 30 sec for up to 4 minutes. The activity of superoxide dismutase enzyme expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%, each 50% inhibition equal to one unit (1 unit/g tissue).

Glutathione peroxidase enzyme activity (GPx; EC 1.1.1.9) was assayed according to the method of Chiu et al. (1976) [36] with some modifications. GPx catalyzes the oxidation of reduced glutathione using cumene hydroperoxide as a substrate. The total reaction volume was 1ml and contained 50μl of the S9 fraction, 0.75 ml of 0.05 M Tris-HCl buffer (pH 7.6), 0.1 ml of 1.5 mM GSH, and 10μl cumene hydroperoxide mixed and incubated for 5 min at 37˚C. In another tube contained the same mixture except for cumene hydroperoxide, the control sample incubated for 5 min at 37˚C. For both control and sample tubes, 1.0 ml of TCA (15%) was added while 10 μl cumene hydroperoxide was added to the control only. Both tubes were incubated for 10 min at 37˚C and centrifuged at 3000 xg for 20 min. Two ml Tris-HCl buffer (pH 8.9) and 0.1 ml 1.5 mM of 5,5'-dithio-bis-2-(nitrobenzoic acid) were added to 1 ml of the supernatant of both sample and control tubes. The optical density of 5-thio-2-nitrobenzoic acid was measured at 412 nm within 5 min. Results were expressed as U/mg protein. A unit of enzyme activity defined as the amount of enzyme that catalyzes the formation of one nmole of GSH conjugate/ mg protein/minute under the assay conditions.

Catalase (CAT; EC 1.11.1.6) was assayed according to the method of Beers and Sizer 1952 [37], with some modifications. The principle of the assay was based on the degradation of H2O2, which measured spectrophotometrically at 240 nm. The reaction mixture contained 2.5 ml H2O2 buffer (0.15M sodium-potassium phosphate buffer pH 7.0) and 50 μl of S9 as the enzyme source. The absorbance was determined spectrophotometrically at 240 nm after 20 and 40 sec intervals against blank. The decrease in absorbance of hydrogen peroxide recorded to determine the activity catalase enzyme in terms of μmoles H2O2/mg protein/min.

Reduced glutathione level estimated in the supernatant of liver tissue homogenate using sulfosalicylic acid for protein precipitation and bis-(3-caboxy-4-nitrophenyl)-disulfide for color development [38]. Glutathione reductase activity assayed by monitoring the oxidation of NADPH at 340 nm using the method of James et al., 1980 [39]. A unit of enzyme activity represents 1 nmole of NADPH oxidized/min/mg protein. GST activity was determined according to the method of Lee et al., 1981 [40]. The conjugate of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 340 nm using a double beam spectrophotometer. A unit of enzyme activity is defined as the amount of enzyme that catalyzed the formation of 1 mmole of CDNB conjugate/mg protein/min under the assay conditions. The molar extinction coefficient of 9.6
mM$^{-1}$ cm$^{-1}$ used for calculation of GST activity. The hepatic lipid peroxidation product, malondialdehyde (MDA), measured as thiobarbituric acid reactive substance (TBARS), according to the method of Tappel and Zalkin, (1959) [41]. The color intensity of the reactants (MDA) measured at 532 nm. An extinction coefficient of 156,000 M$^{-1}$cm$^{-1}$ used for the calculation. Protein concentration was measured according to the method of Lowry et al. (1951) [42] using bovine serum albumin as standard.

The larger proportion of liver supernatant was centrifuged at 105,000 xg for 1 h at 4˚C to yield a microsomal pellet, which was then resuspended in 0.1 M phosphate buffer, pH 7.4. Total microsomal CYP and cytochrome b$_5$ contents were determined according to the method of Omura and Sato, 1964 [43]. The molar extinction coefficient 91 and 185 mM$^{-1}$ Cm$^{-1}$ for the reduced CYP-CO complex and reduced cytochrome b$_5$ respectively used for calculation of such contents.

