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Original Article

Effects of lemongrass oil and citral on hepatic drug-metabolizing enzymes, oxidative stress, and acetaminophen toxicity in rats

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ABSTRACT

The essential oil from a lemongrass variety of *Cymbopogon flexuosus* [lemongrass oil (LO)] is used in various food and aroma industry products and exhibits biological activities, such as anticancer and antimicrobial activities. To investigate the effects of 200 LO (200 mg/kg) and 400 LO (400 mg/kg) and its major component, citral (240 mg/kg), on drug-metabolizing enzymes, oxidative stress, and acetaminophen toxicity in the liver, male Sprague-Dawley rats were fed a pelleted diet and administered LO or citral by gavage for 2 weeks. After 2 weeks of feeding, the effects of LO and citral on the metabolism and toxicity of acetaminophen were determined. The results showed that rats treated with 400 LO or citral had significantly reduced hepatic testosterone 6β-hydroxylation and ethoxyresorufin *O*-deethylation activities. In addition, NAD(P)H:quinone oxidoreductase 1 activity was significantly increased by citral, and Uridine 5′-diphospho (UDP) glucurosyltransferase activity was significantly increased by 400 LO in the rat liver. Treatment with 400 LO or citral reduced lipid peroxidation and reactive oxygen species levels in the liver. After acetaminophen treatment, however, LO and citral treatment resulted in little or no change in plasma alanine aminotransferase activity and acetaminophen-protein adducts content in the liver. Our results indicate that LO and citral may change the activities of drug-metabolizing enzymes and reduce oxidative stress in the liver. However, LO and citral may not affect the detoxification of acetaminophen.

1. Introduction

Lemongrass is an aromatic plant belonging to the Gramineae family [1]. The essential oil isolated from the lemongrass variety (*Cymbopogon flexuosus*) is used in various food and aroma industry products. Lemongrass oil (LO) contains mainly citral [2], a natural combination of two isomeric aldehydes, namely, the isomers geranial (α-citral) and neral (β-citral) [3]. Studies have shown that LO and citral have various biological

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activities, such as anticancer and antimicrobial activities [4–7]. Moreover, citral has also been shown to be renoprotective and hepatoprotective in mice through its antioxidative and anti-inflammatory effects [8,9]. However, little is known about the effect of LO and citral on drug-metabolizing enzymes. Nakamura et al [10] indicated that citral could induce total and pi-class-specific activity of glutathione S-transferase (GST) in a normal rat liver epithelial cell line (RL34 cells). Therefore, it has been suggested that LO or citral may play a role in reducing oxidative stress and detoxifying reactions. Increasing evidence shows that phytochemicals derived from foods or herbal medicines may modulate drug-metabolizing enzymes and thereby influence the pharmacological activity of drugs and their toxicities [11]. Thus, administration of natural products enriched in phytochemicals, such as LO, should be carefully monitored when given together with therapeutic drugs.

During the phase I cytochrome P450 (CYP) oxidation reaction, the production of reactive oxygen species (ROS) can cause lipid peroxidation and oxidative stress [12]. Phase II conjugation enzymes function to eliminate the electrophiles and ROS generated by phase I reactions, thereby preventing the increase of oxidative stress [13]. Increased antioxidants and/or antioxidant enzyme activities, which are involved in the detoxification of various pathological conditions, are one of the proposed mechanisms for the therapeutic effect of herbal medicines [14]. Therefore, in addition to understanding their effects on drug metabolism, evaluating the effects of herbal medicines on the antioxidant system and oxidative stress is needed.

Acetaminophen (*N*-acetyl-p-aminophenol or APAP) is an antipyretic and analgesic drug. An overdose can induce severe hepatotoxicity as a result of the CYP-mediated metabolism of APAP into a reactive metabolite, *N*-acetyl-p-benzoquinone imine (NAPQI), which exerts its toxicity by covalent binding to cellular macromolecules such as proteins, lipids, and DNA [15]. Moreover, NAPQI also reacts with glutathione (reduced form of GSH), leading to cellular GSH depletion and the production of ROS in the liver. Today, APAP overdose is the most common cause of acute hepatic failure in many countries [16].

