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Research Paper

# Protective effects of coffee-derived compounds on lipopolysaccharide/D-galactosamine induced acute liver injury in rats

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## Abstract

**Objectives** The protective effects of coffee-derived compounds on lipopolysaccharide/D-galactosamine (LPS/D-GalN) induced acute liver injury in rats were investigated.

**Methods** Wistar rats were orally administered saline (control) or one of the test compounds (caffeine, chlorogenic acid, trigonelline, nicotinic acid or eight pyrazinoic acids) at a dose of 100 mg/kg, respectively. This was followed by intraperitoneal injection with LPS (100 $\mu$g/kg)/D-GalN (250 mg/kg) 1 h after administration of the test compounds. Blood samples were collected up to 12 h after LPS/D-GalN injection, followed by determination of plasma aspartate aminotransferase, alanine aminotransferase, tumour necrosis factor $\alpha$ (TNF-$\alpha$) and interleukin 10 (IL-10) levels.

**Key findings** Plasma aspartate aminotransferase and alanine aminotransferase levels were significantly increased after LPS/D-GalN-treatment, but were suppressed by pretreatment with caffeine ($n$ = 5), nicotinic acid, non-substituted pyrazinoic acid or 5-methylpyrazinoic acid ($n$ = 6, respectively) 12 h after LPS/D-GalN-treatment ($P < 0.01$, respectively). Moreover, the animals pretreated with these test compounds showed significantly higher survival rates (83–100%) compared with the control (23%). Only pretreatment with caffeine significantly suppressed the LPS/D-GalN induced elevation of plasma TNF-$\alpha$ levels 1 and 2 h after LPS/D-GalN-treatment ($P < 0.01$, respectively). Pretreatment with caffeine, nicotinic acid or non-substituted pyrazinoic acid activated the LPS/D-GalN induced elevation of plasma IL-10 levels at 1 and 2 h, although there were no statistically significant differences in IL-10 levels between control and nicotinic acid or non-substituted pyrazinoic acid treated rats.

**Conclusions** The results suggest that caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid can protect against LPS/D-GalN induced acute liver injury, which may be mediated by the reduction of TNF-$\alpha$ production and/or increasing IL-10 production.

**Keywords** caffeine; coffee; inflammation; liver injury; Maillard reaction products; pyrazine

## Introduction

Several epidemiological studies have shown that coffee consumption reduces the elevation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are markers of liver injury,[1–3] as well as reducing the risk of chronic liver disease and liver cirrhosis.[4,5] Caffeine, one of the methylxanthines, is naturally found in beverages such as coffee.[6] Caffeine has been studied for its biochemical and physiological effects and more recently for its anti-inflammatory effects.[7–9]

The immunomodulatory effect of caffeine has been suggested to be mediated by two likely mechanisms, inhibition of cAMP phosphodiesterase and activation of the cAMP/protein kinase A pathway, followed by a consequential increase in intracellular cAMP concentrations.[7] It has been shown that activation of the cAMP/protein kinase A pathway inhibits lipopolysaccharide (LPS) stimulated production of pro-inflammatory cytokines such as tumour necrosis factor $\alpha$ (TNF-$\alpha$) and interleukin (IL)-12, and promotes that of anti-inflammatory cytokine IL-10.[10,11] It has also been shown that cAMP phosphodiesterase inhibitors suppress the production of pro-inflammatory cytokines such as TNF-$\alpha$, IL-2 and interferon $\gamma$, and stimulate the anti-inflammatory cytokine IL-10.[12,13] In animal studies, it has been shown that long-term dietary supplementation with caffeine or coffee

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suppresses LPS induced elevation of serum AST and ALT levels in D-galactosamine (D-GalN) sensitised rats.[14,15]

In the present study, we investigated the effects of caffeine with single-time administration on LPS/D-GalN induced acute liver injury in rats, by examining survival rates and plasma AST, ALT, TNF-$\alpha$ and IL-10 levels. In addition, we investigated the effect of several coffee-derived compounds, such as chlorogenic acid, trigonelline, nicotinic acid and eight pyrazinoic acids (Figure 1), as well as caffeine, on LPS/D-GalN induced acute liver injury. Pyrazinoic acids have been shown to be generated by metabolism of methylpyrazines, which are Maillard reaction products naturally found in coffee.[16]

## Materials and Methods

### Materials

Caffeine, nicotinic acid, and trigonelline were obtained from Wako Pure Chemical Industries (Osaka, Japan). Chlorogenic acid was obtained from Aldrich (Tokyo, Japan). Commercially available pyrazinoic acids such as non-substituted pyrazinoic acid and 5-methylpyrazinoic acid were obtained from Wako Pure Chemical Industries. 3-Methyl-, 6-methyl-, 3,5-dimethyl-, 3,6-dimethyl-, 5,6-dimethyl- and trimethylpyrazinoic acid were prepared by Kanto Chemical (Tokyo, Japan). LPS and D-GalN hydrochloride were purchased from Wako Pure Chemical Industries and Aldrich, respectively. All other reagents were of the best available grade.

