Comparative Evaluation of Hepatoprotective Activities of Geniposide, Crocins and Crocetin by CCl\textsubscript{4}-Induced liver Injury in Mice

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Abstract

Iridoid glycosides (mainly geniposide) and crocetin derivatives (crocins) are the two major active constituents in Gardenia jasminoides Ellis. In the present study, geniposide, crocins, crocin-1 and crocetin were separated from gardenia chromatographically. Then, mice were orally administrated with geniposide (400 mg/kg b.w.), crocins (400 mg/kg b.w.), crocin-1 (400 mg/kg b.w.) and crocetin (140 mg/kg b.w.) once daily for 7 days with CCl\textsubscript{4}. Hepatoprotective properties were evaluated by biochemical parameters: Administration of geniposide, crocins, crocin-1 and crocetin significantly lowered serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) levels in CCl\textsubscript{4}-treated mice. The reduced glutathione (GSH) levels and antioxidant enzymes (SOD and CAT) activities were also increased by geniposide, crocins, crocin-1 and crocetin. Histopathological examination of livers showed that these components reduced deformability, irregular arrangement and rupture of hepatocyte in CCl\textsubscript{4}-treated mice. These biochemical results and liver histopathological assessment demonstrated that geniposide, crocetin derivatives and crocetin show comparative beneficial effects on CCl\textsubscript{4}-induced liver damage via induction of antioxidant defense. Therefore, contents of geniposide and crocetin derivatives should be both considered for hepatoprotective efficacy of Gardenia jasminoides Ellis.

Key Words: Gardenia jasminoides Ellis, Hepatoprotective, Geniposide, Crocin-1, Crocetin, Histopathological examination

INTRODUCTION

Gardenia jasminoides Ellis (Rubiaceae) is an evergreen shrub widely distributed in the tropical and subtropical regions, growing on mountain slopes or on road sides as an ornamental plant. The dried ripe fruits of this plant have been recorded as Fructus Gardeniae (Chinese herbal name is “zhizi”) in Chinese Pharmacopoeia and included in Traditional Chinese Medicine (TCM) formulations for diuretic, cholagogue, anti-inflammatory, and antipyretic effects (National Commission of Chinese Pharmacopoeia, 2010). Iridoid glycosides (mainly geniposide) and crocetin derivatives (crocins) are the two major active constituents in gardenia fruits (Fig. 1), of which components, characteristics, and activities have been investigated by many researchers (Choi et al., 2001; Wang et al., 2004). In addition, it has been reported that genipin (aglycone of geniposide) has hepatoprotective effect (Kim et al., 2010) and an in vitro study showed that crocetin has protective effect on hepatocyte (Tseng et al., 1995). However, to the best of our knowledge, there is no published literature heretofore concerning the roles of geniposide and crocetin derivatives from gardenia fruits on the CCl\textsubscript{4}-induced hepatic injury in vivo.

The liver is the major site of xenobiotic metabolism and its injury can be caused by toxic chemicals, drugs, and virus in filtration from ingestion or infection (Lee et al., 2007; Mihailović et al., 2013). Conventional drugs used in pharmacotherapy, such as steroids, vaccines, and antiviral drugs, have shown limited therapeutic benefits and are usually associated with serious risks of toxicity. In the absence of a reliable liver protective drug in the modern system of medicine, natural extracts from me-
dicinal plants are considered to be effective and safe for the treatment of liver disorders (Jaishree and Badami, 2010). The present study, therefore, focused on the investigation of the biological activities of geniposide and crocetin derivatives from gardenia fruits on the CCl4-induced hepatic injury. Additionally, all crocins are hydrolyzed to yield the same aglycone (crocetin) in intestine before they are absorbed into the blood (Asai et al., 2005). Whether the pharmacokinetics process could influence biological effects of crocetin derivatives, however, has remained unknown. Therefore, current investigation also aimed at comparatively evaluating hepatoprotective effect of orally administered crocins and its aglucone crocetin.

**MATERIAL AND METHODS**

**Plant materials**

The dried gardenia (*Gardenia jasminoides* Ellis) fruits were harvested in Yibin City, Sichuan Province, in November 2012, and identified by Hao Zhang. The voucher specimens are deposited at the Department of Pharmacognosy, West China School of Pharmacy, Sichuan University, Sichuan Province, China.