In this study, we first evaluated the effects of LO and citral on drug-metabolizing enzymes and oxidative stress in rat livers, and then investigated whether LO and citral could protect the liver from APAP-induced hepatotoxicity in rats.

2. Materials and methods

2.1. Materials

APAP, testosterone, methoxyresorufin, resorufin, p-nitrophenol, 4-nitrocatechol, NADPH, GSH, 1-chloro-2,4-dinitrobenzene, and heparin were obtained from Sigma (St. Louis, MO, USA). 6β-Hydroxytestosterone was purchased from Ultrafine Chemicals (Manchester, UK). All other chemicals and reagents were of analytical grade and were obtained commercially. LO, the essential oil from a lemongrass variety of *C. flexuosus*, was purchased from Tai Gold Herbal Technology Co., LTD (Taoyuan, Taiwan) and contained 60% citral as determined by the High performance liquid chromatography (HPLC) method [17]. Citral (purity > 99%) was purchased from Sigma.

2.2. Animal studies

Experiment 1: We investigated the effect of LO and citral on drug-metabolizing enzymes and oxidative stress in rat livers. Male Sprague-Dawley rats (6 weeks) obtained from BioLASCO Taiwan (Ilan, Taiwan) were used. Rats were housed in plastic cages in a room kept at 23 ± 1°C and 60 ± 5% relative humidity with a 12-hour light-dark cycle. Food and drinking water were available *ad libitum*. The animals were fed a pelleted laboratory diet and administered 200 LO [200 mg/kg body weight (BW)], 400 LO (400 mg/kg BW), or citral (240 mg/kg body BW) by gavage for 2 weeks. The citral given to rats was equal to the citral content given to rats administered 400 LO. Corn oil was used as a vehicle for dissolving the LO and citral. The animals in all four groups were sacrificed by exsanguination via the abdominal aorta while under carbon dioxide (70:30, CO_2/O_2) anesthesia. Heparin was used as the anticoagulant and the plasma was separated from the blood by centrifugation (1750*g*) at 4°C for 20 min. The liver from each animal was immediately removed after exsanguination, weighed, and stored at −80°C. Microsome preparation and enzyme assays were performed within 2 weeks after liver collection. Drug-metabolizing enzymes, antioxidant systems, and oxidative stress in the liver were evaluated.

Experiment 2: To investigate the effect of LO and citral on the metabolism and toxicity of APAP, male Sprague-Dawley rats (6 weeks old) were randomly divided into four groups with eight rats in each group. The animals were fed a pelleted laboratory diet with vehicle (corn oil, Groups 1 and 2), LO (400 mg/kg BW LO, Group 3), or citral (240 mg/kg BW citral, Group 4) for 2 weeks. At the end of the experiment, food was withdrawn for 12 hours and a single 1000-mg/kg dose of APAP [18,19], as a solution in polyethylene glycol 400/water (50/50, v/v), was intraperitoneally injected into each animal in Groups 2, 3, and 4. At 12 hours after the APAP injection, the animals in all four groups were sacrificed. The plasma was separated from blood and the livers were removed immediately as described in Experiment 1. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were measured immediately by use of a commercial kit (Randox Laboratories, Antrum, UK).

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the China Medical University. The animals were maintained in accordance with the guide for the care and use of laboratory animals as translated by The Chinese-Taipei Society for Laboratory Animal Sciences, Taiwan.