Microsomal NDMA-dl activity was determined according to the method of Venkatesan et al. 1968 [44]. The substrate concentration was 4 mM DMN, which represents the saturation level for DMN-dl. The amount of formaldehyde formed was determined. The enzymatic activity of DMN-dl expressed as nmole of formaldehyde per mg protein per hour. Microsomal aryl hydrocarbon hydroxylase (AHH) activity was determined according to the method of Wiebel and Gelboin, 1975 [45]. Briefly, the volume of the incubation mixture was 1ml containing 50 mM Tris-HCl buffer, pH 7.4; 3 μmole MgCl$_2$; 0.6 μmole NADPH; 100 nmole benzo(a)pyrene; 0.1ml of microsomal protein (10 mg/ml). The reaction mixture incubated at 37˚C for 10 min. 1 ml acetone added to stop the reaction. The 3-hydroxy benzo(a)pyrene was extracted with 2 ml hexane. The fluorescence intensity measured at excitation and emission wavelengths of 396 and 522 nm respectively. Ethoxycoumarin hydroxylase activity assayed according to the method of Greenle & Poland (1978) [46]. The intensity of 7-hydroxycoumarin fluorescence measured at excitation and emission wavelengths of 338 and 458 nm respectively. Ethoxyresorufin O-deethylase activity was determined according to the method of Greenlee, and Poland, 1978 [46].

### 2.4 Western immunoblotting

From the pooled sample of each treatment, 40 μg of the microsomal fraction of liver microsomal proteins were prepared and subjected to 10% SDS-polyacrylamide gel electrophoresis. Proteins were transblotted to Hybond-C nitrocellulose membranes (Amersham, UK). The nonspecific binding sites of the membranes blocked with 5% Bovine Serum Albumin (BSA) for 1 h at 37˚C. The membranes were then incubated overnight using anti-rabbit primary antibody (anti-CYP 2E1, CYP 3A4, CYP 2B1/2) using the dilution of 1:1,000 in 20 ml TBS at room temperature, followed by 2 hours incubation period with anti-rabbit (secondary antibody-HRP) using dilution of 1:15,000 in 20 ml TBS at room temperature. Signals of CYP 2E1, 3A4, and 2B1/2 were visualized after binding with their specific monoclonal antibodies. Chemiluminescence signals of the protein bands had detected according to the manufacturer’s instructions (Abcam, UK) (Matsudaira, 1987) [47]. The band intensity was measured using quantity one software program (version 4.6.9, Bio-Rad Co., California, USA).

### 2.5 Histopathological examination

Small proportions of both liver and kidney tissues from each treatment were fixed in 10% formaldehyde solution, embedded in paraffin wax, and cut with the microtome for a section with 5 μm thickness. These sections stained with Hematoxylin and Eosin (H&E) stains, and microscopically studied to evaluate the morphological changes [48].
2.6 Statistical analysis

All data presented as means ± standard errors. Mean of different treatment groups tested for significance using one-way analysis of variance (ANOVA), and were compared using posthoc Duncan’s Multiple Range Test (DMRT). Difference were considered significant at P<0.05. SPSS 17 statistical software package used in the statistical analyses.

3. Results

The changes in activities of ALT, AST, LDH, gamma-GT, CK-MB, and levels of creatinine, urea, and cholesterol, TG, HDL and LDL were insignificant in all treated groups after a single-dose treatment of rats with tramadol (Table 2). However, repeated dose treatment of rats with tramadol increased the activities of ALT, AST, LDH and γ-GT compared to the control group (Table 2). Meanwhile, pretreatment of rats with Curcumin and Gallic acid prior to administration of tramadol insignificantly increased and restored ALT, AST, and LDH and γ-GT activities to their normal levels compared to the control group (Table 2). There was a significant increase in creatinine and urea concentration in tramadol treated group compared to the control group (Table 2). On the other hand, pretreatment of rats with Curcumin and Gallic acid prior to tramadol administration significantly decreased creatinine concentration compared to the tramadol-treated group (Table 2). Total cholesterol, triglycerides, and LDL levels decreased significantly in tramadol-treated group compared to the control group. However, pretreatment of rats with Curcumin and Gallic acid prior to tramadol administration significantly decreased CK-MB compared to the tramadol-treated group (Table 2).

The activities of phase 1 drug-metabolizing enzyme did not change significantly after single-dose treatments in all treated groups (Table 3). However, repeated-dose of tramadol showed a significant increase in DMN-dI activity compared to the control group. However, pretreatment of rats with Curcumin and/or Gallic acid prior to administration of tramadol significantly decreased DMN-dI and AHH activity compared to the control group (Table 3). The results of DMN-dI activity confirmed by data of western blotting of CYP 2E1.