**Extraction and isolation**

Separation and preparation of geniposide, crocins, crocin-1 and crocetin were conducted according to our previous study with minor modification (Chen et al., 2008a, 2008b). The dried ripe gardenia fruits (2.5 kg) were ground to coarse powder and extracted with 25 L of 40% ethanol (v/v) by cold percolation. After concentration by rotary evaporator, the extract was dissolved in water and then subjected to a HPD-100 macroporous resin. The column was eluted with water containing increasing amounts (0, 25, 40, 60%, v/v) of ethanol. The dried residue of 25% alcohol-eluted fraction was dissolved in water and then subjected to HPD-100 macroporous resin column eluting with water, 10, 25 and 35% ethanol. The 35% alcohol-eluted fraction was evaporated to dryness and pure geniposide (1) was obtained. The 60% alcohol-eluted fraction (crocins, 2) was subjected to silica gel column chromatography, and eluted with ethylacetate, containing increasing amounts (10, 20, 30, 50%, v/v) of methanol-water (16:13 v/v). A red powder obtained from the fraction of ethylacetate containing 30% methanol-water was crocin-1 (3). Crocetin (4) was prepared by alkaline hydrolyzation with 10% potassium hydroxide solution of the total crocins (Qian et al., 2010).

**HPLC analysis**

The HPLC was performed as described previously (Jia et al., 2005), on a Shimadzu HPLC system equipped with two LC-10AT VP pumps, CTO-10AS VP column oven, UV-vis SPD-10A VP detector, SCL-10A VP system controller and fitted with a ODS column (4.6x150 mm, 5 μm; Agilent, USA). For determination of crocins and crocetin, the mobile phase was consisted of A (acetonitrile) and B (pure water), which was programmed as follows: from 0 to 15 min, 10 to 30% A. 15.0-20.0 min, linear increase from 30 to 35% A. 20.0-25.0 min, linear increase from 35 to 70% A. 25.0-30.0 min, 70% A. 30.0-40.0 min, linear decrease from 70 to 10% A. For determination of geniposide, isocratic elution of 15% acetonitrile was used according to the literature (National Commission of Chinese Pharmacopoeia, 2010). The flow rate was 1.0 ml/min while chromatogram were recorded at 440 nm for crocetin derivatives and 238 nm for geniposide, respectively.

**Animals and treatment**

All the animal experimental procedures were approved by the Animal Care and Use Committee of Sichuan University for Nationalities (Chengdu, China), and were conducted upon receipt of the approval, numbered 2014-231, from the Animal Care and Use Committee of Sichuan University. Male Kunming mice (20-22 g) were obtained from the Experimental Animal Center of Sichuan University (Chengdu, China). The animals were housed at 25 ± 2°C under a 12 h light/12 h dark cycle with access to food and water ad libitum. After acclimation for 1 week, the animals were randomly divided into seven groups comprising six mice (n=6) in each group as follows:

- **Group A**: sterile distilled water (10 ml/kg b.w., i.g), served as a normal control.
- **Group B**: sterile distilled water (10 ml/kg b.w., i.g) served as a CCl4 control.
- **Group C**: 400 mg/kg (b.w.) of Geniposide.
- **Group D**: 400 mg/kg (b.w.) of Crocins.
- **Group E**: 400 mg/kg (b.w.) of Crocin-1.
- **Group F**: 140 mg/kg (b.w.) of Crocetin.
- **Group G**: 100 mg/kg (b.w.) of biphenylidicarboxylate pills (BP).

2 h after the oral administration on the sixth day, all mice except those in the normal control group were given simultaneously a CCl4-peanut oil mixture (1:1, v/v intraperitoneally, 2 ml/kg b.w.), while the normal control group received peanut oil alone. Then all the animals were fasted for 18 h and were sacrificed by cervical dislocation with the last oral administr-
tion 1 h before. Livers were dissected out immediately and blood was collected, allowed to clot, and serum was separated for assessment of enzyme activity. The weights of the liver and kidney were measured. Some specimens were properly stored at -80°C for pending tests. Part of the liver tissue was immediately transferred into 10% formalin for histopathological investigation.

