RESEARCH

Caffeine metabolite as a biomarker in carbon tetrachloride-induced hepatotoxicity in rats

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ABSTRACT

Liver injury is a form of trauma, also known as liver laceration, which represents 5% of all traumas of the abdomen. Caffeine, which is metabolized mainly in the liver, is a widely consumed stimulant that exists in many commonly consumed beverages. Glycyrrhizin (GZ) and boswellic acids (BA) are naturally occurring compounds and isolated from licorice and Olibanum gum, respectively. They proved to have both hepatoprotective and immunomodulatory activities and present potential drugs for liver disorders. The aim of this study is to investigate the effects of GZ and BA on hepatic intoxication in CCl_4-induced hepatotoxicity and to investigate whether caffeine metabolites concentrations in serum can be used as biomarkers for liver function. Rats treated with CCl_4 showed a significant increase in the average of all liver enzymes levels in serum in comparison with those in the control group which received only caffeine. On the other hand, rats treated with CCl_4 and receiving BA and BA + GZ showed a slight reduction in enzymes levels. Theobromine concentration in rats' sera which received CCl_4 was significantly lower in comparison to the non-treated rats. Caffeine/theobromine (C/T) ratio could be accurately used as a biomarker for liver injury and/or early fibrosis without any interactions of false positive common liver function tests. Also, these results confirmed the hepatoprotective effect of both pentacyclic triterpenes, GZ and BA, tested in this model.

KEYWORDS: Biomarker, liver injury, caffeine metabolism, glycyrrhizin, Boswellic acids

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INTRODUCTION

The liver is a vital organ where most of the body metabolism occurs. Liver injury is a common problem in many populations due to many causes including hepatitis C virus (HCV) infection (Karidis et al., 2015), hepatocellular carcinoma (Luedde & Schwabe, 2011), drug-induced liver injury (Friedrich et al., 2016), herb-induced liver injury (Cho et al., 2017) and even physical injuries (Dandin et al., 2016). Caffeine (1,3,7-trimethylxanthine) is a stimulant that is found naturally in coffee, tea, chocolate, and used as an additive in other beverages and as adjuvant analgesic in some pain medications (Mandel, 2002; Sawynok, 2011; Shively & Tarka, 1984). Apart from that it is commonly consumed all over the world. Almost all caffeine consumed is metabolized with less than 3% excreted (Begas et al., 2007; Kot & Daniel, 2008). The main hepatic metabolism for caffeine in humans, which are illustrated in Figure 1, are the demethylation of; N3 to form paraxanthine, N1 to form theobromine and N7 to form theophylline which accounts for 80, 11 and 4%, respectively, of the caffeine metabolism in humans (Begas et al., 2007; Benowitz et al., 1995; Kot & Daniel, 2008; Tassaneeyakul et al., 1994). Caffeine acts through multiple mechanisms, the most important of which is the antagonism of adenosine receptors (Sawynok, 2011). Recent studies have suggested a role for caffeine in neuroprotection and as a potential treatment for Parkinson's disease (Prediger, 2010). Low consumption of caffeine is mildly rewarding, as it stimulates cognitive performance and memory retention (Borota et al., 2014; Nehlig, 1999; Smith, 2002). Consumption of more than 2 cups of coffee per day was found to be associated with a lower risk of advanced liver fibrosis, particularly in patients with HCV infection (Modi et al., 2010).

Natural products have been the most valuable source of organic compounds for drug development and discovery (Harvey et al., 2015). Frankincense Oil demonstrated immunostimulant activity, which is an added value to the reported anti-inflammatory, immunomodulatory, and anti-leukotriene activity of the Olibanum oleogum resin (Bishai et al., 2003). Olibanum gum resin, as a medicinal plant, is traditionally used to treat various diseases including inflammatory ailment, arthritis, cardiac disorder and pain (Śallakī, 2001). Several studies showed that boswellic acids (BA) (Figure 2, B) are the major constituents of the Olibanum gum resin and they have anti-inflammatory, anti-cancerous and anti-ulcerous activities (Azadmehra et al., 2014). *Boswellia carterii* total extract showed high immunostimulant activity of up to 90% Lymphocyte transformation (Badria et al., 2003). Olibanum showed protective effects against CCl_4 and D-galactosamine-induced hepatotoxicity in the Institute of Cancer Research (ICR) mice (Chang et al., 2014).