Table 2. Effect of single- and repeated-dose treatments of male rats with Curcumin, Gallic acid or their combination before administration of tramadol on lipid profiles, and biomarkers of liver and kidney functions.

| Parameters                  | Single dose treatment | Repeated doses treatment |
|-----------------------------|-----------------------|--------------------------|
|                             | Control               | Tramadol                 | Tramadol+ Curcumin | Tramadol+ Gallic acid | Tramadol+ Curcumin+ Gallic acid | Control | Tramadol | Tramadol+ Curcumin | Tramadol+ Gallic acid | Tramadol+ Curcumin+ Gallic acid |
| ALT (IU/L)                  | 32.97±3.32*           | 34.82±5.03*              | 26.73±2.57*         | 32.72±2.30*          | 28.77±2.77*                   | 35.22±0.94* | 51.21±1.94* | 37.02±1.68*       | 37.11±2.50*          | 35.73±0.97*               |
| AST (IU/L)                  | 117.46±7.9*           | 99.80±8.21*              | 102.46±3.34*        | 108.91±2.96*         | 105.91±3.09*                  | 100.77±5.50* | 134.63±6.13* | 102.96±2.89*      | 112.43±8.22*         | 99.97±0.73*              |
| LDH (IU/L)                  | 482.07±37.5*          | 443.59±35.9*             | 498.01±50.4*        | 465.59±55.0*         | 432.59±46.3*                  | 610.32±37.0* | 1663.15±118.3* | 912.03±124.3*     | 852.34±65.6*         | 728.50±56.3*             |
| γGT (U/L)                   | 3.66±0.43*            | 3.39±0.87*               | 2.77±0.53*          | 2.45±0.56*           | 2.48±0.46*                    | 4.49±0.88* | 18.97±13.2*    | 3.53±0.69*         | 1.98±0.30*           | 1.94±0.20*               |
| CK-MB (U/L)                 | 71.82±2.6*            | 77.53±0.03*              | 50.13±2.3*          | 50.71±3.3*           | 35.17±2.16*                   | 49.16±1.53* | 84.36±4.39*    | 68.23±7.30*        | 57.58±4.05*         | 50.40±5.11*              |
| Creatinine (mg/dl)          | 0.67±0.08*            | 0.45±0.07*               | 0.79±0.13*          | 0.53±0.11*           | 0.45±0.06*                    | 0.66±0.08* | 1.09±0.12*     | 0.87±0.12*         | 0.75±0.05*           | 0.64±0.07*              |
| Urea (mg/dl)                | 33.15±1.5*            | 35.10±1.72*              | 26.11±1.3*          | 27.67±1.6*           | 28.35±1.66*                   | 29.18±2.3* | 41.37±3.54*    | 34.82±2.40*        | 34.67±1.7*          | 34.07±1.80*              |
| Cholesterol (mg/dl)         | 182.29±15*            | 156.21±12.6*             | 119.77±7.09*        | 114.35±12.2*         | 105.06±3.10*                  | 208.71±21.1* | 169.66±13.2*  | 158.88±18*         | 117.81±11.3*       | 143.2±12.89*             |
| TG (mg/dl)                  | 96.56±11.8*           | 84.64±5.89*              | 92.24±7.22*         | 104.04±10.10*        | 91.46±8.66*                   | 107.73±11.4* | 81.60±5.77*    | 87.92±6.85*        | 78.11±3.69*         | 81.33±5.58*              |
| HDL (mg/dl)                 | 47.53±3.61*           | 48.44±3.12*              | 45.61±2.63*         | 46.57±3.93*          | 58.39±2.71*                   | 59.30±4.16* | 48.70±3.59*    | 49.14±3.99*        | 51.50±3.30*         | 47.08±2.51*              |
| LDL (mg/dl)                 | 91.89±1.33*           | 89.10±4.34*              | 93.67±4.15*         | 81.99±4.83*          | 83.61±4.37*                   | 88.41±3.73* | 59.30±1.58*    | 86.84±2.85*        | 98.91±4.25*         | 79.97±4.78*              |

abc Mean values with different superscript letters are significantly different, P<0.05; whereas means with the same superscript letter are not significantly different, P>0.05.

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expression since this isozyme is a marker for DMN-N-dl activity. Pretreatment of rats with Curcumin, Gallic or their mixture prior to administration of tramadol didn’t change the protein expression of CYP 2E1 after single dose treatments (Fig 1A). However, such treatments restored the induced protein expression of CYP2E1 caused by tramadol to its normal level (Fig 1A). On the other hand, the protein expression of CYP2B1/2 was inhibited after administration of tramadol either alone or after administration of Curcumin, Gallic or their mixture as repeated doses, whereas single-dose treatments of rats with these compounds didn’t change such expression (Fig 1B). The results of ECOD activity confirmed by data of western blotting (Fig 1B). There was no change in CYP3A4 expression compared to the control group in the single-dose treatment (Fig 1C). However, administration of tramadol as repeated dose down regulated the protein expression of CYP3A4 compared to the control group. Interestingly, pretreatment of rats with Curcumin, Gallic acid or their mixture prior to administration of tramadol restored the down-regulated protein expression of CYP3A4 to its normal level (Fig 1C).