### Animals and treatment

Male Wistar rats (5–6 weeks old) were purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan). The rats were housed in a temperature-controlled (23 ± 1°C) room under a 12-h light–dark cycle (lights on: 0700–1900 hours) for 1 week. The rats were fed a standard rat chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and had free access to tap water while being housed. The experiments using rats were conducted in accordance with the guidelines of, and with the approval of the Animal Research Ethics Committee at Tokyo University of Pharmacy and Life Sciences.

The animals were orally administered saline (control rats) or one of the test compounds (caffeine, chlorogenic acid, trigonelline, nicotinic acid or eight pyrazinoic acids) at a dose of 100 mg/kg, respectively, suspended in methylcellulose, after fasting for 18 h. At 1 h after the administration of saline or test compounds, the animals were intraperitoneally injected with LPS (100 $\mu$g/kg)/D-GalN (250 mg/kg). Blood samples were collected from the tail vein before the first injection and after LPS/D-GalN injection up to 12 h, using a 50-$\mu$l heparinised micropipette (Vitrex Medical, Copenhagen, Denmark), and were centrifuged at 805$g$ for 10 min at 25°C. The plasma samples obtained were stored at −80°C until used.

### Determination of AST and ALT levels

Plasma AST and ALT levels were determined using an enzymatic test kit (transaminase CII test; Wako Pure Chemical Industries). A microplate reader (LS-PLATE manager 2001 Windows version 2.10; Wako Pure Chemical Industries) was used to determine the UV absorption spectra.

### Determination of TNF-$\alpha$ and IL-10 levels

Plasma TNF-$\alpha$ and IL-10 levels were determined by a commercially available enzyme-linked immunosorbent assay kit (R&D systems, Minneapolis, MN, US). The UV absorption spectrum was determined as described above.

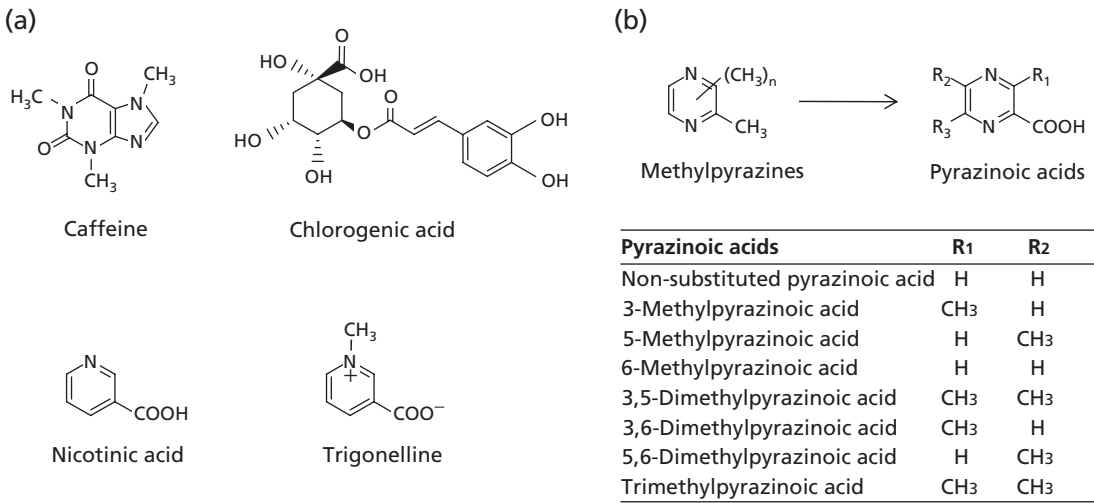
### Statistical analysis

The results are presented as means ± SE. Data comparisons were carried out using Fisher's exact test for 24-h survival rates and one-way analysis of variance and Dunnett's test for AST, ALT and cytokine levels; $P < 0.05$ was considered significant.