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin that is widely used to induce toxic liver injury in a range of laboratory animals. CCl_4-induced hepatotoxicity is believed to involve two phases. The initial phase involves the metabolism of CCl_4 by cytochrome P450 to the trichloromethyl radical ($CCl_3^•$), which leads to lipid peroxidation (Edwards et al., 1993). Heme oxygenase-1, the rate-limiting enzyme in heme catabolism, is known to be induced by oxidative stress and to confer protection against oxidative stress injuries (Nakahira et al., 2003). The second phase of CCl_4-induced hepatotoxicity involves the activation of Kupffer cells, which is accompanied by the production of pro-inflammatory mediators (Planaguma et al., 2005). With liver function declined due to CCl_4 intoxication, caffeine metabolism gets ceased or at least decreased. The aim of this study was to investigate the use of serum caffeine metabolites as biomarkers of liver toxicity and to evaluate hepatoprotective activities of GZ and BA in CCl_4-induced hepatotoxicity.

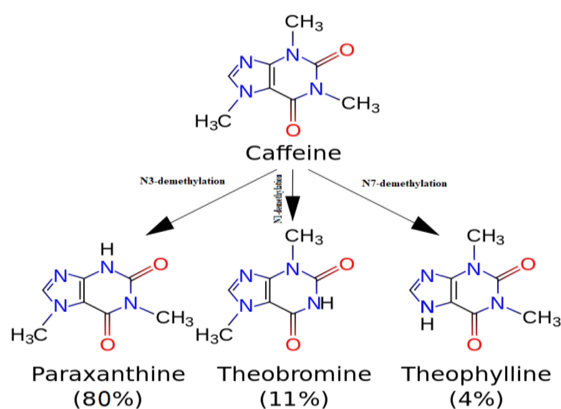

Figure 1. Caffeine metabolism in the liver ("Caffeine," 2018).

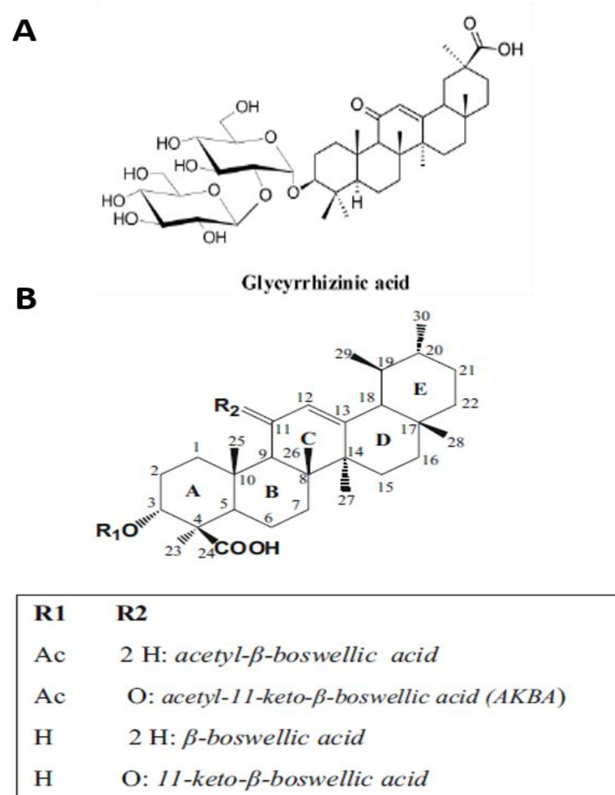

Figure 2. Chemical structure of glycyrrhizinic acid (A) and major bioactive triterpenoids isolated from the oleogum resin of *Boswellia carterii* (B) (Yusif et al., 2016).