In repeated-dose treatment, there was a significant decrease in the hepatic content of CYP450, cytochrome b$_5$, and the activities of NADPH-cytochrome c-reductase and ECOD in the tramadol-treated group compared to the control group (Table 3). However, pretreatment of rats with Curcumin and Gallic acid prior to tramadol administration significantly increased such activities compared to the tramadol-treated group (Table 3). The activities of GR, GPx, GST, catalase, and SOD in all treated groups did not change in liver and kidney of rats after single-dose treatment (Tables 4 & 5). However, in the single dose experiment of the tramadol-treated rats there was a significant increase in free radical levels, measured as TBARS in the S9 fraction compared to the control group (Table 4). However, in repeated-dose of tramadol showed a significant increase in the TBARS level in both liver and kidney compared to the control group (Tables 4 & 5). However, pretreatment of rats with Curcumin and Gallic acid prior to tramadol administration, significantly decreased TBARS level compared to tramadol group (Tables 4 & 5).

### Table 3. Effect of single- and repeated-dose treatments of male rats with Curcumin, Gallic acid or their combination before administration of tramadol on the activities of phase I-drug metabolizing enzymes in liver microsomes of male rats.

| Parameters                  | Single dose treatment | Repeated doses treatment |
|-----------------------------|-----------------------|--------------------------|
|                             | Control | Tramadol | Tramadol+ Curcumin | Tramadol+ Gallic acid | Tramadol+ Curcumin+ Gallic acid | Control | Tramadol | Tramadol+ Curcumin | Tramadol+ Gallic acid | Tramadol+ Curcumin+ Gallic acid |
| CYP P450                    | 5.3±0.376$^a$       | 5.2±0.428$^a$          | 6.9±0.904$^a$       | 6.3±0.872$^a$       | 4.3±0.427$^{ab}$          | 3.76±0.439$^{bc}$   | 5.748±0.807$^{ac}$ | 5.981±0.450$^a$       | 6.287±0.590$^a$       |
| Cytochrome b$_5$            | 5.6±0.303$^a$       | 3.8±0.454$^a$          | 3.9±0.436$^a$       | 4.6±0.477$^a$       | 4.7±0.489$^a$            | 3.7±0.416$^a$       | 2.68±0.245$^{bc}$   | 2.941±0.356$^a$       | 3.876±0.406$^a$       | 4.454±0.638$^a$       |
| Cyt-C Red.                  | 49.4±2.61$^a$       | 43.7±3.87$^a$          | 40.2±4.09$^a$       | 44.6±4.92$^a$       | 35.7±6.13$^a$            | 54.4±7.24$^a$       | 29.54±2.85$^a$      | 52.04±5.66$^a$        | 58.92±8.5$^a$         | 63.15±4.37$^a$        |
| DMN-N-dl                    | 33.4±3.52$^a$       | 32.1±2.07$^a$          | 32.9±2.94$^a$       | 33.6±2.83$^a$       | 33.6±3.43$^a$            | 31.8±2.98$^a$       | 60.28±4.93$^a$      | 15.09±1.12$^a$        | 18.58±2.28$^a$        | 19.26±1.24$^a$        |
| AHH activity                | 678.7±45.$^a$       | 611.8±41.5$^a$         | 624.4±59.1$^a$      | 660.9±70.77$^a$     | 613.0±36.27$^a$          | 692.0±29.59$^b$     | 875.0±37.56$^a$     | 540.61±56.38$^c$      | 589.49±40.33$^{bc}$   | 581.19±25.76$^{bc}$   |
| ECOD activity               | 1.15±0.02$^a$       | 1.07±0.16$^a$          | 1.0±0.12$^a$        | 1.15±0.14$^a$       | 1.0±0.22$^a$             | 1.17±0.184$^a$      | 0.457±0.05$^b$      | 0.553±0.06$^b$        | 0.591±0.057$^b$       | 0.398±0.055$^b$       |

CYP P450 (nmole CYP450/mg protein); cytochrome b$_5$ (nmole CYP b$_5$/mg protein); NADPH-cytochrome c-reductase (nmole Cyt.c reduced/mg protein/min); DMN-N-dl (nmole HCHO/mg protein/min); AHH (pmole 3 OH B(a)P/mg protein/min); ECOD (nmole OH-coumarin/mg protein/min).