2.3. Preparation of liver microsomes

Liver microsomes were separated by the method reported previously [20]. The frozen liver was thawed and then homogenized (1:4, w/v) in ice-cold 0.1M phosphate buffer (pH 7.4) containing 1mM ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 10,000*g* for 15 min at 4°C. The supernatants were then centrifuged at 105,000*g* for

60 min. The resulting microsomal pellets were suspended in a 0.25M sucrose solution containing 1mM EDTA and were stored at −80°C until use. The microsomal protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.4. Drug-metabolizing enzyme activity assays

The CYP enzyme activities were determined by the method reported previously [20]. Ethoxyresorufin (2μM), methoxyresorufin (5μM), and pentoxyresorufin (5μM) were respectively used as the probe substrates for ethoxyresorufin O-deethylation (CYP1A1), methoxyresorufin O-demethylation (CYP1A2), and pentoxyresorufin O-dependentylation (CYP2B), and diclofenac (4μM), dextromethorphan (5μM), p-nitrophenol (50μM), and testosterone (60μM) were respectively used as the probe substrates for diclofenac 4-hydroxylation (CYP2C), dextromethorphan O-demethylation (CYP2D), p-nitrophenol 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A). Microsomal protein concentrations (0.2 mg/mL) and incubation times (15 min) were the same for all metabolic reactions. The metabolites of all CYP enzyme reactions were determined by HPLC/MS as reported previously [20]. Enzyme activities were expressed as pmol of metabolite formation/min/mg protein. The microsomal Uridine 5′-diphospho-glucuronosyltransferase (UGT) activity was determined by using p-nitrophenol as the substrate where the rate of formation of p-nitrophenol glucuronic acid was measured by High performance liquid chromatography-mass spectrometry (HPLC/MS) [21].

2.5. Determination of GSH, GST, and reduce form of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H):quinone oxidoreductase 1 activities

Liver homogenate was prepared by homogenizing each gram of liver with 10 mL of ice-cold 1.15% KCl and centrifuging the homogenate at 10,000*g* for 15 minutes at 4°C. The resulting supernatant was used to determine the GSH content and GST activity. The GSH and oxidized glutathione (GSSG) contents in liver homogenates were determined by HPLC/MS [22]. GST [23] and NAD(P)H:quinone oxidoreductase 1 (NQO1) [24] activities were determined spectrophotometrically. The protein concentration in tissue homogenates was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.6. Determination of APAP-protein adducts in the liver

To determine hepatic APAP-protein adducts, liver homogenate was filtered through Nanosep centrifugal devices (Pall Life Sciences, Ann Arbor, MI, USA) with a membrane molecular weight cutoff of 30 kDa to remove low molecular weight compounds with the potential to interfere in the assay. The filtrate was then digested for 16 hours with proteases to free the APAP-cysteine from APAP-protein adducts [25]. The resulting APAP-cysteine level was determined by HPLC-MS [26].

2.7. Statistical analysis

Statistical differences among groups were calculated by using one-way ANOVA (SAS Institute, Cary, NC, USA). The differences were considered to be significant at $p < 0.05$ as determined by independent-sample t tests.

3. Results

Table 1 shows the effect of LO and citral on drug-metabolizing enzyme activity in rat livers. Rats treated with 400 mg/kg LO (400 LO group) and 240 mg/kg citral (citral group) for 2 weeks had significantly reduced ($p < 0.05$) testosterone 6β-hydroxylation activity (CYP3A). The ethoxyresorufin O-deethylation activity (CYP1A1) was slightly but significantly lower in both LO treatment groups and the citral group ($p < 0.05$). No significant difference ($p > 0.05$) was found in the diclofenac 4-hydroxylation (CYP2C), methoxyresorufin O-demethylation (CYP1A2), dextromethorphan O-demethylase (CYP2D), nitrophenol 6-hydroxylation (CYP2E1), and pentoxyresorufin O-dependentylation (CYP2B) activities among the groups. In addition, UGT activity was higher in the 400 LO group ($p < 0.05$). NQO1 activity was increased only in the citral

Table 1 – Changes in hepatic drug-metabolizing enzyme activities in rats fed the lemongrass oil (LO) or citral diet for 2 weeks.