MATERIAL AND METHODS

Animal care and treatments

All procedures with rats were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Research Ethics Committee at the Faculty of Pharmacy, Mansoura University (Mansoura, Egypt). Young albino male Sprague Dawley rats were obtained from Theodor Bilharz Institute, Imbaba, Cairo, Egypt at 6–8 weeks old with an average body weight of 150g. Rats were housed in well-ventilated opaque polypropylene cages. All animals had free access to balanced laboratory diet and water ad libitum. Animals were acclimatized to the housing conditions (12-h light/dark cycle, temperature 25°C, relative humidity (40%–60%) for at least 5 days prior to the initiation of experiment and were then divided randomly into five groups of 6-8 rats; group 1 (control which received only caffeine), group 2 (rats supplemented with caffeine and CCl_4), group 3 (rats supplemented with caffeine, CCl_4 and BA), group 4 (rats supplemented with caffeine, CCl_4, BA and GZ) and group 5 (rats supplemented with caffeine, BA and GZ). Caffeine, GZ, and BA were introduced orally to the animal at 2.857 mg/kg for caffeine and 20 mg/kg for both GZ and BA daily for 4 weeks. The doses were determined from lethal median dose (LD50) analysis conducted in our lab (the results are not included). CCl_4 was administered orally once a week for four weeks with doses 0.16, 0.24, 0.32 and 0.4 ml/kg for the first, second, third and fourth week, respectively (Pilon et al., 1988). All CCl_4 doses were mixed with corn oil (1:1 v/v) before administration to alleviate irritation.

Biochemical evaluation of liver function

After 4 weeks of intoxication, rats were sacrificed, and blood was collected for biochemical analysis. Serum was separated for the estimation of alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) activities according to Reitman and Frankel methods (Reitman & Frankel, 1957) and alkaline phosphatase (AP) activity according to Belfield, et al. method (Belfield & Goldberg, 1971).

Samples preparation for caffeine and theobromine detection

Each serum sample (500 µl) was mixed and vortexed thoroughly with 200 µl of 0.01 M HCl. The mixture was extracted with 4.0 ml of ethyl acetate and isoamyl alcohol (49:1 v/v) and finally centrifuged at 3000 rpm for 15 min. One milliliter of the organic layer was separated, concentrated, and evaporated under vacuum at 40^0C. The obtained residue was reconstituted in 200 µl of toluene, isoamyl alcohol and isolectin (83:13:3v/v) to be used for GC-MS.

Gas chromatography-mass spectrometry (GC/MS)

GC/MS (Biochemistry Department, University of Saint Paul, MN, USA) was performed using Varian Saturn 3Ms ion trap with star 3400 CX GC. The GC conditions were as the following; DB5 MS 30-meter column with 0.25 mm internal diameter and 0.25 µ film thickness, temperature program was 60°C to 260°C with a flow rate of 10 µl per minute, 4.5 minutes at maximum temperature, and both injection port and Xfer line temperatures were 230^0C. MS scan was for mass from 51 to 220 Dalton with 0.31 second per scan time for 10 to 20 minutes.

Caffeine and theobromine retention times were determined using standard solutions of both. Standard curves were established for both of them and the curves equations were used to determine caffeine and theobromine concentrations in the plasma samples by solving the equation for x values (µvolt) at the specific retention time (3.696 and 3.794 minutes for caffeine and the theobromine, respectively) to get y values which represent caffeine and theobromine concentrations in the injected volumes. Caffeine to theobromine ratio (C/T ratio) was calculated for each animal group.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.01. One-Way ANOVA was used to calculate the significance of the results (P value) with α = 0.05 (95% confidence interval). Dunnett test was used and results were compared with the caffeine group.

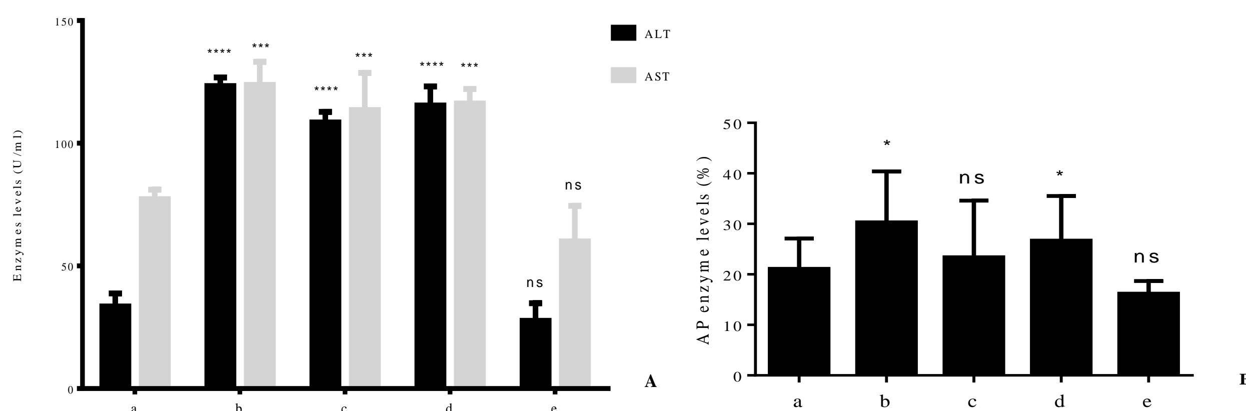

Figure 3. Comparison of mean values of ALT, AST (A) and AP (B) levels in animals' sera in different groups; a. Caffeine, b. Caffeine + CCl_4, c. Caffeine + CCl_4 + BA, d. Caffeine + CCl_4 + BA + GZ, e. Caffeine + BA + GZ