**Measurement of serum biochemical markers**

Collected blood samples were stored at 4°C for 2 h and centrifuged at 3000 rpm for 10 min at 4°C to obtain the serum. The level of total protein (TP) and the activities of ALT, AST and ALP were determined using commercial reagent kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) according to the instruction manuals.

**Measurement of SOD, CAT and GSH in liver homogenate**

Liver homogenates (10.0%, w/v) were prepared with 50 mmol/L cold potassium phosphate buffer (pH 7.4). The resulting suspension was centrifuged at 2000 rpm for 10 min, and the supernatant was collected for further analysis. All treatments were done at 4°C. Protein and GSH concentration and the activities of SOD and CAT were assayed using commercial reagent kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China) according to the instruction manuals.

**Histopathological examination**

Liver slices were fixed with 10% formalin in phosphate buffered saline for 24 h and embedded in paraffin. Sections of 5 μm in thickness were cut, deparaffinized, dehydrated, stained with haematoxylin-eosin (HE) and observed under microscope to evaluate histopathological lesions in the livers. Photographs of each of the slides were taken at 100× magnification.

**Table 1. Percentage of each ingredient**

| Ingredients | Geniposide | Crocins | Crocin-1 | Crocetin |
|-------------|------------|---------|----------|----------|
| Percentage of weight | 96.6% | 93.8% | 96.9% | 98.1% |

![Fig. 2. The HPLC-UV chromatograms. (A) geniposide, (B) crocins, (C) crocin-1 and (D) crocetin.](http://dx.doi.org/10.4062/biomolther.2015.094)
Statistical analysis
The data were analyzed using SPSS 19 (SPSS Inc., Chicago, USA), expressed as the means ± SD and statistically analyzed by one way analysis of variance (ANOVA) test and \( p<0.05 \) was considered significant.

**RESULTS**

HPLC/MS analysis for the purified compounds and fractions
In current study, separation of 2.5 kg of gardenia fruits yielded components 1-4. Identification of the purified compounds and fraction was carried out by TLC, HPLC-UV, LC-MS and NMR, in comparison with those of reference chemicals. The main chemical in component 1 was geniposide, the major fragmentation of which was confirmed by \([\text{M+Na}]^+\) at 411.1260 (Calcd for \(\text{C}_{17}\text{H}_{24}\text{O}_{11}\text{Na} [\text{M+Na}]^+\), 411.1267). In HRESIMS spectrum of component 3, 999.3671 in \([\text{M+Na}]^+\) indicated the presence of crocin-1 (Calcd for \(\text{C}_{44}\text{H}_{64}\text{O}_{24}\text{Na} [\text{M+Na}]^+\), 999.3685). In addition, 351.1549 in \([\text{M+Na}]^+\) of component 4 confirmed the presence of crocetin (calcd for \(\text{C}_{20}\text{H}_{24}\text{O}_{4}\text{Na} [\text{M+Na}]^+\), 351.1572).

These components were further identified by TLC, HPLC and NMR with reference chemicals obtained in our previous study. Additionally, the purities of components 1, 2, 3 and 4 were determined, respectively, by HPLC with reference chemicals geniposide, crocin-1, crocin-2, crocin-3 and crocetin (Table 1, Fig. 2).

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**Table 2. Effect of geniposide, crocins, crocin-1 and crocetin on liver and kidney index in CCl₄-induced liver damage model**

| Groups    | Administration | Doses (mg/kg) | n  | Body weight (g) | Liver (%) | Kidney (%) |
|-----------|----------------|---------------|----|-----------------|-----------|------------|
| A         | Normal control | -             | 6  | 30.6 ± 1.04     | 5.31 ± 0.16 | 1.59 ± 0.10 |
| B         | CCl₄ model     | -             | 6  | 28.6 ± 0.82**   | 6.17 ± 0.27** | 1.65 ± 0.06** |
| C         | Geniposide     | 400           | 6  | 29.5 ± 0.69     | 5.86 ± 0.33 | 1.52 ± 0.06** |
| D         | Crocins        | 400           | 6  | 30.2 ± 1.74     | 5.70 ± 0.11** | 1.47 ± 0.06** |
| E         | Crocin-1       | 400           | 6  | 29.2 ± 1.18     | 5.95 ± 0.16 | 1.52 ± 0.06** |
| F         | Crocetin       | 140           | 6  | 29.8 ± 0.95*    | 5.67 ± 0.18* | 1.47 ± 0.05** |
| G         | Biphenyldicarboxylate | 100       | 6  | 28.7 ± 1.28     | 6.05 ± 0.24 | 1.55 ± 0.04** |

