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Quercetin Prevents Methotrexate-induced Hepatotoxicity without Interfering Methotrexate Metabolizing Enzymes in Liver of Mice

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Abstract

Drug-herb interactions are of great concern that may lead to increased side effects and decreased efficacy of the drugs. This study examined the effect of quercetin (QE) on the microsomal drug metabolizing and antioxidant systems in methotrexate (MX) treated mice, and also determined whether these substance affect the toxicity or detoxification of MX.

Administration of MX at 10 mg/kg/day, i.m. for consecutive 3 days (on 7, 8 and 9. days of experiment) impaired antioxidant mechanisms and caused substantial increases in the levels liver injury markers in serum. Pretreatment with QE (50 mg/kg/day, p.o. for 10 days) inhibited MX-induced liver injury and oxidative stress by decreasing microsomal lipid peroxidation, increasing cellular glutathione content and maintaining the levels of antioxidant enzymes close to control values. MX markedly ($P<0.05$) decreased the activity of the hepatic phase I enzymes cytochrome P450 reductase, cytochrome b5 reductase, aniline hydroxylase and aldehyde oxidase. In general, administration of QE in combination with MX did not significantly ($P>0.05$) affect these enzyme activities. This is especially noteworthy in terms of aldehyde oxidase which is the key cytosolic enzyme in MX metabolism. Administration of QE along with MX significantly ($p<0.05$) increased the activity of the phase II enzymes glutathione S-transferase and DT-diaphorase relative to the MX treated group. Based on these results, the combination of QE or VA with MX therapy may represent a novel and highly effective strategy for reducing MX hepatotoxicity, without altering the outcome of MX metabolisim.

Keywords: Methotrexate; quercetin; drug metabolizing enzymes; herb-drug interactions; oxidative stress

INTRODUCTION

Drug-herb interactions are of great concern that may lead to increased side effects and decreased efficacy of the drugs[1]. The extensive parts of drug interactions arise via interaction of compounds with drug metabolizing enzymes, of which the most important are the cytochrome P450s, mixed-function mono-oxygenases and cytochrome C reductase. Most anticancer drugs undergo Phase I and/or II metabolism, induction and inhibition of these enzymes is considered an important mechanism for herb-drug interactions. All these systems are located in microsomes, each of them responsible for the degradation of one or a few substances [2].

Methotrexate, a folic acid antagonist, is a chemotherapeutic agent widely used in the treatment of some types of cancers and various inflammatory diseases such as psoriasis and rheumatoid arthritis. However, the efficacy of this agent in high doses has been associated with hepatotoxicity [3,4]. The mechanisms of MX toxicity are not yet fully understood but recent studies have shown that a decrease in the levels of cellular glutathione (GSH) and oxygen radicals are linked with the development of MX induced toxicity [3,4,5,6]. It was demonstrated that cytosolic nicotineamide adenosine diphosphate (NADP) dependent dehydrogenases are inhibited by MX, suggesting that the drug decreases the availability of NADPH in cells [7]. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of GSH, an important cytosolic antioxidant that protects cells against reactive oxygen species (ROS). Thus, the significant reduction in GSH levels induced by MX therapy leads to suppression of the antioxidant enzyme defense system, sensitizing the cells to ROS. Considering the relationship between GSH and the deleterious effects of MX, interest has focused on antioxidant compounds that can either stimulate GSH synthesis or act as direct antioxidants [8].

Quercetin (3,3',4',5-7-pentahydroxyflavone) is one of the most widely distributed flavonoids, present in fruits, vegetables, and many other dietary sources and it is is also available as a dietary supplement [9]. Due to its antioxidant, antitumor and antiinflammatory activity, QE has been studied at depth as a chemoprevention agent in several cancer models and its hepatoprotective effects have been reported [10,11,12,13]. Dihydrofolate reductase (DHFR) is the primary target enzyme for antifolate drugs because it catalyzes the reaction of 7,8-dihydrofolate and NADPH to form 5,6,7,8-tetrahydrofolate and $NADP^+$. MX is a potent inhibitor of this enzyme, causing depletion of the intracelluler tetrahydrofolic pool [14]. The glucuronide quercetin-3-xyloside is also a strong inhibitor of DHFR [15]. Also, Pirouzpanah et al. [16] demonstrated that QE reduced inhibition of aldehyde oxidase (AO), which plays a major role in the metabolism and detoxification of MX. On the basis of this synergistic relationship between MX and QE may be considered that QE would be useful in MX therapy.