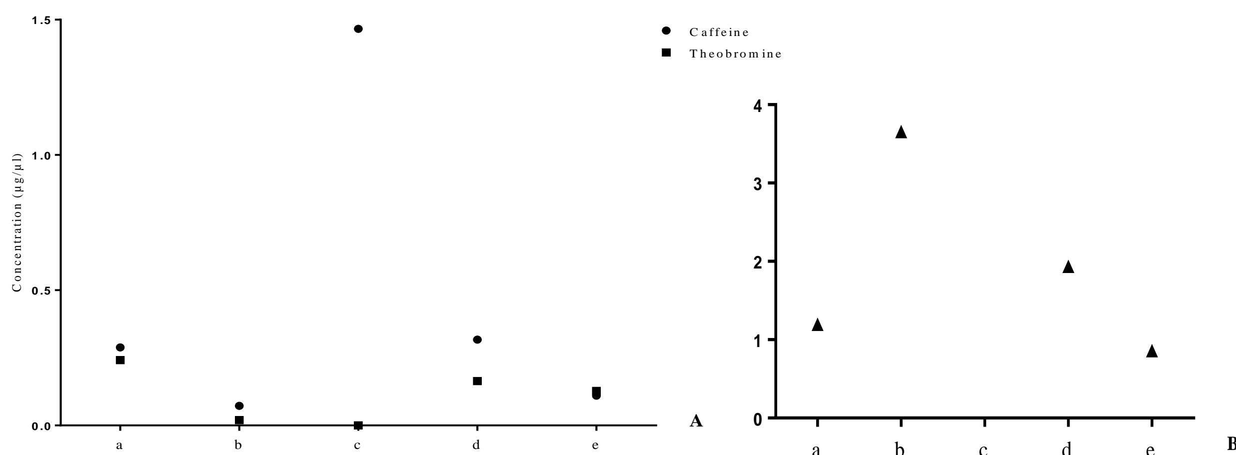

Figure 4. Concentrations of caffeine and theobromine (A) and C/T ratios (B) in sera in different groups; a. Caffeine, b. Caffeine + CCl_4, c. Caffeine + CCl_4 + BA, d. Caffeine + CCl_4 + BA + GZ, e. Caffeine + BA + GZ.

Table 1. Mean values of liver enzymes levels in rats' serum for each of the groups.[a]

Groups	ALT (U/ml)		AST (U/ml)		AP (%)	
Caffeine	33.5 ± 5.3		77.2 ± 3.9		21.1 ± 6.0	
Caffeine + CCl_4	123.3 ± 3.5	****	123.8 ± 9.4	***	30.3 ± 10.1	*
Caffeine + CCl_4 + BA	108.5 ± 4.3	****	113.5 ± 15.2	***	23.4 ± 11.2	ns 0.27
Caffeine + CCl_4 + BA + GZ	115.4 ± 7.8	****	116.2 ± 5.9	***	26.7 ± 8.8	*
Caffeine + BA + GZ	27.6 ± 7.2	ns 0.15	60.0 ± 14.5	ns 0.19	16.2 ± 2.5	ns 0.06

[a] Data is represented as mean ± standard deviation (SD); ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant (P value is represented).

RESULTS

Rats treated with CCl_4 showed a significant increase in the average level of all liver enzymes in serum in comparison with those in the control group who received only caffeine as presented in Table 1. On the other hand, all rats treated with CCl_4 and receiving BA and BA + GZ showed a slight reduction in enzymes levels. Rats treated with either caffeine or BA only and combined BA and GZ without receiving CCl_4 exhibited a

reduction in liver enzymes when compared to the control group as shown in Figure 3.

Table 2. Caffeine and theobromine concentrations results in rats' serum for each of the groups determined by GC/MS, and C/T ratio.