Values are mean ± SD (n=6). "**p<0.01 vs. Normal, "*p<0.05 vs. Normal; "**p<0.01 vs. CCl₄-treated group, "*p<0.05 vs. CCl₄-treated group.

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**Fig. 3.** Effects of geniposide and crocin derivatives on serum enzymes in CCl₄-induced hepatotoxicity in mice (n=6). "a" \( p<0.01 \); "b" \( p<0.05 \) vs. CCl₄ control.
Effect of chemicals on the animal vital signs and tissue index

After the administration of carbon tetrachloride, there was no death case in normal control group and the therapies groups, while three deaths occurred in model control group. Moreover, apathetic condition with emaciation and the rising of liver and kidney indexs (liver and kidney weight as a percentage of body weight) in mice were mostly observed in the model control group, and the therapies groups significantly improved those symptoms of liver damaged mice, among which, crocetin group showed the best effect. Liver index and kidney index in each group were listed in Table 2. It was observed that the treatment with crocins and crocetin resulted in a significant reduction in liver index ($p<0.05$), and interestingly, all drugs treated animals shown a remarkable reduction in kidney index ($p<0.01$).

In vivo antioxidant and hepatoprotective activity

Administration of animals with carbon tetrachloride resulted in an acute hepatotoxicity which can be revealed from the levels of serum marker enzymes and the liver antioxidant levels (Fig. 3, 4). Significant increase ($p<0.01$) in ALT, AST and ALP levels (Fig. 3) in the serum were observed in CCl$_4$-intoxicated group (ALT 95.38 ± 3.60 IU/L; AST 122.86 ± 6.62 IU/L; ALP 18.79 ± 1.44 IU/L) if compared with the normal control group (ALT 52.13 ± 2.36 IU/L; AST 32.48 ± 2.70 IU/L; ALP 11.55 ± 1.04 IU/L) (Fig. 3). However, the levels of these enzymes were significantly decreased in mice treated with geniposide (400 mg/kg) (ALT 70.32 ± 2.58 IU/L; AST 89.39 ± 2.29 IU/L, $p<0.01$; ALP 15.23 ± 2.88 IU/L, $p<0.05$), crocins (400 mg/kg) (ALT 72.60 ± 1.59 IU/L; AST 104.07 ± 10.47 IU/L, $p<0.01$; ALP 14.39 ± 2.18 IU/L, $p<0.01$), crocin-1 (400 mg/kg) (ALT 76.20 ± 2.00 IU/L; AST 111.92 ± 7.42 IU/L, $p<0.01$; ALP 16.74 ± 1.74 IU/L, $p<0.05$) and crocetin (140 mg/kg) (ALT 59.48 ± 1.81 IU/L; AST 84.59 ± 5.07 IU/L, $p<0.01$; ALP 13.20 ± 1.75 IU/L, $p<0.01$) if compared with CCl$_4$-intoxicated group, although this decrease was minimum in the group receiving crocin-1. On the other hand, the model control group had considerably lower TP level (40.05 ± 0.76 mg/ml) than normal control group (49.33 ± 2.15 mg/ml, $p<0.01$). However, crocins (400 mg/kg) (47.49 ± 4.59 mg/ml, $p<0.01$) and crocetin (140 mg/kg) (48.12 ± 3.01 mg/ml, $p<0.01$) treated groups resulted in significant

Fig. 4. Effects of geniposide and crocetin derivatives on liver antioxidant levels in CCl$_4$-induced hepatotoxicity in mice (n=6). *$p<0.01$; $p<0.05$ v.s. CCl$_4$ control.