$^a,b,c$ Mean values with different superscript letters are significantly different, $P<0.05$; whereas means with the same superscript letter are not significantly different, $P>0.05$.

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Activities of GSH, GR, GPx, GST, catalase and SOD were lower in the liver and kidney of rats treated with tramadol as repeated dose compared to the control group. Whereas, pretreatment of rats with Curcumin and Gallic acid prior to tramadol administration, significantly

Table 4. Effect of single- and repeated-dose treatments of male rats with Curcumin, Gallic acid or their combination before administration of tramadol on the activities of phase II-drug metabolizing, antioxidant enzyme and free radical levels in supernatant of liver homogenates of male rats.

| Parameters                  | Single dose treatment                                                                 | Repeated doses treatment                                                                 |
|-----------------------------|----------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
|                             | Control                                  | Tramadol                                  | Tramadol+ Curcumin                                  | Tramadol+ Gallic acid                                  | Control                                  | Tramadol                                  | Tramadol+ Curcumin                                  | Tramadol+ Gallic acid                                  |
| TBARS                       | 3.167±0.407ab                          | 5.544±0.462ab                           | 5.5125±0.315a                                    | 4.450±0.278abc                                      | 2.627±0.265c                                    | 3.839±0.461b                                    | 5.870±0.554b                                    | 4.185±0.340b                                    | 3.713±0.260b                                    | 3.936±0.409b                                      |
| GSH                         | 1.01±0.22a                             | 0.94±0.08a                              | 1.19±0.23a                                      | 0.91±0.13a                                         | 1.16±0.13a                                      | 1.47±0.28ab                                      | 0.40±0.22a                                      | 0.80±0.10bc                                      | 0.91±0.11b                                      | 1.01±0.13ab                                      |
| GR                          | 5.77±0.92a                             | 3.98±0.30a                              | 4.27±0.36a                                      | 5.06±0.62a                                         | 5.41±0.35a                                      | 4.06±0.47bc                                      | 2.07±0.07a                                      | 2.89±0.46b                                      | 4.47±0.48b                                      | 5.93±0.51ab                                      |
| GPX                         | 21.06±2.19b                            | 22.72±1.68b                             | 21.50±2.13b                                     | 22.21±3.11ab                                       | 28.16±3.05a                                     | 16.36±1.44b                                      | 10.25±0.40a                                     | 19.85±1.82a                                     | 20.4±2.46b                                      | 23.82±2.51ab                                     |
| GST                         | 66.78±9.48ab                           | 51.44±9.5b                             | 66.01±7.05abc                                    | 82.54±5.40a                                       | 78.38±3.82a                                     | 66.75±2.47b                                      | 20.64±1.97a                                     | 52.31±7.21b                                     | 50.17±4.16b                                     | 66.67±7.89ab                                     |
| Catalase                    | 55.76±6.89a                            | 47.11±0.08ab                           | 48.91±4.08a                                      | 53.96±5.05a                                        | 45.64±1.13a                                     | 72.81±2.42a                                      | 37.52±1.39b                                     | 60.13±7.41a                                     | 62.80±4.93b                                     | 74.67±3.81ab                                     |
| SOD                         | 2.33±0.29a                             | 2.49±0.41a                             | 1.79±0.30a                                      | 1.68±0.23a                                         | 2.60±0.23a                                      | 4.27±0.27ab                                      | 0.61±0.04a                                      | 2.71±0.39b                                      | 3±0.44b                                         | 3.54±0.36ab                                      |

TBARS (μmole/ TBARS/g tissue); GSH (μmole GSH/g tissue); GR (μmole NADPH oxidized/mg protein/min); GPx (U/mL); GST (μmole CDNB conjugate/mg protein/min); Catalase (μmole H₂O₂/mg protein/min); SOD (U/mg protein).

abc Mean values with different superscript letters are significantly different, P<0.05; whereas means with the same superscript letter are not significantly different, P>0.05

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Fig 1. Western blotting data showed changed in protein expression of CYP 2E1, Cyp2B1/2, and CYP 3A4. In all Fig 1A, 1B and 1C, Lane 1 represents the microsomal proteins of matched control group. Followed by represented pooled protein samples of repeated dose treatments of rats with tramadol (lane 2), tramadol+ Curcumin (lane 3), tramadol+ Gallic acid (lane 4), tramadol + Curcumin+ Gallic acid (lane 5) respectively. The last four lanes on the right hand represented the pooled protein samples of single dose treatment of rats with tramadol, tramadol + Curcumin, tramadol + Gallic acid, tramadol + Curcumin + Gallic acid respectively. The probability values P<0.05; P<0.01; P<0.001 represented by one, two and three stars respectively are significantly different from those of the control groups.