To assess the interactions of the metabolism of the herb and drug, the effects of QE on the activities of phase I enzymes, such as NADPH cytochrome P450 reductase (Cyt P450R), NADH-cytochrome b5 reductase (Cyt b5R), aniline hydroxylase (AH) and phase II enzymes, such as glutathione S-transferase (GST) and DT-diaphorase (DTD) were studied in the liver of Swiss albino mice. The activity of the antioxidant enzymes [cytosolic superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)], microsomal lipid peroxidation (LP), the level of GSH, (which mitigates against MX toxicity), and AO, which is a key cytosolic enzyme in MX metabolism, were also assesed.

MATERIALS AND METHODS

Drugs and chemicals

Methotrexate was purchased from Ebewe (Unterach, Avusturya), and Quercetin and all other chemicals and reagents were purchased from Sigma-Aldrich Scientific International, Inc. (Hampton, NH, USA).

Animals and treatments

All animal handling procedures and the study design were approved by the Ethics Committee of Ondokuz Mayis University in Samsun, Turkey, via number and date of registration: 34/ HADYEK (27th Dec 2007). Six groups of adult male Swiss albino mice (eight mice/group were used. They were housed in cages under laboratory conditions (natural day/night cycle, 21±1 °C, standard food rations and tap water freely available) during the experiment. QE was administered orally by gastric gavage and MX was injected intramusculary.

Control: For the control, a total of 1 mL/kg of 0.9% NaCl solution was administered orally by intragastric incubation at the same time every day for 10 days until the mice were euthanized. **QE:** QE (50 mg/kg/day) for 10 days. **MX**: MX (10 mg/kg/day) on days 7, 8 and 9. **QE+MX:** QE (50 mg/kg/day) for 10 days and MX on days 7, 8 and 9.

Preparation of homogenates, cytosol and microsome fractions

After 10 days, the animals were euthanized by cervical dislocation, and their livers were removed, rinsed and homogenized with (B. Brown homogenizer) in 3 volumes of 0.25 M-sucrose/0.05 M Tris/ HCl buffer at pH 7.4. An aliquot of this homogenate (0.5 ml) was used for assaying reduced GSH levels. The remaining homogenate was centrifuged at 15,000 g for 30 min. The resulting supernatant was then centrifuged at 105,000 g for 60 min in a Beckman ultracentrifuge (Model L5-75B) to yield microsomal pellets. Washed microsomes were then resuspended with a hand-operated Potter Elvehjem homogenizer in 10 mM Tris–HCl buffer (pH 7.4) containing 1mM EDTA and 20% (v/v) glycerol, and fractions were immediately frozen in liquid nitrogen and stored at − 80 °C until use. The supernatant cytosolic fraction was used for the assay of GST, DTdiaphorase, AO and the antioxidant enzymes (SOD, CAT and Gpx), whereas the microsomal fraction was used for assaying Cyt P450R, Cyt b5R, AH and microsomal LP.

Microsomal and cytosolic protein were assayed by employing the method of Lowry et al. [17], using bovine serum albumin as the standard.

Biochemical assays

Measurement of liver injury markers

The levels of the liver injury markers ALT and AST in the serum were measured with an autoanalyzer (COBAS Integra 800, Roche Diagnostics, Basel, Switzerland).

Cytosolic antioxidant enzymes and glutathione levels

CAT activity in the cytosolic fraction of the liver was measured spectrophotometrically at 240 nm by calculating the rate of degradation of H_2O_2 [18]. Superoxide dismutase (EC 1.15.1.1) activity was measured by the method of Winterbourn et al. [19], with one unit of SOD activity defined as the amount of enzyme activity causing 50% inhibition of nitroblue tetrazolium (NBT) reduction. The rate of inhibition of NBT reduction by superoxide generated by the photoreduction of riboflavin was determined by measuring the absorbance at 560 nm with a T7 spectrophotometer. Glutathione peroxidase (EC 1.11.1.9) activity was measured by the coupled assay method described by Lawrance and Burk [20]. One unit of enzyme activity was defined as nanomoles of NADPH consumed per minute per milligram of protein, based on an extinction coefficient of 6.22 $mM.^{-1}$ cm^{-1}. The GSH level in the liver homogenate was determined by the method of Moron et al. [21] and based on its reaction with 2.2′-dithiobis (2-nitrobenzoic acid) to produce a compound with maximum absorption at 412 nm and results were calculated using an extinction coefficient of 13,600 $M.^{-1}cm.^{-}$