## Results

### Survival rates

The 24-h survival rates of the rats administered test compounds and LPS/D-GalN are shown in Table 1. LPS/D-GalN-treatment




| Pyrazinoic acids | R1 | R2 | R3 |
|---|---|---|---|
| Non-substituted pyrazinoic acid | H | H | H |
| 3-Methylpyrazinoic acid | $CH_3$ | H | H |
| 5-Methylpyrazinoic acid | H | $CH_3$ | H |
| 6-Methylpyrazinoic acid | H | H | $CH_3$ |
| 3,5-Dimethylpyrazinoic acid | $CH_3$ | $CH_3$ | H |
| 3,6-Dimethylpyrazinoic acid | $CH_3$ | H | $CH_3$ |
| 5,6-Dimethylpyrazinoic acid | H | $CH_3$ | $CH_3$ |
| Trimethylpyrazinoic acid | $CH_3$ | $CH_3$ | $CH_3$ |

**Figure 1** Caffeine and coffee-derived compound structures. Chemical structures of (a) caffeine, chlorogenic acid, trigonelline and nicotinic acid and (b) pyrazinoic acids.

**Table 1** Survival rates

| Group | Number of rats (living/tested) | Survival rate (%) | *P* value |
|---|---|---|---|
| Control | 3/13 | 23 | – |
| Caffeine | 5/5 | 100 | $P < 0.01$ |
| Chlorogenic acid | 0/6 | 0 | NS |
| Trigonelline | 0/6 | 0 | NS |
| Nicotinic acid | 6/6 | 100 | $P < 0.01$ |
| Non-substituted pyrazinoic acid | 5/6 | 83 | $P < 0.05$ |
| 3-Methylpyrazinoic acid | 0/6 | 0 | NS |
| 5-Methylpyrazinoic acid | 5/6 | 83 | $P < 0.05$ |
| 6-Methylpyrazinoic acid | 4/6 | 67 | NS |
| 3,5-Dimethylpyrazinoic acid | 2/6 | 33 | NS |
| 3,6-Dimethylpyrazinoic acid | 2/6 | 33 | NS |
| 5,6-Dimethylpyrazinoic acid | 3/6 | 50 | NS |
| Trimethylpyrazinoic acid | 0/6 | 0 | NS |

Effect of oral administration of 100 mg/kg caffeine, chlorogenic acid, trigonelline, nicotinic acid and eight pyrazinoic acids on the 24-h survival rates of lipopolysaccharide/D-galactosamine induced acute liver injury rats. Data comparison between control and treated groups was carried out using Fisher's exact test; $P < 0.05$ was considered as significant (NS, not significant).

caused high lethal toxicity to rats, resulting in a 23% survival rate in the control rats within 24 h. LPS/D-GalN toxicity was significantly prevented by caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid ($P < 0.01$, 0.01, 0.05 and 0.05, respectively), resulting in 100, 100, 83 and 83% survival rates, respectively. In the group of rats pretreated with chlorogenic acid, trigonelline or 3-methyl- and trimethylpyrazinoic acid, all animals died within 24 h after LPS/D-GalN-treatment.

## Plasma AST and ALT levels

The effects of the test compounds on plasma AST levels in LPS/D-GalN induced acute liver injury rats are shown in Table 2. In the control rats ($n = 13$), plasma AST levels were significantly increased 6 and 12 h after LPS/D-GalN-treatment (by 18- and 166-fold at 6 and 12 h, respectively) ($P < 0.05$). Pretreatment with 100 mg/kg of caffeine ($n = 5$), nicotinic acid or seven pyrazinoic acids ($n = 6$, respectively) significantly decreased the plasma AST levels compared with the control at 12 h ($P < 0.05$ or 0.01). Of these compounds, caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid showed extremely strong effects on lowering the plasma AST levels, resulting in approximately 2, 22, 21 and 8% of the control values, respectively. Caffeine, non-substituted pyrazinoic acid, and 5-methylpyrazinoic acid also showed significant AST-lowering effects compared with the control at 6 h ($P < 0.01$, 0.05 and 0.01, respectively).

The effects of the test compounds on plasma ALT levels in LPS/D-GalN induced acute liver injury rats are shown in Table 2. In the control rats, plasma ALT levels were significantly increased 6 and 12 h after LPS/D-GalN-treatment (by 23- and 226-fold at 6 and 12 h, respectively). Pretreatment with 100 mg/kg of caffeine, nicotinic acid, non-substituted pyrazinoic acid or 5-methyl- or 6-methylpyrazinoic acid significantly decreased plasma ALT levels compared with the control at 12 h ($P < 0.05$ or 0.01). As with the results for plasma AST levels, caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid showed the greatest lowering effects, approximately 1, 24, 19 and 9% of the control ALT values, respectively. Caffeine and 5-methylpyrazinoic acid also showed significant ALT lowering effects compared with the control at 6 h ($P < 0.01$ and 0.05, respectively).

