Original Article

Antioxidant and protective effect of an oleanolic acid-enriched extract of *A. deliciosa* root on carbon tetrachloride induced rat liver injury

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The ethanol-water extract of *A. deliciosa* root (EEAD) was fractionated into n-hexane (EEAD-He), ethyl acetate (EEAD-Ea), n-butanol (EEAD-Bu) and aqueous (EEAD-Aq) fractions according to their different polarity and solubility. Among the four extracts, it was found that EEAD-Bu was enriched with oleanolic acid (OLA). The antioxidant and hepatoprotective activities of various EEAD fractions and OLA were carefully investigated by the methods of ferric thiocyanate (FTC) and thiobarbituric acid (TBA), as well as the model of CCL_4-induced liver toxicity in rats. The results showed that the EEAD-Bu had higher *in vitro* antioxidant and *in vivo* hepatoprotective activities than those of the other types of extracts ($p < 0.05$). When the CCL_4-induced rats were treatment with 120 mg/kg EEAD-Bu, the activities of alanine transaminase (ALT) and aspartate transanimase (AST) in rat serum decreased 90 % and 81 %, respectively, as compared with those of the CCL_4 control rats. Furthermore, the lipid peroxidation (MDA) decreased 42 % and glutathione (GSH) increased 114 % in the rats liver homogenate, as compared with those of the control. The results also indicated that the hepatoprotective activity of the EEAD-Bu (at the dose of 120 mg/kg) was higher than that of the reference drug *silymarin* (at the dose of 60 mg/kg), and OLA acted as an important role in dose-dependent protection against CCL_4 hepatotoxicity. The findings indicate that the OLA-enriched EEAD-Bu extract had significant and concentration dependent hepatoprotective effect for the carbon tetrachloride induced rat liver injury.

Key Words: *A. deliciosa* root, oleanolic acid-enriched extract, CCL_4, antioxidant, hepatoprotective

Introduction

A. deliciosa (*A. Chev.*) *C. F. Luing et A. R. Ferguson* (*ADF*) is a subfamily of the genus *Actinidias*, which was also named as *Chinese gooseberry*, *kiwifruit*, *Yangtao*, etc, in Chinese.The subfamily consists of 55-60 species. Among them, *ADF* is intensely cultivated all over the world and the fruit has been acclaimed for its native and medicinal values.[1]

In China, the *ADF* root has been used for long time as the traditional drugs[1], such as agents of anti-hepatotoxic, anti-pyorrhea and anti-gingival inflammation. Furthermore, the ethanol extracts of *ADF* root had been proven to possess anticancer properties *in vitro*[2] and *in vivo*.[3-4] It was found that the main constituents of ethanol extracts from *Actinidia* root was triterpenoid.[2-3] Oleanolic acid (OLA) (Fig 1), one of the triterpenoid constituents of *ADF* root, could protect mice against carbon tetrachloride (CCl_4)-induced hepatotoxicity,[5-7] and inhibit lipid peroxidation in rat liver microsomes.[8] However, few studies have been reported on the effect of ethanol extracts from *ADF* root on liver damage caused by hepatotoxicants. In order to evaluate the hepatoprotective value of plant root on liver dysfunction, in this study, the hepatoprotective and antioxidant effects of oleanolic acid-enriched fraction, obtained from *ADF* root, on CCl_4-induced liver injury in rats was carefully investigated. The hepatoprotective activity of oleanolic acid was also investigated for comparison.

Materials and methods

Design

The ethanol-water extract of *A. deliciosa root* (EEAD) was fractionated into different fractions, of which OLA quantification, *in vitro* antioxidant activity and *in vivo* hepatoprotective activity were assessed. Moreover, the hepatoprotective effect of OLA-enriched fraction was further investigated *in vivo*, compared with that of OLA.

Subjects

Wistar albino rats (140 ± 20 g) of either sex, procured from Nanjing Medical University (Nanjing China) were used for the study. The animals were housed in large polypropylene cages and allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 20 ± 2 ^{o}C and 50 ± 5 % relatively humidity with a 12-hour dark/

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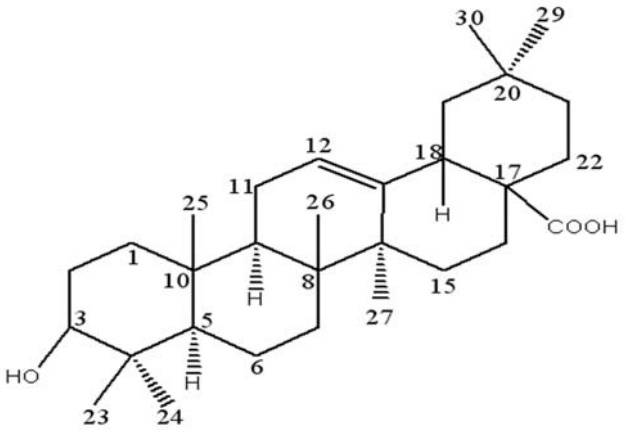