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Table 5. Effect of single- and repeated-dose treatments of male rats with Curcumin, Gallic acid or their combination before administration of tramadol on the activities of phase II-drug metabolizing, antioxidant enzyme and free radical levels in the supernatant of kidney homogenates of male rats.

| Parameters       | Single dose treatment | Repeated doses treatment |
|------------------|-----------------------|--------------------------|
|                  | Control | Tramadol | Tramadol+ Curcumin | Tramadol+ Gallic acid | Tramadol+ Curcumin+ Gallic acid | Control | Tramadol | Tramadol+ Curcumin | Tramadol+ Gallic acid | Tramadol+ Curcumin+ Gallic acid |
| TBARS (μmole/ TBARS/g tissue) | 4.33±0.45a | 4.11±0.38a | 3.33±0.34a | 3.42±0.30a | 4.07±0.25a | 5.45±0.37b | 7.83±0.95a | 4.65±0.22b | 4.29±0.20b | 5.45±0.37b |
| GSH (μmole g tissue) | 0.45±0.07a | 0.38±0.02a | 0.44±0.05a | 0.47±0.07a | 0.42±0.03a | 0.67±0.05ab | 0.44±0.05c | 0.61±0.07b | 0.62±0.07b | 0.83±0.03b |
| GST (μmole CDNB conjugate/mg protein/min) | 8.560±2.605a | 10.64±2.28a | 9.331±1.482a | 8.620±1.114a | 11.035±1.633a | 20.159±2.365a | 10.731±1.088b | 24.721±5.055b | 28.789±4.071b | 23.976±4.406a |
| Catalase (U/mL) | 64.47±2.39a | 41.37±3.54a | 34.82±2.40b | 34.67±1.72b | 34.07±1.80b | 81.93±2.56a | 36.13±5.07c | 47.69±2.49b | 56.75±5.17b | 54.59±4.23b |
| SOD (U/mg protein) | 0.146±0.22a | 0.184±0.269a | 0.153±0.0231a | 0.115±0.038a | 0.183±0.071a | 0.328±0.025b | 0.169±0.0107c | 0.409±0.0299c | 0.4365±0.100a | 0.409±0.0305a |

TBARS (μmole/ TBARS/g tissue); GSH (μmole g tissue); GR(μmole NADPH oxidized/mg protein/min); GPx (U/mL); GST(μmole CDNB conjugate/mg protein/ min); Catalase (μmole H2O2/mg protein/min); SOD (U/mg protein).

abcd Mean values with different superscript letters are significantly different, P<0.05; whereas means with the same superscript letter are not significantly different, P>0.05.

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Enhancement of scavenging ability of antioxidant defense system [49,50]. Inhibition of endogenous antioxidant defense system and increased oxidant levels increased the kidney damage [49]. The present study could provide a new evidence for the kidney damage since urea and creatinine levels markedly increased in tramadol-treated rats. In addition, the histopathological study of kidney tissues showed renal tubular vacuolization, mononuclear cell infiltration, and focal necrosis after treatment of rats with tramadol. However, pretreatment of rats with...
Curcumin and/or Gallic acid or their combination as repeated-dose prior to tramadol admin-
istration ameliorated changes in the kidney structures, restored urea and creatinine levels, and
increased SOD, CAT and GPX activities. In addition, lipid peroxidation decreased significantly
in the plasma and kidney tissues of rats pretreated with Curcumin or Gallic acid or their com-
bination before administration of tramadol as repeated dose. The finding of the present study
is in accordance with the finding of the previous studies [51,52]. Gallic acid decreased the level
of TBARS and increased the level of GSH and activities of GST and GPx in rats [53, 54]. In the
present study, administration of tramadol as repeated dose inhibited the activities of antioxi-
dant enzymes. The mechanism of inhibition of antioxidant enzymes (SOD, GPx) might be due
to the binding of tramadol or its metabolites with transition metals that act as cofactors for
antioxidant enzymes [55].