## Plasma TNF-$\alpha$ and IL-10 levels

The effects of caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid on plasma TNF-$\alpha$ levels in LPS/D-GalN induced acute liver injury rats are shown in Figure 2a. In the control rats, plasma TNF-$\alpha$

**Table 2** Plasma aspartate aminotransferase and alanine aminotransferase levels

| Group | Aspartate aminotransferase (Karmen units) | | | Alanine aminotransferase (Karmen units) | | |
|---|---|---|---|---|---|---|
| | 0 h | 6 h | 12 h | 0 h | 6 h | 12 h |
| Control | 135 ± 8 | 2414 ± 346 | 22 425 ± 1393 | 26 ± 4 | 607 ± 103 | 5956 ± 635 |
| Caffeine | 146 ± 33 | 282 ± 48** | 345 ± 67** | 22 ± 4 | 51 ± 6** | 66 ± 19** |
| Chlorogenic acid | 147 ± 13 | 1998 ± 621 | 18 934 ± 160 | 25 ± 4 | 571 ± 179 | 7338 ± 200 |
| Trigonelline | 185 ± 23 | 2829 ± 500 | 18 578 ± 971 | 31 ± 7 | 773 ± 157 | 5959 ± 647 |
| Nicotinic acid | 162 ± 16 | 1346 ± 208 | 4991 ± 1274** | 28 ± 5 | 355 ± 51 | 1413 ± 367** |
| Non-substituted pyrazinoic acid | 236 ± 27 | 899 ± 181** | 4808 ± 2034** | 27 ± 7 | 192 ± 44 | 1108 ± 461** |
| 3-Methylpyrazinoic acid | 143 ± 15 | 2325 ± 383 | 16 317 ± 619** | 29 ± 7 | 683 ± 97 | 5446 ± 766 |
| 5-Methylpyrazinoic acid | 171 ± 10 | 639 ± 187** | 1851 ± 1605** | 25 ± 2 | 176 ± 61* | 523 ± 474** |
| 6-Methylpyrazinoic acid | 196 ± 21 | 1131 ± 379 | 12 887 ± 2012** | 41 ± 11 | 293 ± 95 | 3776 ± 772* |
| 3,5-Dimethylpyrazinoic acid | 195 ± 26 | 2474 ± 657 | 18 600 ± 1025 | 43 ± 9 | 596 ± 129 | 6089 ± 683 |
| 3,6-Dimethylpyrazinoic acid | 177 ± 14 | 2331 ± 507 | 15 621 ± 1140** | 22 ± 5 | 425 ± 75 | 4106 ± 426 |
| 5,6-Dimethylpyrazinoic acid | 171 ± 20 | 2167 ± 531 | 14 965 ± 437** | 31 ± 7 | 600 ± 214 | 3872 ± 476 |
| Trimethylpyrazinoic acid | 180 ± 11 | 1879 ± 198 | 17 873 ± 460* | 48 ± 18 | 511 ± 88 | 5186 ± 518 |

Effect of oral administration of 100 mg/kg caffeine, chlorogenic acid, trigonelline, nicotinic acid and eight pyrazinoic acids on plasma aspartate aminotransferase and alanine aminotransferase levels in lipopolysaccharide/D-galactosamine induced acute liver injury rats. The number of animals used was 13 (control), five (caffeine) or six (other compounds). Data are expressed as means ± SE. *$P < 0.05$ and **$P < 0.01$, statistically significant compared with the control.