Phase I and II enzymes and microsomal lipid peroxidation assay

The level of the phase I enzyme aniline hydroxylase which indicates the activity of cytochrome P-450 2E1 was determined by measuring the formation of the end product p-aminophenol from aniline [22,23]. Specific activity was calculated from a standard curve prepared with the reaction product 4-aminophenol (one nmol of p-aminophenol in the standarad assay mixture gave a $\Delta A_{630\ nm}$ value of 0.040). The microsomal NADPH-cytochrome P450 reductase level was measured according to the method of Omura and Takesue [24] using the rate of oxidation of NADPH at 340 nm. The enzyme activity was calculated using an extinction coefficient 6.22 $mM.^{-1}$ cm^{-1}. NADH-cytochrome b5 reductase was assayed according to the method of Mihara and Sato [25] by measuring the rate of reduction of potassium ferricyanide at 420 nm by NADH. The enzyme activity was calculated by using an extinction coefficient of 1.02 $mM.^{-1}$ cm^{-1}. Benzaldehyde was assayed spectrophotometrically to determine hepatic cytosolic aldehyde oxidase activity by monitoring the decrease in absorbance at 247 nm, following the oxidation of 0.05 mM benzaldehyde (extinction coefficient, 17.54 $mM.^{-1}$ $cm.^{-1}$) to benzoic acid [26]

The activity of the phase II enzyme glutathione S-transferase was determined spectrophotometrically at 25°C, with 1-chloro-2.4-dinitrobenzene as a general substrate [27]. The specific activity of glutathione S-transferase was expressed as µmole of GSH-CDNB conjugate formed/min/mg protein by using an extinction coefficient of 9.6 $mM.^{-1}$ cm^{-1}. DT-diaphorase activity was determined by the method of Benson et al.[28] by using 2,6-dichloroindophenol (DCPIP) as the electron acceptor. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µmole of DCPIP/min. The activity was expressed as nmol DCPIP reduced/min/mg protein, using

an extinction coefficient of 21 $mM.^{-1}$ cm^{-1}. Microsomal lipid peroxidation testing was done by the method of Wright et al. [29], with results expressed as nmol MDA formed/mg protein, using a molar extinction coefficient of 1.56×10^5 $M.^{-1}$ $cm.^{-1}$

Statistical analyses

Results are expressed as mean ± SD, and all statistical comparisons were performed by using one-way analysis of variance (ANOVA), followed by Tukey's HSD multiple comparison test. The computer program (SPSS 12) was used for statistical analysis and $p < 0.05$ was regarded as statistically significant.

RESULTS

At the outset, it should be noted that none of the mice treated with MT or QE, either alone or in combination, showed signs of morbidity or mortality during the study.

The effects of QE pretreatment on the levels of the liver injury markers AST and ALT in mouse serum

As shown in Table 1, MX treated mice had significantly ($p<0.05$) increased levels of AST and ALT in serum when compared with control mice. Administration of QE (50mg/kg day) in combination with MX resulted in significantly ($p<0.05$) lower levels of AST and ALT than those of mice treated with MX alone.

The effects QE pretreatment on cytosolic antioxidant enzyme activity and GSH levels in the liver

Treatment of mice with QE did not affect SOD and CAT activity when compared to the untreated control group ($p>0.05$). However, mice treated with QE alone had significantly ($p< 0.05$) increased GPx activity when compared to the control. Significant ($p<0.05$) decreases in the activity of the enzymatic antioxidants (SOD, CAT, GPx) and in the glutathione level were observed in MX treated mice. Administration of QE with MX significantly ($p<0.05$) increased the levels of enzymatic antioxidants and glutathione in the liver when compared to the MX treated group, except for CAT activity ($P>0.05$) (Table. 2).