The current study also showed that levels of total cholesterol, triglycerides, HDL and LDL
decreased after administration of tramadol as repeated-doses for 30 consecutive days, which are
in agreement with the finding of El-Gaafarawi (2006) [56]. Lipid peroxidation of cell mem-
branes lead to loss of membrane fluidity increased the membrane permeability, which lead to
leakage of ALT, AST, and γGT enzymes from the liver cells into the plasma. In the present
study, the liver function markers (ALT, AST, γGT) increased in the plasma of tramadol-treated
rats. Elevation in activities of the hepatic marker enzymes (ALT, AST, and LDH) in tramadol-
treated rats might be due to enhancement of lipid peroxidation in the liver tissues that subse-
quently increased the leakage of these biomarkers into the plasma [56]. The histopathological
changes (hydropic degeneration of hepatocytes, hepatic congestion, hemorrhage, loss of archi-
tecture, and necrosis) supported the changes in the activities of liver biomarker enzymes after
treatment of rats with tramadol [51]. These changes restored to their normal levels after pre-
treatment of rats with Gallic acid or Curcumin and/or their mixture prior to administration of
tramadol, which are in agreement with the finding of the previous study [57]. Supporting our
finding, it has found that Gallic acid and Curcumin decreased the activity of both ALT, and
AST in sodium fluoride- and lipopolysaccharide/D-galactosamine-treated rats respectively [58].

Cytochrome P450 (CYP) is a multi-enzyme complex and plays a great role in the metabo-
lism of chemical carcinogens as well as endogenous compounds. Therefore, the deleterious
effects of chemical carcinogens (N-nitrosamines) are correlated with the alterations in the
activity of hepatic microsomal DMN-dI as well as with the changes in the protein expression
of CYP 2E1 [59–61]. Therefore, a balance in CYP450 activity is important in cancer prevention
through increasing the degree of cellular safety [60]. In the present study, DMN-dI activity
and the expression of CYP 2E1 markedly induced in the liver microsomes after administration
of tramadol, whereas such activity and 2E1 expression were decreased after pretreatment of
rats with Curcumin and/or Gallic acid as repeated doses before administration of tramadol.
Fig 3. A and B are photomicrographs of kidney sections of the control group that showed normal renal tissues of glomeruli and tubules (H&E 200). Fig 3C is the photomicrograph of kidney section of tramadol-treated group after single dose treatment and showed mild tubular vacuolation (pink arrow) (H&E 400). Fig 3D is the photomicrograph of kidney section of tramadol-treated group after repeated dose that showed moderate tubular vacuolation (yellow arrow), mesangial expansion (yellow star), and cloudy swelling (pink arrow) (H&E 400). Fig 3E is the photomicrograph of kidney section of tramadol + Curcumin after single dose treatment that showed mild tubular vacuolation (orang arrow) (H&E 200). Fig 3F is the photomicrograph of kidney section of tramadol + Curcumin after repeated-dose treatment that showed mild tubular vacuolation (black arrow) (H&E 200). Fig 3G is the photomicrograph of kidney section of tramadol + Gallic acid-treated group that showed mild tubular vacuolation (orange arrow), mesangial expansion (black star) and cloudy swelling (red arrow) after single dose treatments (H&E 200). Fig 3H is the photomicrograph of kidney section of tramadol + Gallic acid-treated group that showed mild tubular vacuolation (pink arrow), mesangial expansion (black star) and cloudy swelling (red arrow) after single dose treatments (H&E 200). Fig 3I is the photomicrograph of kidney section of tramadol + Curcumin + Gallic acid-treated group that showed mild tubular vacuolation (orange arrow) after repeated-dose treatment (H&E 200). Fig 3J is the photomicrograph of kidney section of tramadol + Curcumin + Gallic acid-treated group that showed mild tubular vacuolation (orange arrow) after repeated-dose treatment (H&E 200).

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Induction of DMN-d1 activity could lead to an increase in the production of reactive alkylating agents that could bind with DNA and other macromolecules [60]. Therefore, the deleterious effects of N-nitrosamines might be increased in the liver and probably other organs after long-term use of tramadol treatment since N-nitrosamines are already present in tobacco smoke and other environmental sources [62,63]. In accordance with this finding, it had found that inhibition of DMN-d1 was effective in decreasing the tumorigenicity that induced in rodents by N-nitrosamines [64]. Meanwhile, repeated-dose treatment of rats with tramadol had found to down-regulate the CYP3A4 expression compared to the control group. Inhibition of CYP3A4 expression could lead to enhancement of the deleterious effects of carcinogens through accumulation of their toxic metabolites. In accordance with this finding, administration of Curcumin as repeated doses enhanced the bioavailability of tamoxifen that was mainly due to inhibition of the CYP3A4-mediated metabolism of tamoxifen [65]. However, the expression of CYP3A4 in rats was limited after administration of Curcumin as a single oral dose [66].