	Control	200 LO	400 LO	Citral
Testosterone 6β-hydroxylase (CYP3A) (pmol/min/mg protein)	872.1 ± 222.1	667.6 ± 140.3	562.8 ± 217.8*	590.1 ± 153.5*
Diclofenac 4-hydroxylase (CYP2C) (pmol/min/mg protein)	15.0 ± 3.0	16.9 ± 0.8	14.6 ± 1.6	16.5 ± 2.5
Nitrophenol 6-hydroxylase (CYP2E1) (pmol/min/mg protein)	257 ± 37.6	264.6 ± 27.6	284.1 ± 53.5	286.9 ±52.7
Dextromethorphan O-demethylase (CYP2D) (pmol/min/mg protein)	121.4 ± 19.7	122.6 ± 15.9	112.1 ± 13.7	127.2 ± 30.5
Ethoxyresorufin O-deethylase (CYP1A1) (pmol/min/mg protein)	26.8 ± 1.0	25.0 ± 0.8*	23.6 ± 1.3*	22.7 ± 0.9*
Methoxyresorufin O-demethylase (CYP1A2) (pmol/min/mg protein)	21.3 ± 2.1	22.1 ± 3.0	21.5 ± 2.5	21.9 ± 3
Pentoxyresorufin O-dependentylase (CYP2B) (pmol/min/mg protein)	20.0 ± 2.7	19.9 ± 1.5	20.8 ± 1.6	20.4 ± 2.2
NAD(P)H:quinone oxidoreductase 1 (nmol/min/mg protein)	71.7 ± 15.9	73.3 ± 17.4	81.8 ± 14.1	138.2 ± 30.6*
Glutathione S-transferase (nmol/min/mg protein)	501.2 ± 87.3	479.4 ± 41.6	535.9 ± 91.2	451.2 ± 40.6
UDP-glucurosyltransferase (nmol/min/mg protein)	3.5 ± 0.7	3.5 ± 0.8	5.1 ± 1.1*	4.5 ± 1.3

Animals were administered 200 (200 LO) or 400 mg/kg (400 LO) body weight LO or 240 mg/kg body weight citral by gavage for 2 weeks. Results are expressed as the mean ± standard deviation of 8 rats.
CYP = cytochrome P450.
* Significantly different from control group, $p < 0.05$.

group ($p < 0.05$). Treatment with LO or citral had no significant effect on hepatic GST activity ($p > 0.05$). No significant difference in plasma ALT and AST activities was observed, indicating that LO and citral caused no hepatotoxicity (data not shown). There were also no significant differences in BW or liver weight in rats after LO or citral treatment (data not shown).

As shown in Table 2, after 2 weeks of treatment, LO and citral had no significant effect on GSH content compared with the control group. In the 400 LO and citral groups, however, the GSSG level was decreased ($p < 0.05$) and the GSH/GSSG ratio in the liver was increased ($p < 0.05$). An increase in hepatic GSH reductase activity was observed in rats treated with LO and citral ($p < 0.05$). Furthermore, GSH reductase activity was increased significantly more in the citral group than in the LO group ($p < 0.05$). Neither LO nor citral caused a significant change in GSH peroxidase activity in the liver ($p > 0.05$). Levels of thiobarbituric acid reactive substances and ROS in the liver were significantly lower in both the 400 LO group and the citral treatment group ($p < 0.05$).

After a single dose of APAP, plasma ALT and AST activities were increased in APAP-treated rats compared with control animals ($p < 0.05$; Figures 1A, 1B). Treatment with either LO or citral had little or no effect on the elevations of plasma ALT and AST activities induced by APAP ($p > 0.05$). Hepatic GSH content was lower after APAP treatment ($p < 0.05$; Figure 2A). Treatment with LO and citral treatment had little or no effect on APAP-protein adducts content in the liver ($p > 0.05$; Figure 2B).

4. Discussion

Many phytochemicals have been shown to prevent chemical-induced tissue damage and carcinogenesis in animals, mainly as a result of the modulation of drug-metabolizing enzymes after their administration [27,28]. In this study, rats treated with LO and citral changed the activities of some CYP and phase II detoxifying enzymes. Treatment with either 400 LO or citral reduced oxidative stress in the liver. However, neither LO nor citral had a significant effect on APAP-induced hepatotoxicity in rats.