The effects of QE pretreatment on Phase I and II enzyme activity and microsomal lipid peroxidation level in the liver

The results of treatments on phase I and II enzymes are shown in Table 3. MX treatment resulted in significant ($p<0.05$) decreases in the activity of the hepatic phase I enzymes Cyt P450R, Cyt b5R and AH, as compared to the controls. Furthermore, QE supplementation either alone or together with MX significantly ($p<0.05$) decreased enzyme activity compared to the control group. However, the inhibition of Cyt b5R and AH activity in the MX+QE group did not significantly ($p>0.05$) differ from the MX treated group. Inhibitions were seen in phase I enzymes in MX treated animals and this inhibitions were partly ameliorated by QE ($p>0.05$). Also, Cyt P450R activity was normalized by the effects of QE ($p<0.05$) (Table 3).

AO activity significantly ($p<0.05$) decreased in all the treatment groups compared to the control group. However, the largest decrease AO activity was seen in the MX treated group (about 1.7 times). The inhibition of AO seen in MX treated animals was partly reduced by QE ($P>0.05$). There was also no significant ($p>0.05$) difference in AO activity between the MX and the QE+MX groups (Fig 1).

Table 1. The effect of QE (50 mg/kg) 10-day pretreatment on the levels of the liver injury markers aspartate and alanine transaminase (AST and ALT) in the serum of mice treated with MX (10 mg/kg, consecutive 3 days).

	ALT (U/L)	AST (U/L)
C	56.17±3.92	79.50±6.19
QE	55.33±4.5	72.35±5.63
MX	186.47±11.36 [a]	254.40±19.80 [a]
MX+QE	107.22±8.67 [b]	130.67±7.42 [b]

Values are expressed as mean ± SD for eight mice in each group. [a]Significantly different from control group, [b]significantly different from MX treated group ($p < 0.05$).

Table 2. The effect of QE (50 mg/kg) pretreatment on cytosolic antioxidant enzymes (SOD, CAT, GPx) activity and GSH level in the liver of mice treated with MX (10 mg/kg, 3 consecutive days).

	SOD	CAT	GPx	GSH
C	7.57±0.20	96.47±11.56	45.88±5.65	1.99±0.15
QE	7.83±0.53	100.44±11.78	56.61±6.99[a]	2.14±0.13
MX	5.17±0.48[a]	58.52±9.29[a]	24.72±3.21[a]	1.27±0.13[a]
MX+QE	6.84±0.23[ab]	74.87±10.83[ab]	42.71±5.26[b]	1.73±0.11[ab]

Values are expressed as mean ± SD for eight mice in each group. [a]Significantly different from control group, [b]significantly different from MX treated group ($p < 0.05$). SOD activity is expressed as 50% inhibition of nitroblue tetrazolium (NBT) reduction/min/mg of protein; CAT activity is expressed as µmole of H_2O_2 consumed/min/mg of protein; GPx activity is expressed as nmoles of NADPH consumed/minute /mg of protein; and GSH level is expressed as µmole of GSH/g tissue.

Table 3. The effect of QE (50 mg/kg) on microsomal phase I enzyme (Cyt 450R, Cyt b5R, AH) activity and cytosolic Phase II enzymes (GST and DTD) in the livesr of mice treated with MX (10 mg/kg, 3 consecutive days).

	Cyt 450R	Cyt b5R	AH	GST	DTD
C	0.15±0.01	1.83±0.17	0.03±0.00	1.24±0.24	14.26±1.37
QE	0.13±0.02[a]	1.69±0.15	0.02±0.00[a]	1.42±0.18	15.02±1.42
MX	0.11±0.02[a]	1.36±0.10[a]	0.02±0.00[a]	0.97±0.13[a]	10.66±1.58[a]
MX+QE	0.14±0.01[b]	1.46±0.16[a]	0.02±0.00[a]	1.21±0.19[b]	13.59±2.01[b]

Values are expressed as mean ± SD for eight mice in each group. [a]Significantly different from control group, [b]significantly different from MX treated group ($p < 0.05$). Cyt P450R activity is expressed as µmole of oxidized NADPH/min/mg protein; Cyt b5R activity is expressed as reduction of µmole of ferricyanide/min/mg protein; AH activity is expressed as nmol p-aminophenol/min/mg protein; GST is expressed as µmole of GSH-CDNB conjugate formed/min/mg protein, and DTD is expressed as nmol of 2.6-dichloroindophenol reduced/ min/mg protein.