The rate-limiting step in the activation and detoxification of chemical carcinogens is dependent on the rate of reduction of cytochrome P450-substrate complex, which in turn is dependent on the activation and turnover rates of NADPH-cytochrome c reductase, cytochrome b5, and on the total cytochrome P450 content [67]. In the present study, the hepatic activity of NADPH-cytochrome c reductase, cytochrome b5, and cytochrome P450 content potentially decreased after repeated-dose treatment of rats with tramadol. However, administration of Curcumin and Gallic acid as repeated dose prior to tramadol administration increased the hepatic content of cytochrome b5, the total cytochrome P450, and NADPH-cytochrome c reductase activity. On the other hand, the hepatic activity of AHH potentially induced after repeated-dose treatment of rats with tramadol, whereas such activity decreased after Curcumin and/or Gallic acid treatment as repeated doses.

Curcumin plays a role in cancer chemoprevention and chemotherapy in patients, which might be due to inhibition of several cell-signaling pathways at multiple levels. Curcumin inhibited the DNA-alkylation reaction and carcinogen-DNA adducts catalyzed by CYP1A1, 1A2 A in rats [68]. Moreover, Curcumin has shown its effect in hampering CYP1A1 activity in DMBA-treated cells, and also inhibited the metabolic activation of DMBA and decreased the DMBA-induced cytotoxicity [69,70]. Moreover, the nuclear levels of aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) decreased after administration of Curcumin to mice [71]. Therefore, Curcumin might be responsible for the prevention of malignant transformation via inhibition of AhR and ARNT [72].
In Phase II reactions, the conjugation of xenobiotic with small hydrophilic endogenous substances increases the solubility and facilitates their excretions. Moreover, the critical and the significant strategy for cancer treatment and suppression could be due to activation of glutathione S-transferase activity [69]. Several earlier studies reported that turmeric/Curcumin played a significant role in the prevention of cancer via activation of the protein and the gene expression of glutathione S-transferase [72,73]. Moreover, Curcumin induced antioxidant response via GST expression through induction of signaling of the nuclear erythroid-derived 2-related factor 2 (NRF-2) and NF-B [74].

Creatine kinase- (CK), creatine kinase-MB (CK-MB) and LDH play a major role in the differential diagnosis and in monitoring of myocardial infarction patients [75]. In the present study, there was a significant increase in CK-MB and LDH activity in the tramadol-treated group compared to the control group in repeated-dose treatment. However, pretreatment of rats with Curcumin and Gallic acid prior to tramadol administration significantly decreased CK-MB and LDH compared to the tramadol-treated group. Recently, some strategies have proposed to reduce the incidence of myocardial infarction through reduction of oxidative stress, which mediated by eating of fresh vegetables and fruits. It has been found that flavonoids, anthocyanin and polyphenol compounds are the major constituents of vegetables and fruits, which possessed antioxidant capacity [76].

Increasing of ROS production and opening of the mitochondrial permeability transition pore (mPTP) were mainly due to the imbalance between pro-oxidants (free radicals) and antioxidant capacity [75]. Therefore, increment in LDH, creatine-phosphokinase-myocardial (CPK-MB) and creatine kinase (CK) activities could be due to induction ROS levels and inhibition of antioxidant enzymes activities, which resulted from tramadol treatment [77]. On the other hand, combined pretreatment of rats with Curcumin and Gallic acid was more protective and effective than one of them, and this effect was highly significant in CK-MB and TBARs levels.

In conclusion, the data indicated that pretreatment of rats with Curcumin, Gallic acid, and/or their mixture prior to administration of tramadol alleviated the changes in the antioxidant capacity, cardiac, liver and kidney marker enzymes caused by tramadol. The protective role of Curcumin or Gallic acid against the toxicity caused by tramadol could be due to enhancement of the antioxidant defense mechanisms, probably through scavenging of ROS, and suppression of the oxidative stress. Moreover, patients whom are using tramadol for a long period recommended taking Curcumin and/or Gallic acid to alleviate such toxicities. Further study using different dose of Curcumin and Gallic acid as well as other antioxidants are highly recommended to show the maximum protection capacity against the toxicity caused by tramadol.

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