Figure 1. The structure of oleanolic acid (OLA)

light cycle, and acclimatized for at least one week before use. The roots of *ADF* were collected from the plants grown in the campus of School of Medicine, Jishou University (Hunan Province, China) in November, 2004, and identified by Prof. Liu Zhonghua, the Department of Botany, Jishou University. A voucher specimen had been deposited in The Key Laboratory of Food Science & Safety, Ministry of Education, Southern Yangtze University, Wuxi China, Vide accession No. 2004036.

Preparation and quantification of the extracts

The dried and powdered *ADF* root (1 kg) was extracted with 60 % ethanol-water (v/v) (6L ×3) under 45 ^{o}C for 8 hours each time. The combined extract was cooled to room temperature (25 ^{o}C) and filtered through muslin. Then the filtrate was concentrated under the environment of reduced pressure (45 ^{o}C, 0.1 MPa, 3 hours) and freeze-dried (24 hours) to produce a 60 % ethanol crude extract (EEAD) (206 g). The EEAD yield on the dry root was 20.6 %. The EEAD (100 g) so obtained was suspended in water (300 mL) and then extracted successively with n-hexane, ethyl acetate and n-butanol (3 × 500 mL each) to obtain four fractions: the n-hexane extract (EEAD-He) (8.6 g, yield, 8.6%), the ethyl acetate extract (EEAD-Ea) (22.5 g, yield, 22.5%), the n-butanol extract (EEAD-Bu) (35.8 g, yield, 35.8%) and the residual aqueous portion (EEAD-Aq) (25.6 g, yield, 25.6%) after removal of the solvent under vacuum. The OLA content of various extracts fractions were estimated by the colorimetric method,[9] using authentic OLA as the standard (Table 1).

Estimation of the different extracts on CCl_4-induced hepatotoxicity (in vivo)

The animals were divided into eight groups, each group with six animals. Group I served as normal control and received saline water (1 mL/kg, p.o.) daily for 5 days and received olive oil (1 mL/kg, s.c.) on days 2 and 3 .[13] Group II served as CCl_4 control and received saline water (1 mL/kg, p.o.) daily for 5 days and received CCl_4: olive oil (1:1, 2 mL/kg, s.c.) on days 2 and 3. Group III was treated with the reference drug *silymarin* (60 mg/kg, p.o.) daily for 5 days and received CCl_4: olive oil (1:1, 2 mL/kg, s.c.) on days 2 and 3, 30 min after administration of reference drug. Groups IV–VIII were treated with the extracts, at doses of 60 mg/kg (p.o.), respectively, for 5 days and received CCl_4: olive oil (1:1, 2 mL/kg, s.c.) on days 2 and 3, 30 min after administration of extracts.

Estimation of EEAD-Bu and OLA on CCl_4-induced hepatotoxicity (in vivo)

The animals were treated as mentioned above except that Groups IV–VI were treated with EEAD-Bu at doses of 30, 60 and 120 mg/kg (p.o.), Groups VII-VIII were treated with OLA at doses of 30 and 100 mg/kg (p.o.), respectively, for 5 days and received CCl_4:olive oil (1:1, 2 mL/kg, s.c.) on days 2 and 3, 30 min after administration of extract.

Estimation of in vitro antioxidant activity

The extracts antioxidant activity was determined based on the ferric thiocynate (FTC) method of Kikuzaki et al. (1993)[10], and the thiobarbituric acid (TBA) method of Ottolenghi (1959)[11]. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

$$\text{Percent inhibition} = [\frac{A_0 - A_1}{A_0}] \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of sample at 500nm.[12]

Biochemical estimations

The rats were sacrificed on the sixth day by cervical decapitation and blood was collected in plain tubes. The serum was obtained by centrifugation. After bleeding, the livers were frozen quickly in dry ice/methanol and stored at -70 ^{o}C until analysis. The activities of serum aspartate transaminase (AST), alanine aminotransferase (ALT) were assayed by the standard method using commercially available kits (Nanjing Biomedical.Co., Ltd., China) on an auto-biochemical analyzer (BTS-370 plus, Spain). The hepatic parameters GSH and lipid peroxidation (MDA) were assayed by the standard method using commercially available kits (Nanjing Biomedical.Co., Ltd., China).