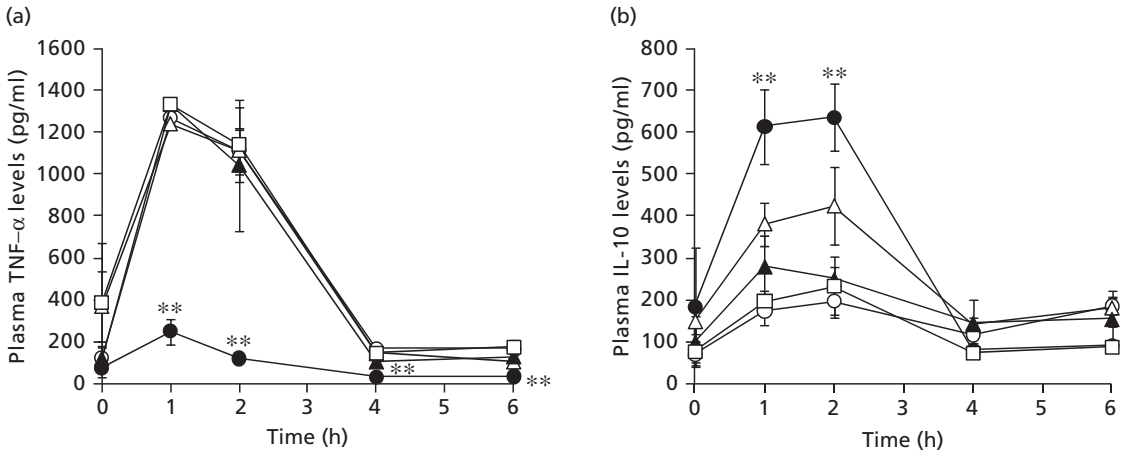



**Figure 2** Plasma tumour necrosis factor $\alpha$ and interleukin-10 levels. Effect of oral administration of 100 mg/kg caffeine (●), nicotinic acid (△), non-substituted pyrazinoic acid (▲) and 5-methylpyrazinoic acid (□) on (a) plasma tumour necrosis factor $\alpha$ (TNF-$\alpha$) and (b) interleukin 10 (IL-10) levels in lipopolysaccharide/D-galactosamine (LPS/D-GalN) induced acute liver injury rats. Blood sampling was carried out up to 6 h after LPS/D-GalN administration ($n$ = 4), and cytokine levels were measured by enzyme-linked immunosorbant assay. Bars represent the means ± SE. $^{**}P < 0.01$, statistically significant compared with control (○).

levels significantly increased 1 and 2 h after LPS/D-GalN-treatment (by 10.2- and 9.0-fold at 1 and 2 h, respectively). Caffeine-pretreatment significantly decreased plasma TNF-$\alpha$ levels compared with the control at 1, 2, 4 and 6 h ($P < 0.01$, respectively). However, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid did not have an effect on plasma TNF-$\alpha$ levels.

The effects of caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid on plasma IL-10 levels in LPS/D-GalN induced acute liver injury rats are shown in Figure 2b. In the control rats, plasma IL-10 levels significantly increased 1 and 2 h after LPS/D-GalN-treatment (by 2.5- and 2.8-fold at 1 and 2 h, respectively). Caffeine pretreatment significantly increased plasma IL-10 levels compared with the control at 1 and 2 h ($P < 0.01$, respectively). Pretreatment with nicotinic acid and non-substituted pyrazinoic acid also increased plasma IL-10 levels compared with the control by 119 and 61% at 1 h and 117 and 28% at 2 h, respectively, although the effects were not statistically significant.

## Discussion

We investigated the effects of single-time oral administration of caffeine or several coffee-derived compounds on LPS/D-GalN induced acute liver injury in rats. First, we examined the effects of the test compounds on plasma AST and ALT levels in LPS/D-GalN injected rats. LPS is a toxic component of the cell walls of Gram-negative bacteria,[17] while D-GalN has been known to sensitise animals to LPS followed by development of lethal liver injury.[18] Co-administration of LPS and D-GalN has been used for developing an experimental model of acute liver injury.[19] In the present study, when 100 $\mu$g/kg of LPS and 250 mg/kg of D-GalN were concomitantly given intraperitoneally to the rats, the animals showed a survival rate of only 23%. Thus, we considered this dose to be suitable to assess the protective effects of the test compounds against LPS/D-GalN induced acute liver injury. We found that pretreatment with caffeine, nicotinic acid, non-substituted pyrazinoic acid or 5-methylpyrazinoic acid significantly suppressed the LPS/D-GalN induced elevation of plasma AST and ALT levels, leading to significantly high survival rates (83–100%) compared with the control. The results suggest that these compounds showed protective effects against LPS/D-GalN induced acute liver injury. Chlorogenic acid has been reported to have protective effects against carbon tetrachloride ($CCl_4$) induced liver injury in an in-vitro assay.[20] However, in the present study, this compound had no protective effect against liver injury. This discrepancy in the results may be due to differences in experimental methods between the study by Basnet *et al.*[20] and the present study, especially the use of different toxic compounds ($CCl_4$ *vs* LPS/D-GalN).