Groups	Caffeine (C)				Theobromine (T)				C/T ratio	
	Peak area (μvolt)	Volume injected (μl)	Split ratio	Concentration (μg/μl)	Peak area (μvolt)	Volume injected (μl)	Split ratio	Concentration (μg/μl)		
Caffeine	5810	5	1:10	0.2883	6720	5	1:10	0.2412	1.1953	
Caffeine + CCl_4	1460	5		0.0726	553	5		0.0199	3.6565	****
Caffeine + CCl_4 + BA	35400	6		1.4665	-	-		-	-	-
Caffeine + CCl_4 + BA + GZ	3830	3		0.3173	2740	3		0.1639	1.9358	****
Caffeine + BA + GZ	2640	6		0.1098	4260	6		0.1274	0.8583	***

****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant (*P* value is represented).

Contrary to the obtained biochemical data; caffeine/theobromine (C/T) ratio of the sera of intoxicated rats revealed an increase in caffeine concentration and very low metabolite concentration (theobromine) when compared with normal rats as presented in Figure 4. Rats receiving caffeine, CCl_4, BA, and GZ had an improved C/T ratio in comparison with those treated with CCl_4 as shown in Table 2. Also, rats receiving caffeine, BA and GZ had a lower C/T ratio than the control group. On the other hand, rats receiving Caffeine, CCl_4 and only BA had no traceable concentrations of theobromine in their serum.

DISCUSSION

The correct staging of liver function is critical for guiding the treatment of liver injury or fibrosis (Huang et al., 2015). Abnormal liver function tests are associated with other disorders e.g. acute heart failure (Biegus et al., 2016), cardiogenic shock (Jantti et al., 2017), non-alcoholic fatty liver (Stollenwerk et al., 2016), Lyme disease associated with erythema migrans (Horowitz et al., 1996) and hemolysis (Jamjute et al, 2009). As a consequence, it is vital to find a more reliable and noninvasive method to determine liver function with high accuracy and minimum interactions. Caffeine and theobromine concentrations in rats' serum were calculated accurately using GC/MS. The significant difference in C/T ratios between control animals

and those treated with CCl_4 indicates the possibility of using caffeine and its metabolites concentrations in serum to accurately determine the status of the liver. The promise of using caffeine metabolism as an indicator of liver function test is not only due to the ease of the method, but also because it determines the metabolic function of the liver without interaction with other illnesses or disorders at variance of liver enzymes as ALT and AST which only determine the physical integrity of the liver cells and their abnormal levels are associated with many other disorders. C/T ratio is calculated using noninvasive assays with minimum implications on the patient because caffeine is widely consumed in many food and beverage items (Begas et al., 2007). Also, caffeine was proven to be associated with a lower risk of liver fibrosis in patients with HCV infection (Modi et al., 2010).

The present study also revealed that BA and GZ together have an improving and protective effect on liver function in rats treated with CCl_4, which is known to induce liver injury. BA in rats receiving CCl_4 caused no significant improvement in liver function. These findings ensure the significance of natural compounds in the field of drug discovery. The results are in full agreement with published data of Badria et al., which showed that both GZ and BA extracts exhibited hepatoprotective action in rats treated with CCl_4 with a significant reduction in the hepatic damage (Badria et al., 2003). Also, BA may just have a protective and anti-inflammatory effect on the liver (Azadmehra et al., 2014), but doesn't influence the metabolic mechanisms of the liver. Results also correlate with the reported protective mechanisms of BA and GZ on acute liver injury induced by CCl_4 (Azadmehra et al., 2014; Lee et al., 2007). Due to the minimum side effects of natural compounds and the wide consumption of their sources, these results may have clinical implications in the near future.

Further studies should be conducted to determine the *in vivo* mechanism of BA and GZ, bioavailability and their biochemical targets. C/T ratio can be used as a liver function after further improvement, determination of the reference values in populations, and determination of optimal doses ingested before the test. Also, more practical assays for the determination of caffeine and its metabolites should be established.

CONCLUSION

This study revealed the usefulness of using GC-MS in measuring Caffeine/theobromine (C/T) ratios as a biomarker for liver injury or intoxicated liver and/or early fibrosis without any interactions of false positive common liver function tests. Also, the obtained results confirmed the hepatoprotective effect both pentacyclic triterpenes, GZ and BA, in this model.

Conflict of interest statement

The author has declared that no competing or conflict of interests exists.

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