Fig. 5. Effect of geniposide and crocetin derivatives on hepatic cells in liver tissue of CCl$_4$-induced liver damage mice (n=6). Sections are 5 μm thick and photomicrographs are taken at 100×. (A) Normal control, (B) CCl$_4$ control, (C) geniposide 400 mg/kg b.w.+CCl$_4$, (D) crocins 400 mg/kg b.w.+CCl$_4$, (E) crocin-1 400 mg/kg b.w.+CCl$_4$, (F) crocetin 140 mg/kg b.w.+CCl$_4$, (G) biphenyldicarboxylate 100 mg/kg b.w.+CCl$_4$. 

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improvement in TP level (Fig. 3). Generally, treatments with geniposide, crocins, crocin-1 and crocetin showed significant hepatoprotective activity and, crocins and crocetin seemed more effective.

The effects of geniposide, crocins, crocin-1 and crocetin treatments on the activities of SOD and CAT and the cellular antioxidant GSH in the liver are shown in Fig. 4. The activities of SOD and CAT and the levels of GSH in the CCl₄-treated group were significantly \((p<0.05)\) decreased \((34.13 \pm 2.59 \text{ U/mg protein, } 21.89 \pm 2.35 \text{ U/g protein and } 3.54 \pm 0.23 \text{ g GSH/L; } p<0.01)\) when compared with the normal control mice. This decreasing activities were restored very significantly in the geniposide \((400 \text{ mg/kg})\) \((74.73 \pm 1.77 \text{ U/mg protein, } 97.74 \pm 12.08 \text{ U/g protein and } 4.37 \pm 0.73 \text{ g GSH/L})\), crocins \((400 \text{ mg/kg})\) \((66.97 \pm 3.36 \text{ U/mg protein, } 92.27 \pm 9.44 \text{ U/g protein and } 4.16 \pm 0.36 \text{ g GSH/L})\), crocin-1 \((400 \text{ mg/kg})\) \((61.30 \pm 2.49 \text{ U/mg protein, } 114.27 \pm 4.12 \text{ U/g protein and } 4.04 \pm 0.31 \text{ g GSH/L})\) and crocetin \((140 \text{ mg/kg})\) \((54.02 \pm 2.72 \text{ U/mg protein, } 100.53 \pm 8.89 \text{ U/g protein and } 4.36 \pm 0.61 \text{ g GSH/L})\) treated groups \((p<0.01\text{ except crocin-1 and crocetin in GSH model } p<0.05)\).

Histopathological examination

In order to assess the liver histological changes, hematoxylin and eosin staining of liver tissue sections from each group were examined. There were no pathological changes in normal control livers of healthy lobular architecture with central veins and radiating hepatic cords (Fig. 5). In the CCl₄ model group, severe liver pathological changes characterized by deformability, irregular arrangement and rupture of hepatocyte. In addition, condensation of nucleus, nuclear membrane shrinkage and destruction occurred, and many vesicles appeared in the cytoplasm. These pathological changes reflected CCl₄-induced acute liver injury. Compared with the CCl₄-model group (Fig. 5), the pathological changes of hepatocytes induced by CCl₄ was obviously improved by the treatment of compounds.

DISCUSSION

The whole separation of crocins and geniposide is a crucial and challenging task for pharmacological investigation of gardenia fruits. In our previous study, crocin-1 and crocetin were separated from gardenia fruits by various chromatographic means (Chen et al., 2008a, 2008b, 2010). In the present work, crocetin was prepared by alkaline hydrolyzation of the total crocins. Therefore, the yield of crocetin is significantly higher than that of the previous method. Additionally, high purities of all obtained components were achieved with the aid of the multiple chromatographic steps and the purified components were characterized and identified by HRESIMS, ¹H NMR and ¹³C NMR (data not shown).