In the present study, treatment with the higher dose of LO or citral resulted in significantly lower CYP3A and 1A1 activities. However, LO and citral had little or no effect on the activities of CYP1A2, 2B, 2C, 2D, 2E1, or GST. These results suggest that 400 LO or citral treatment might reduce the biotransformation of xenobiotics catalyzed by CYP3A and 1A1. However, the reduction of CYP3A activity by 400 LO (−35.4%) and citral (−32.3%) was more significant than the reduction of CYP1A1 activity (400 LO: −11.9%; citral: −15.3%). These results suggest that CYP3A, a major CYP enzyme responsible for metabolizing more than 50% of clinical drugs in the human liver [29], is likely to be a target enzyme modulated by treatment with LO and citral. The citral content in the 400 LO group was equivalent to that in the citral group. Therefore, the inhibition of CYP3A and CYP1A1 activity after treatment with 400 LO might have been due to the citral.

It was interesting to note that LO and citral increased the activities of phase II detoxifying enzymes. Despite an equal dose of citral present in both the 400 LO and citral groups, in this study, only the citral group showed increased NQO1 activity in the liver. Similarity, higher UGT activity was observed only in the 400 LO group. These results indicate that, in addition to citral, other components in LO may down-regulate NQO1 activity and up-regulate UGT activity. This phenomenon is possibly due to the multiple constituents in LO that may have different physiological activities and component-component interactions that may have influenced its overall effects. Similar results were also observed in some Traditional Chinese medicines [30]. It has been reported that NQO1 can protect cells from oxidative stress induced by reactive and damaging quinines [24]. Lack of NQO1 increases susceptibility to benzo(*a*)pyrene- and 7,12-dimethylbenz(*a*)anthracene-induced skin carcinogenesis [31]. UGT is critical for regulating nutrients, hormones, and endobiotics, as well as for detoxifying xenobiotics. UGT has been shown to catalyze the conjugation of the metabolites of major tobacco carcinogens, such as benzo(*a*)pyrene, to facilitate their excretion [32]. Therefore, consumption of LO or citral may aid in the detoxification of carcinogens by increasing the activity of phase II detoxifying enzymes.

Concerning the role of LO and citral on oxidative stress in the liver, in this study, LO and citral did not affect the GSH

Table 2 – Effects of lemongrass oil (LO) or citral on GSH, lipid peroxidation, and antioxidative enzyme activity in rat livers.

	Control	200 LO	400 LO	Citral
GSH (μmol/g liver)	6.6 ± 2.1	5.8 ± 1.2	5.5 ± 0.5	6.4 ± 0.9
GSSG (μmol/g liver)	0.5 ± 0.1	0.4 ± 0.1	0.3 ± 0.1*	0.3 ± 0.1*
GSH/GSSG	15 ± 6.4	16.6 ± 5.8	22.3 ± 5.9*	26.8 ± 7.9*
Glutathione peroxidase (nmol/min/mg protein)	237.2 ± 39.2	216.4 ± 41.1	232.2 ± 28.1	262.9 ± 47.7
Glutathione reductase (nmol/min/mg protein)	33.2 ± 8	50.3 ± 3.3*	49.7 ± 6.5*	82.2 ± 12.8*,**
ROS (nmol/g liver)	172.1 ± 3.8	166.8 ± 4.0	165 ± 5.2*	154.6 ± 5.9*
TBARS (nmol/g liver)	9.8 ± 2.2	7.8 ± 1.7	6.1 ± 1.4*	6.4 ± 0.9*

Animals were administered 200 (200 LO) or 400 (400 LO) mg/kg body weight LO or 240 mg/kg body weight citral by gavage for 2 weeks. Results are expressed as the mean ± standard deviation of 8 rats.
GSH = reduced glutathione; GSSG = oxidized glutathione; ROS = reactive oxygen species; TBARS = thiobarbituric acid reactive substances.
* Significantly different from control group, $p < 0.05$.
** Significantly different from the 200 and 400 LO groups, $p < 0.05$.