Statistical analysis

The data were expressed as mean ± S.E.M. (n = 6). Results were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison using SPSS software student's version. The difference was considered

Table 1. Oleanolic acid (OLA) content in various extracts of *ADF* root

Extract	OLA Content (mg/g)
EEAD (Ethanol crude extract)	126
EEAD-He (n-hexane extract)	10
EEAD-Ea (ethyl acetate extract)	139
EEAD-Bu (n-butanol extract)	259
EEAD-Aq (aqueous portion)	22

The OLA content of various extracts fractions were estimated by the colorimetric method, using authentic oleanolic acid as standard

significant if $p < 0.05$.

Results and discussion

Table 1 shows OLA in various extracts of ADF root. Both the ethyl acetate and n-butanol extracts were enriched with OLA, which were 13.9 % and 25.9 % (wt %), respectively. However, only 1.0 % and 2.2 % OLA was detected in the n-hexane and water extracts.

Figure 2 shows the antioxidant activity of different extracts by FTC method. The absorbance values decreased, along with the increase of the antioxidant activities of the samples. A highest absorbance value of 0.892 was achieved for the control group, followed by 0.619, 0.583, 0.548, 0.537 and 0.403 for EEAD-He, EEAD-Aq, EEAD, EEAD-Ea and EEAD-Bu, respectively. Based on the results, the highest percent inhibition 54.8 ± 3.2 % was calculated for EEAD-Bu, followed by EEAD-Ea (39.8 ± 1.6 %), EEAD (38.5 ± 1.9 %), EEAD-Aq (34.6 ± 1.2 %) and EEAD-He (30.6 ± 1.6 %). As a result, EEAD-Bu shows higher antioxidant activity than that of other extracts ($p < 0.01$).

Figure 3 shows the antioxidant activity of different extracts by TBA method. The results are similar as those detected by FTC method. The control group had the highest absorbance value (0.375) followed by EEAD-He (0.245), EEAD-Aq (0.227), EEAD (0.208), EEAD-Ea (0.207), EEAD-Bu (0.162). Based on the results, EEAD-Bu had the highest percent inhibition of 56.8 ± 3.1 %, followed by EEAD-Ea (44.8 ± 2.0 %), EEAD (44.5 ± 1.6 %), EEAD-Aq (39.5 ± 1.4 %) and EEAD-He (34.7 ± 1.8 %).

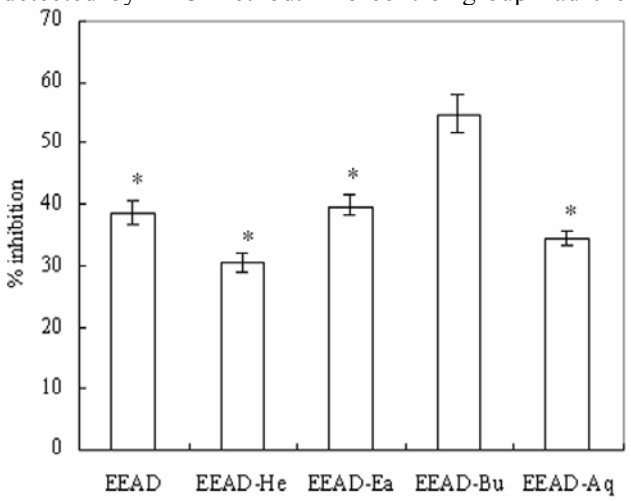

Figure 2. *In vitro* antioxidant activity of different extracts of *ADF* root by FTC method. * $p < 0.05$ compared to EEAD-Bu (one-way ANOVA followed by Tukey's multiple comparison test). Data represents mean ± S.E.M. of six samples.

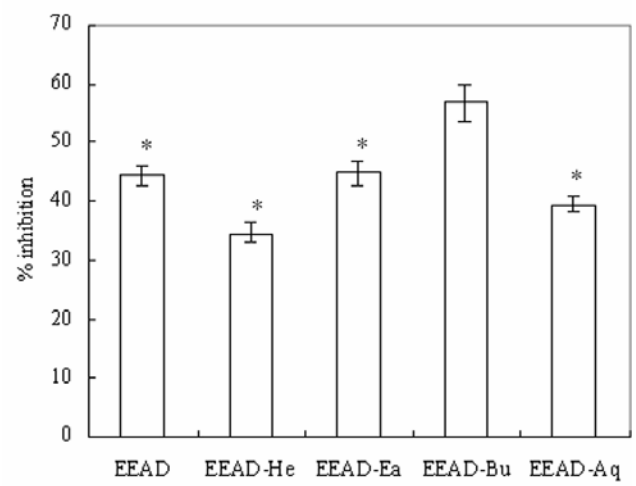

Figure 3. *In vitro* antioxidant activity of different extracts of *ADF* root by TBA method. * $p < 0.05$ compared to EEAD-Bu (one-way ANOVA followed by Tukey's multiple comparison test). Data represents mean ± S.E.M. of six samples.