To further investigate the hepatoprotective effects of the test compounds, we examined the plasma cytokine levels in rats treated with LPS/D-GalN and the test compounds. It has been shown that LPS activates macrophages in the liver, followed by stimulation of the synthesis and release of inflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$ and IL-6.[19] Since TNF-$\alpha$ is known to play a critical role in LPS/D-GalN induced acute liver injury among these cytokines,[21,22] we focused on TNF-$\alpha$ as the inflammatory cytokine in this study. We also examined the plasma levels of IL-10, which is an anti-inflammatory cytokine, since LPS intoxication has also been shown to induce release of IL-10 from macrophages.[23] In this study, caffeine significantly suppressed LPS/D-GalN induced elevation of plasma TNF-$\alpha$ levels. In addition, caffeine significantly increased plasma IL-10 levels in LPS/D-GalN treated rats. It has been shown that exogenous dibutyryl cAMP, a stable and permeable cAMP analogue, had protective effects against LPS induced acute liver injury in mice, with decreasing circulating TNF-$\alpha$ and increasing

circulating IL-10 levels.[23,24] Since caffeine has been shown to increase intracellular cAMP levels,[7] caffeine may have exerted hepatoprotective effects through cAMP induced immunomodulation in this study.

The dose of caffeine in this study was fixed at 100 mg/kg, a high dose that would not be achieved by normal coffee consumption in humans. It has been shown that caffeine has biphasic effects on liver injury depending on the dose.[25] A high dose of caffeine (100 mg/kg) can inhibit concanavalin A induced liver injury in mice, while lower doses of caffeine (10 or 20 mg/kg) are shown rather to enhance inflammatory damage by antagonising the adenosine receptor A2AR, which is a $G_s$-coupled receptor that induces intracellular cAMP upon ligand binding.[25] Considering the biphasic effects of caffeine, the hepatoprotective effects of caffeine observed in the present study might not be exerted at lower doses in LPS/D-GalN treated rats as in concanavalin A treated mice. However, it has also been shown that this liver injury by acute caffeine pretreatment through binding to A2AR was reduced by chronic caffeine consumption.[25] Hence, further investigation regarding the effects of a lower dose of caffeine on the LPS/D-GalN induced liver injury in caffeine-naive or habitual caffeine-consuming rats may be required.

He *et al.*[14] have shown that dietary supplementation with caffeine for 14 days suppresses LPS/D-GalN induced elevation of serum AST and ALT levels in rats, with reductions in both TNF-$\alpha$ and IL-10 levels. The results of the study by He *et al.*[14] do not agree with our results with regard to the effect of caffeine on IL-10 levels. This contrasting result may be owing to different experimental procedures, such as single-time administration *vs* long-term administration, and forced administration *vs* dietary supplementation.

Unlike the effects of caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid did not suppress LPS/D-GalN induced elevation of plasma TNF-$\alpha$ levels. In contrast, nicotinic acid and non-substituted pyrazinoic acid promoted the elevation of plasma IL-10 levels induced by LPS/D-GalN, although 5-methylpyrazinoic acid did not have an effect on plasma IL-10 levels. There is a possibility that nicotinic acid and non-substituted pyrazinoic acid showed protective effects against LPS/D-GalN induced acute liver injury through increasing plasma IL-10 levels. However, considering the fact that TNF-$\alpha$ plays a critical role in LPS/D-GalN induced acute liver injury,[21,22] the hepatoprotective mechanisms of these tested compounds remain unclear. We have previously reported that non-substituted pyrazinoic acid and 5-methylpyrazinoic acid have intense plasma non-esterified fatty acid lowering effects in rats as well as nicotinic acid, while other pyrazinoic acids have little or no effect.[16] The non-esterified fatty acid lowering effects of these compounds were thought to be mediated by binding to the nicotinic acid receptor GPR109A.[16,26,27] The potency of the hepatoprotective effects of nicotinic and pyrazinoic acids observed in the present study were consistent with those of the non-esterified fatty acid lowering effects of these compounds, suggesting that the hepatoprotective effects of nicotinic and pyrazinoic acids may be related to their binding affinity to GPR109A.

## Conclusions

Caffeine, nicotinic acid, non-substituted pyrazinoic acid and 5-methylpyrazinoic acid suppress the LPS/D-GalN induced elevation of plasma AST and ALT levels in rats, leading to high survival rates in treated animals. Our findings suggest that these compounds showed protective effects on LPS/D-GalN induced acute liver injury. The hepatoprotective mechanisms of these compounds are considered to be mediated, at least partially, by alteration in plasma cytokine levels.

## Declarations

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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