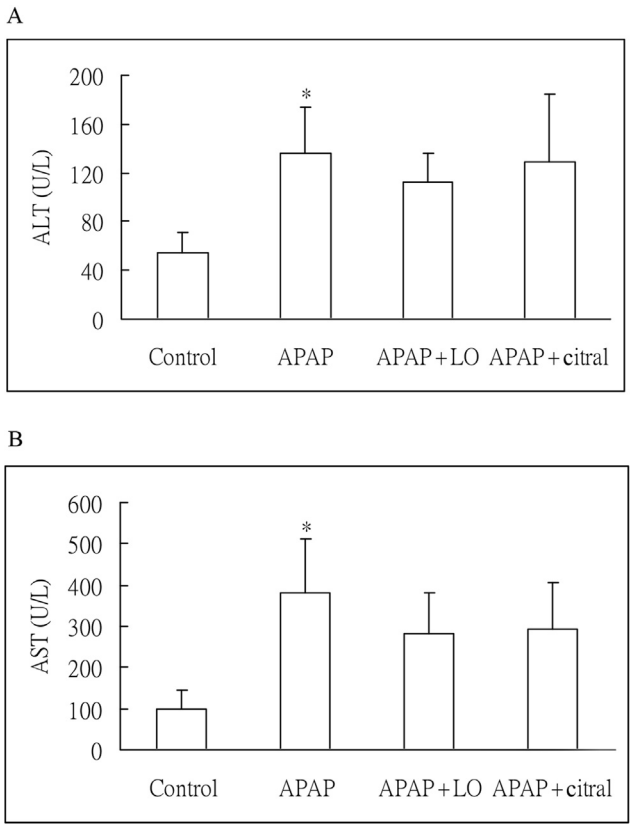

Figure 1 – Effects of LO and citral on plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in rats. Animals were treated with corn oil (vehicle control), LO (400 mg/kg), or citral (240 mg/kg) for 2 weeks and then administered a single 1000-mg/kg dose of APAP. Results are expressed as the mean ± S.D. of 8 rats.* Significantly different from control group, $p < 0.05$. APAP = N-acetyl-p-aminophenol; LO = lemongrass oil; S.D. = standard deviation.

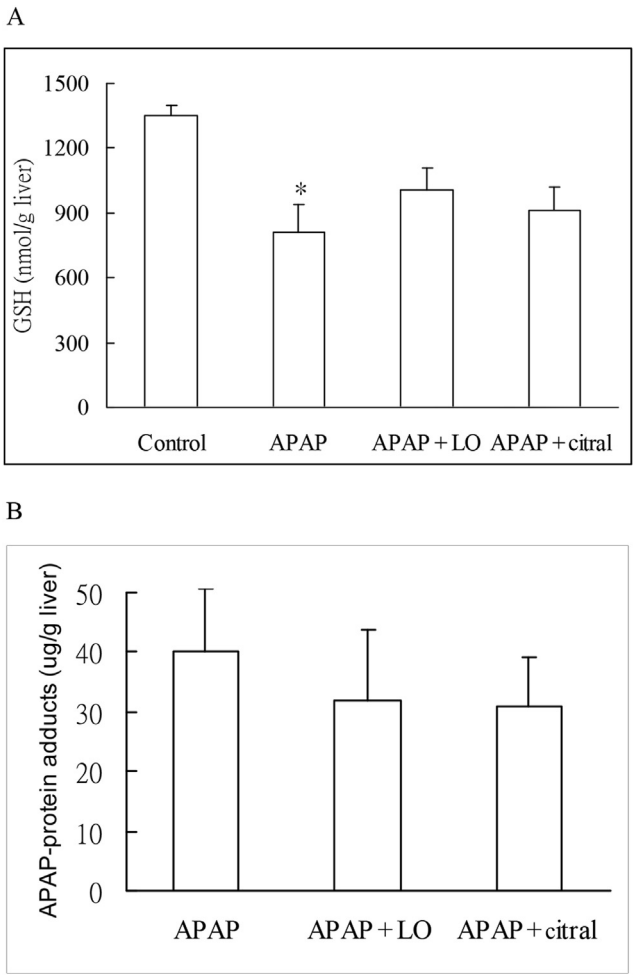

Figure 2 – Effects of LO and citral on GSH and APAP-protein adducts contents in the liver. Animals were treated with corn oil (vehicle control), LO (400 mg/kg) or citral (240 mg/kg) for 2 weeks and then administered a single 1000-mg/kg dose of APAP. Results are expressed as the mean ± S.D. of 8 rats.* Significantly different from control group, $p < 0.05$. APAP = N-acetyl-p-aminophenol; GSH = glutathione; LO = lemongrass oil; S.D. = standard deviation.