CCl₄ is widely used to induce liver injury in laboratory rodents (Seifert et al. 1994; Manubolu et al., 2014; Cao et al., 2015). Increased levels of serum transaminases reflect hepatic injury as the enzymes are released into circulation following the exposure. CCl₄ initially causes necrosis and steatosis and may lead to fibrosis, cirrhosis, and hepatocellular carcinoma when administered at higher dosages (Pierce et al., 1987). Therefore, CCl₄-induced hepatic insult was selected for the hepatoprotective evaluation of components from gardenia. The increased levels of ALT, AST, ALP, and LDH are conventional indicators of liver injury (Thabrew et al., 1987). The present study revealed a significant increase in the activities of ALT, AST and ALP levels in rat serum on exposure to CCl₄, indicating considerable hepatocellular injury (Bhodave et al., 2014; Raj and Gothandam, 2014). However, the serum ALT, AST and ALP activities significantly declined by treatment with geniposide \((400 \text{ mg/kg})\), crocins \((400 \text{ mg/kg})\), crocin-1 \((400 \text{ mg/kg})\) and crocetin \((140 \text{ mg/kg})\), implying that these components can rise up stabilization of plasma membrane and ameliorate biliary dysfunction effectively thereby preserving the structural integrity of cells as well as the repair of CCl₄-induced liver damage, which is further confirmed by the reduced amount of histopathological injury. To compare biological effect of these components in terms of ALT, AST and ALP levels, current data indicated crocins and crocetin feature stronger hepatoprotective effect if compared with geniposide and crocin-1 (Fig. 3). In this study, total protein level in the serum was measured as well. CCl₄ expectedly reduced serum total protein, while only the treatment of crocins \((400 \text{ mg/kg})\) and crocetin \((140 \text{ mg/kg})\) remarkably restored the proteins content \((p<0.05)\).

The body has a set of endogenous antioxidant enzymes as an effective defense mechanism to prevent and neutralize the free radical-induced damage (Bansal et al., 2005). In order to evaluate the antioxidant activities of geniposide, crocins and crocetin in vivo, we determined the activities of antioxidant enzymes (CAT and SOD), as well as the levels of GSH in mice liver. As enzymatic antioxidant systems, both SOD and CAT play important roles in protection against the deleterious effects of hydrogen peroxide and lipid peroxidation in diseases related to oxidative stress (Zhu et al., 2012; Abdelaziz and Ali, 2014). As a non-enzymatic antioxidant, GSH, which plays an important role in maintaining the body’s antioxidant defense mechanism, conjugates with free radicals directly to protect the integrity of cell membranes (He et al., 2012). In the present studies, we observed that the levels of CAT, SOD and GSH were significantly lower in CCl₄-induced liver injury mice as compared with those of normal control group, representing severe oxidative stress status to hepatic cells. Treatment with geniposide \((400 \text{ mg/kg})\), crocins \((400 \text{ mg/kg})\), crocin-1 \((400 \text{ mg/kg})\) and crocetin \((140 \text{ mg/kg})\) resulted in restoration of antioxidant enzymes activity and GSH level in CCl₄-induced liver injury mice. To compare antioxidant activities of these components, the present study implied that geniposide, crocins, crocin-1 and crocetin have comparative in vivo antioxidant effect. A series of crocetin glycosides (crocins), but not crocetin, are the main pigments of gardenia (Chen et al., 2010). Literature showed that orally administered crocetin was rapidly absorbed into the blood circulation and orally administered crocins are hydrolyzed to crocetin before or during intestinal absorption, and absorbed crocetin is partly metabolized to mono- and diglucuronide conjugates which may be biologically active (Asai et al., 2005). Thus the concentration-time profile of plasma crocetin was considerably different from that in crocins-administered mice. In current study, stronger activity of crocetin in comparison with that of crocin-1 indicated the process of this hydrolyzation probably influence hepatoprotective activity of crocins.

In the present investigation, the encouraging findings indicated that geniposide, crocetin derivatives and crocetin, separated and purified from Gardenia jasminoides Ellis, show com-
paratively beneficial effects on CCl4-induced liver damage via induction of antioxidant defense and these biological effects were further confirmed by histological observations. It is demonstrated that hydrolyzation might influence hepatoprotective effect of crocin derivatives. In light of our observations, we may draw a conclusion that geniposide and crocin derivatives are probably both responsible for hepatoprotective properties of gardenia, and therefore, contents of geniposide and crocin derivatives should be considered for hepatoprotective evaluation of Gardenia jasminoides Ellis.

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DECLARATION OF INTEREST

The authors declare that there is no conflict of interest.

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