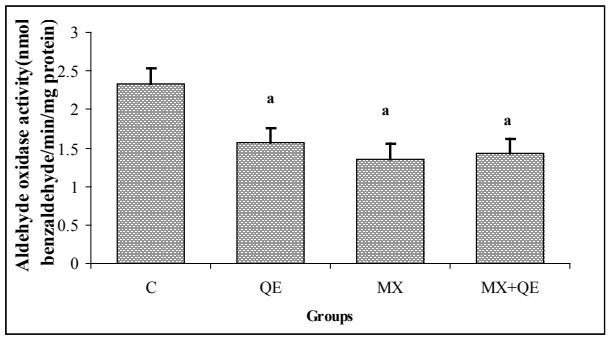

Fig.1. The effects of QE (50 mg/kg) on cytosolic aldehyde oxidase activity in the liver of mice treated with MX (10 mg/kg, 3 consecutive days). Values are expressed as mean ± SD for the eight mice in each group. [a]Significantly different from control group, [b]significantly different from MX treated group ($p < 0.05$). Aldehyde oxidase activity is expressed as nmole of benzaldehyde/min/mg protein.

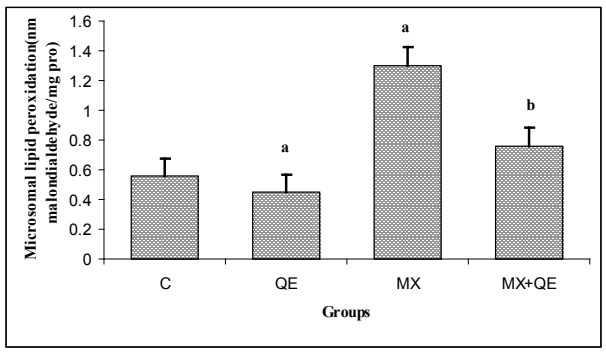

Fig. 2. The effects of QE (50 mg/kg) on MX (10 mg/kg, consecutive 3 days) induced hepatotoxicity in mice. Values are expressed as mean ± SD for eight mice in each group. [a]Significantly different from control group, [b]significantly different from MX treated group ($p < 0.05$). (Microsomal lipid peroxidation is expressed as nmole of malondialdehyde/ mg protein).

MX significantly ($p<0.05$) decreased the activity of GST and DTD but QE combined with MX significantly ($p<0.05$) normalised these enzyme activities. Although not statistically significant ($p>0.05$) GST and DTD activities increased after QE treatments. (Table 3). In the mice exposed to 10 mg/kg/day of MX for 3 consecutive days, a progressive increase in liver microsomal LP that reached more than 2.4 times the control value was observed. This rise was completely amolierated by QE ($p<0.05$) (Fig 2).

DISCUSSION

Administration of QE significantly reduced the levels of hepatic injury marker enzymes in the serum of MX treated mice, which infers that MX induced alterations in the hepatocellular membrane may have been minimized through the membrane stabilizing effects of QE. Previous investigations have shown that QE mitigates the effects of MX and hence the appearance of liver injury markers in serum [10-13].

In the present study, administration the dose of MX of 10 mg/kg/day for 3 consecutive days significantly increased microsomal LP and decreased GSH and cytosolic antioxidant enzyme activity. The decrease in antioxidant enzyme activity and GSH level could have been a response to toxic MX treatment and an adaptation to cope with oxidative stress in the liver. Similar results were previously reported [4,6]. GSH is a low molecular weight thiol antioxidant and a cosubstrate for a variety of antioxidants and xenobiotic enzymes of the phase II pathway of the CYP450 cycle. A decline in the constitutive GSH level adversely affects cellular thiol-redox balance and potentially makes the cells susceptible to a number of internal and environmental stresses [30]. The decreased activity of GPx in the MX treated mice is due to its depletion of GSH which is the substrate for GPx. Due to the diminished activity of GPx, accumulation of H_2O_2 takes place, leading to inhibition of other antioxidant enzymes Sinet and Garba [31]. This phenomenon leads to an increase in the steady state level of oxidants and severe oxidative stress due to accumulation of free radicals in the liver.