These results indicate that the various extracts of *ADF* root exhibit different antioxidant activity by inhibiting the oxidation of linoleic acid with both FTC and TBA methods, and the antioxidant activity of EEAD-Bu was highest among allextracts ($p < 0.01$). Furthermore, the results also demonstrate that this natural product contains antioxidant activity.

Table 2 shows the activities of serum ALT and AST in different treated group rats. CCl_4 treatment significantly increased ($p< 0.01$) ALT and AST activities in the rat serum 624 % and 601 %, respectively, compared with those of the normal control group. The table also indicate that all of the extracts were effectively against the acute CCL_4 induce hepatic damage in rats as evidenced from the recovery of altered parameters. However, among the five different extracts, EEAD-Bu at the dose of 60 mg/kg had highest hepatoprotective effect ($p < 0.05$). With treatment of EEAD-Bu, the ALT and AST activities in the rat serum decreased from 586 ± 61 U/L and 669 ± 34 U/L to 233 ± 20 U/L and 305 ± 24 U/L, and the decreasing rate reached 60 % and 59 %, respectively, as compared with those of the CCL_4 control group. Simultaneously, EEAD-Bu even had higher effect than that of *silymarin* at the dose of 60 mg/kg.

Table 3 shows the effects of different dose of EEAD-Bu and OLA on CCL_4-induced hepatotoxicity in rats. Administration of CCL_4 alone resulted in a significant increase in normal levels of serum and hepatic parameters. Treatment with EEAD-Bu (30-120 mg/kg, p.o.) and OLA (30-100 mg/kg, p.o.) showed a certain degree reduction of elevated levels of ALT and AST in a dose dependent manner. With the increase of the dosage of EEAD-Bu, the ALT and AST activities decreased. The ALT and AST activities reached the lowest level 106 ± 27 U/L and 150 ± 29 U/L, and the decreasing rate reached 90 % and 81 %, respectively, as compared with those of the CCL_4 control group, when 120 mg/kg EEAD-Bu administrated to CCL_4-induced rats. Moreover, ALT and AST were not significantly different from the vehicle control by Dunnett's t test. Rats pretreated with OLA also showed a dose dependent protection against CCL_4 challenge, with ALT reduced by 49 % and 61 %, AST reduced by 37 % and 49 %, respectively, at daily doses of 30 and 100 mg/kg.

The liver GSH and lipid peroxidation (MDA) in each group were also analysed, due to the fact that of the oxidative stress of tissue generally involves with the GSH system and lipid peroxidationand, and the results were illustrated in Table 3. Liver MDA decreased with the increasing of EEAD-Bu, and reached the minimum level (63 ± 5 nmoL/g liver), when CCL_4-induced rats was treated with 120 mg/kg EEAD-Bu. At the same time, the CCl_4 treatment could significantly decrease the GSH. But EEAD-Bu could prevent the depletion of GSH. When rats were treated by CCL_4, GSH decreased from 5.56 ± 0.24 mg/g liver to 2.55 ± 0.16 mg/g liver, with the decreasing

Table 2. The effect of different extracts against CCL_4 induced hepatic injury in rats

Treatment	Dose (mg/kg) p.o.	Serum parameters	
		ALT(U/L)	AST(U/L)
Vehicle control	–	81±16	95±8
Vehicle+ CCL_4	2	586±61[a]	669±34[a]
Silymarin+ CCL_4	60.0	310±20[ab]	376±30[ab]
EEAD+CCL_4	60.0	389±26[abcd]	479±41[acd]
EEAD-He +CCL_4	60.0	413±34[abcd]	620±65[acd]
EEAD-Ea+CCL_4	60.0	268±25[abcd]	316±37[ab]
EEAD-Bu+CCL_4	60.0	233±20[abc]	305±24[abc]
EEAD-Aq+CCL_4	60.0	303±17[abd]	360±52[abd]

Values are expressed as mean±S.E.M. of six animals in each group; symbols represent statistical significance: [a] $p < 0.01$, significantly different from the vehicle control. [b] $p < 0.01$, significantly different from the vehicle+ CCL_4. [c] $p < 0.01$, significantly different from the *silymarin*+ CCL_4. [d] $p < 0.05$, significantly different from the EEAD-Bu.+ CCL_4. Dunnett's t test against the respective control.