levels or GSH peroxidase activity in the liver. However, the LO and citral groups had significantly decreased GSSG levels in the liver, resulting in a higher GSH/GSSG ratio. The higher GSH/GSSG ratio observed in the 400 LO and citral groups indicated a lower oxidative stress status in the liver [33]. In addition, LO and citral treatment increased GSH reductase activity, which may be responsible for the decreased GSSG in the liver. Moreover, lower lipid peroxidation and ROS levels were found in the 400 LO and citral groups. It is known that lipid peroxidation increases when free radical production overwhelms the total antioxidant defense. Taken together, it is reasonable to speculate that treatment with 400 LO may decrease lipid peroxidation and ROS levels in the liver owing to increased GSH reductase activity and a lower GSSG level. The increase in antioxidant defense capacity by LO may be attributed to citral.

Studies have shown that natural products that decrease CYP enzyme activity [34] and increase antioxidant enzyme activity or the GSH level may attenuate APAP-induced liver toxicity [25]. It has been reported that lower CYP3A activity after APAP treatment in the liver can reduce CYP-mediated bioactivation of APAP to generate the toxic electrophile NAPQI [35]. Although citral reduced CYP3A activity and lowered oxidative stress in the liver, treatment with either 400 LO or citral failed to ameliorate APAP-induced hepatotoxicity. LO and citral had little or no effect on APAP-induced elevations of plasma ALT and AST activities (Figure 1) and depletion of hepatic GSH and APAP-protein adduct contents (Figure 2). To our knowledge, the reason for the lack of effect of LO or citral on APAP-induced hepatotoxicity is currently unknown. It is likely that, in this study, the liver damage caused by APAP was much greater than the level of drug toxicity that LO and citral could overcome. Recently, citral has been shown to reduce liver injury in high-fat diet-induced nonalcolholic fatty liver disease in mice [9]. Therefore, further studies are needed to clearly investigate the hepatoprotective effects of LO and citral.

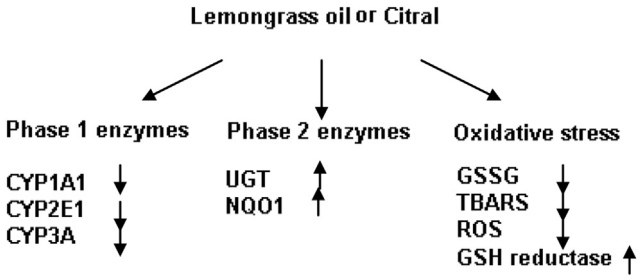

Figure 3 – Effects of lemongrass oil and citral on phase I and phase II drug-metabolizing enzymes and oxidative stress in liver. CYP = cytochrome P450; UGT = uridine 5-diphosphoglucurosyltransferase; NQO1 = NAD(P)H:quinone oxidoreductase 1; GSSG = oxidized glutathione; TBARS = thiobarbituric acid reactive substances; ROS = reactive oxygen species; GSH reductase = glutathione reductase.

In summary, the present study showed that pretreatment with LO reduced CYP1A1 and CYP3A activities and lowered oxidative stress in the liver. Citral may be the major component responsible for these effects. In addition, LO and citral treatment had different effects on increasing phase II detoxifying enzyme activity in the liver (Figure 3). However, LO and citral had little or no effect on APAP-induced hepatotoxicity. Because CYP3A is the most important isoform related to the oxidative biotransformation of numerous medicines in humans, further studies will be needed to investigate possible herb-drug interactions.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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