Among QE's many biological properties is its potent antioxidant activity [32] that may be due to (i) its high diffusion rate into membranes, allowing it to scavenge oxy-radicals at sites throughout the lipid bilayer, and (ii) its pentahydroxyflavone structure, allowing it to chelate metal ions via the ortho-dihydroxyphenolic structure, thereby scavenging lipid alkoxyl and peroxyl radicals [33]. The current study demonstrated in vivo protective effects of QE as a result of increased antioxidant enzyme levels and GSH level and decreased microsomal lipid peroxidation in mice exposed to MX, which may be attributable to their anti-radical and antioxidant effects. These data are in accordance with the findings of recent studies which demonstrated the hepatoprotective effects of and QE [10-13]. Owing to its hepatoprotective ability, QE has clinical potential and may be used to reduce MX hepatotoxicity.

In this study, MX also caused significant alterations in the activity of some drug metabolising enzymes. The high dose of MX (10 mg/kg/day for 3 consecutive days) significantly decreased phase I enzymes (Cyt P450R, Cyt b5R and AH) activity. Lukienko et. al [34] reported that low doses of MX of 0.25 and 1 mg/kg subcutaneously for 6 days decreased Cyt b5R and AH activity and the level of the cytochromes b5 and P-450 in rat livers. However, Cheung et al [35] reported that MX appears to possess little capacity for modulation of hepatic CYP enzymes and did not affect hepatic CYP enzyme levels on the 1st, 2nd, 7th or 14 th day after rats received a single intraperitoneal injection of MX at 4 mg/kg. Aniline hydroxylase is conjugative enzyme of the CYP 2E1 system that is responsible for coupling xenobiotics for excretion. CYP2E1 has a high capacity to leak electrons and therefore promotes the generation of ROS [36]. Inhibition of AH by QE may be an important factor in minimizing oxidative stress in the liver. The effects of QE on the activity of hepatic microsomal enzymes have been demonstrated in previous studies; NADH-cytochrome c reductase activity was inhibited but AH activity was unchanged when rats were injected intraperitoneally with QE at doses of 150 mg/kg body weight/day for 3 days [37]. On the other hand, Siess et al. [38] observed no significant changes in microsomal NADPH/ NADH cytochrome c reductase and cytosolic GST enzyme activity in rats treated with QE at 0.3% weight of their diet for 2 weeks.

The cytosolic enzyme aldehyde oxidase (aldehyde:O2 oxidoreductase EC 1.2.3.1) converts MX to 7-OH MX and plays a major role in the metabolism and detoxification of MX [16]. A synergistic effect was seen between QE and MT and their combination led to inhibition of AO activity in this study. However, when QE was given with MX, the resulting inhibition was not greater than for MX alone, suggesting that QE do not interfere with the detoxification of MX.

In this study, liver GST activity increased after mice were gavaged for 10 days with a 50 mg/kg/day dose of QE. A study by Fischer and Fisher [39] reported an increase in liver GST after 4 weeks of feeding rats 1% and 2% QE diets, which is in agreement with results of this study. Uda et al, [40] demonstrated that QE induced DT diaphorase increase in Hep1c1c7 cells and that the 2.3 double bond in the C ring is essential for induction of this enzyme. In addition, the ortho-orientation of the hydroxyl groups on the B ring of the polyphenol, the same as in QE, has been found to monofunctionally induce DT diaphorase activity [41]. QE induced DT diaphorase gene expression was also demonstrated in MCF 7 cells [32]. QE monofunctionally induced the activity of the Phase II enzymes, GST and DT-diaphorase, without inducing the activity of Phase I enzymes in the present study. Prestera et al, [42] proposed that some dietary anticarcinogens may inhibit carcinogen activation by tipping the balance between carcinogen activating phase I enzymes and phase II detoxifying enzymes in favour of detoxification. Consequently, factors which repress or inhibit phase I enzymes or induce or activate phase II enzymes are likely to have a protective role against cellular damage.

However, reductions in drug toxicity alone are not sufficient justification for the use of alternative therapies as they may have unwanted interactions with the drug, which can lead to adverse effects and treatment failure. The current study demonstrated that elimination of MX induced toxicity by QE (1) improved liver injury enzyme levels and antioxidant parameters, and (2) activated phase II enzymes, without inducing phase I enzymes. Consequently, the resulting strong detoxification mechanism may be considered validated.

QE demonstrated a mitigating effect on the induction of phase I enzymes by MX. Moreover, QE did not prevent detoxification of MX due to its similiar effects on AO activity. Based on the results of the present study, the combination of oral QE with intramuscular MX therapy may represent a novel and highly effective strategy for reducing MX hepatotoxicity without altering the outcome of MX metabolism.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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