Table 3. The effect of different dose EEAD-Bu and OLA against CCL_4 induced hepatic injury in rats

Treatment	Dose (mg/kg) p.o.	Serum parameters		Hepatic parameter	
		ALT(U/L)	AST(U/L)	Lipid peroxidation (MDA:nmoL/g liver)	Glutathione (µmole/g liver)
Vehicle control	–	85 ± 21	122 ± 20	57 ± 3	5.56 ± 0.24
Vehicle+ CCL4	–	1.07×10^3 ± 48[a]	775 ± 50[a]	108 ± 6[a]	2.55 ± 0.16[a]
Silymarin+ CCL_4	60	369 ± 38[ab]	373. ± 47[ab]	74 ± 4[ab]	4.46 ± 0.25[ab]
EEAD-Bu + CCL_4	30.0	430± 44[ab]	406 ±33[ab]	77 ± 3 [ab]	4.24 ± 0.23[ab]
EEAD-Bu + CCL_4	60.0	269± 29[ab]	282 ± 29[ab]	71 ± 4[ab]	4.68 ± 0.34[ab]
EEAD-Bu + CCL_4	120	106 ± 27[bNS]	150 ± 29[bNS]	63 ± 5[bNS]	5.46 ± 0.29[bNS]
OLA+ CCl_4	30.0	546 ± 51[ab]	487 ± 48[ab]	81 ± 6[ab]	3.96 ± 0.26[ab]
OLA+ CCl_4	100	421± 42[ab]	398 ± 41[ab]	74 ± 3[ab]	4.34 ± 0.21[ab]

Values are expressed as mean±S.E.M. of six animals in each group; symbols represent statistical significance: [a] $p < 0.01$, significantly different from the vehicle control. [b] $p < 0.01$, significantly different from the vehicle+ CCL_4. NS: $p > 0.05$, not significantly different from the vehicle control. The doses of oleanolic acid (OLA) were calculated on the basis of OLA content of EEAD-Bu, the low dose (30 mg/kg) of OLA was approximated to the amount of OLA present in EEAD-Bu (at the dose 120 mg/kg), as estimated by colorimetric method.

rate of 54 %. However, when 120 mg/kg of EEAD-Bu was used, GSH decreased 2 % only, from 5.56 ± 0.24 mg/g liver to 5.46 ± 0.29 mg/g liver. The effect of the EEAD-Bu over MDA and GSH (at the dose of 120 mg/kg) was higher than that of the reference drug *silymarin* (at the dose of 60 mg/kg). Rats treated with OLA showed a dose dependent protection against CCL_4 challenge, with hepatic MDA reduced 25 % and 32 %, while hepatic GSH increased by 55 % and 70 %, respectively, at daily doses of 30 and 100 mg/kg.

All results demonstrated that the degree of hepatoprotection by different extract fractions treatment against CCL_4 hepatotoxicity seems to correlate with the OLA of each fraction, as the oleanolic acid-enriched EEAD-Bu (at the dose 120 mg/kg) was shown to be the most protective (Table1 and Table 2, 3). On the other hand, our results indicate that treatment with OLA could produce a dose-dependent protection against CCl_4 hepatotoxicity (Table 3). With regard to this, it should be noted that the protection extent by OLA treatment was far less than that of EEAD-Bu, by considering pure OLA at a dose of 30 mg/kg which was equivalent to the amount of OLA present in EEAD-Bu (at the dose 120 mg/kg). Therefore, the results suggest that OLA may not be solely responsible for the hepatoprotective action of EEAD-Bu.

From the results mentioned above, it could be concluded that the *ADF* root extracts exhibits antioxidant and hepatoprotective activities against CCl_4-induced liver

damage. Among of these extracts, EEAD-Bu was verified as the most effective hepatoprotective ($p< 0.05$). In addition,the hepatoprotective effect might also be due to the enhancement of hepatic glutathione regeneration capacity and the decreased level of lipid per-oxidation, particularly for CCl_4 induced oxidative stress. OLA has a dose-dependent protection effect against CCl_4 hepatotoxicity, but it is not the only anti-hepatotoxic bioactive constituents of EEAD-Bu.

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