EFFECT OF *AQUILEGIA VULGARIS* (L.) ETHYL ETHER EXTRACT ON LIVER ANTIOXIDANT DEFENSE SYSTEM IN RATS

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Abstract

**Introduction:** The ethyl ether extract from *Aquilegia vulgaris* (L.) (Ranunculaceae) contains a lot of phenolic acids. Their hydroxyl groups are capable of donating hydrogen atoms at the initial stage of lipid peroxidation (LPO), which inactivates hydroxyperoxides formed from polyunsaturated fatty acids (PUFAs) and leads to breakdown of the propagation chain.

**Material and methods:** Rats pretreated with acetaminophen (APAP) (600 mg/kg b.w., p.o.) were given ethyl ether extract (100 mg/kg b.w., p.o.) obtained from *A. vulgaris* herb. The study parameters measured were microsomal lipid peroxidation, reduced glutathione, and the activity of hepatic antioxidant enzymes and some drug metabolizing enzymes.

**Results:** The treatment with ethyl ether extract of the herb produced a 87–95% decrease in uninduced and Fe²⁺/ascorbate-stimulated microsomal lipid peroxidation in the liver of rats receiving APAP. Hepatic glutathione level depleted by APAP increased significantly (by 18%) after the extract treatment. Antioxidant enzyme activity in the liver, inhibited by APAP, was found to increase after administration of the extract: catalase by about 36%, glutathione reductase by 27% and glutathione S-transferase by 29%. Glucose-6-phosphate dehydrogenase, which decreased after APAP administration, increased again by 26% after extract treatment. The extract tested did not affect the activity of DT-diaphorase. The cytochrome P450 content, depleted by APAP, increased as much as by 100% after the treatment. The activities of NADPH-cytochrome P450 reductase, aniline hydroxylase and aminopyrine N-demethylase were not affected.

**Conclusions:** The protective effect of the *Aquilegia vulgaris* extract in APAP-induced liver injury was mediated by its antioxidant activity. The extract did not inhibit the formation of reactive intermediate metabolites of APAP.

**Key words:** *Aquilegia vulgaris*, Ethyl ether extract, Antioxidant enzymes, Microsomal lipid peroxidation, Reduced glutathione, Drug metabolizing enzymes

INTRODUCTION

Recently, the naturally occurring compounds that exhibit the antioxidant or free radical scavenging potential have attracted considerable attention. Synthetic antioxidants have been shown to produce numerous side effects. Hence, the therapeutic use of medicinal plants in reducing free radical-induced tissue injury has been considered [1,2]. *Aquilegia vulgaris* (L.) (Ranunculaceae), syn. columbine, is a perennial herb indigenous in the central and southern Europe. Decoction from the leaves and stems of *A. vulgaris* has been used in folk medicine against liver and bile duct disorders, especially for the treatment of jaundice, and chronic skin inflammation. The herb is a component of the immunostimulating preparation Padma 28 and homeopathic drugs [3]. Phytochemical studies of *A. vulgaris* showed the presence of cyanogenic compounds, tannins, anthocyanins [4] and cycloartane derivatives showing immunosuppressive properties [5].
We have isolated and identified several flavonoids [6–9] and phenolic acids [10,11] in the aerial parts of the plant as well as alkaloids in roots [12]. The predominant compound was 4’-methoxy-5,7-dihydroxyflavone 6-C-glucopyranoside (isocytisoside) [6]. Our previous studies have demonstrated that ethanol and ethyl acetate extracts, and isocytisoside isolated from A. vulgaris attenuated the effects of CCl₄- and APAP-induced hepatic injury by restoring the activity of most of the antioxidant enzymes and by inhibiting microsomal lipid peroxidation [13,14].

The present study was undertaken to evaluate the potential protective effects of ether ethyl extract from A. vulgaris on APAP-induced hepatotoxicity. We aimed at elucidating the mechanism of this effect by measuring the level of microsomal lipid peroxidation, reduced glutathione, and the activity of hepatic antioxidant enzymes and some drug metabolizing enzymes.

MATERIALS AND METHODS

Chemicals and plants

The chemicals used were purchased from Sigma Chemical Co. Aquilegia vulgaris (L.) stems and leaves were collected in the Botanical Garden of A. Mickiewicz University, Poznań, Poland in June 1999. A voucher specimen is deposited in the authors’ laboratory (No. KF 1261999).

Preparation of the extract

Air-dried and powdered leaves with stems of A. vulgaris (50 g) were extracted seven times with methanol in hot water bath under reflux, and the extract was evaporated under reduced pressure to afford a dark brown residue. The residue was treated with hot water and the insoluble part was filtered off. The filtrate was extracted with ethyl ether. The ethyl ether extract was evaporated to dryness (0.41 g) [15].

Phytochemical analysis

The extract was analyzed by TLC as described previously [10], and the following phenolic acids were identified: caffeic, ferulic, p-coumaric, protocatechuic, vanillic, sinapic, chlorogenic and p-hydroxybenzoic. Isocytisoside (4’-methoxy-5,7-dihydroxyflavone 6-C-glucopyranoside) was identified by UV and 1H NMR, 13C NMR analysis [6].

Quantitative analysis of isocytisoside and phenolic acids was performed using HPLC. Lachrom-Merck chromatograph equipped with DAD detector and Zorbax SB-C18 column (250×4.6 mm; 5 μm). The mobile phase (flow rate 1 ml/min) was methanol-water-formic acid (25:75:1 v/v) for phenolic acids analysis and (40:60:1 v/v) for isocytisoside analysis. The standard curve for phenolic acids and for isocytisoside was plotted in the range of 2–12 μg. The content of isocytisoside in the extract was 1.35% and the content of phenolic acids was as follows: protocatechuic 0.20%, p-coumaric 0.03%, vanillic 0.03%.

Experimental design

Male Wistar rats (240±10 g) were divided randomly into five groups, eight animals each. The animals were housed in an animal facility at 22±1°C with a 12-h light-dark cycle, controlled humidity and air circulation. The substances tested were administered intragastrically in the mixture of water and olive oil (1:1, v/v) with a drop of Tween 20. Groups I–III were given acetaminophen at a dose of 600 mg/b.w. Then, after 4 h, these groups were treated as follows: group I was given vehicle; group II, ethyl ether extract, group III, α-tocopherol. The other two groups were given vehicle, and after 4 h, group IV was administered ethyl ether extract, and group V, which served as control, received vehicle again. All the substances were given at a dose of 100 mg/b.w. The α-tocopherol, a model antioxidant, was used as a positive control. Nineteen hours after the first treatment, the animals were sacrificed by decapitation. The livers were removed, perfused with ice-cold 1.15% KCl and homogenized in buffered sucrose solution (Tris, pH = 7.55). Microsomal and cytosol fractions were prepared according to standard procedure. Protein concentration in the fractions was determined using Folin-Ciocalteu reagent. The experiment was performed according to the local Animal Ethics Committee guidelines for animal experiments.
Biochemical assays
Microsomal lipid peroxidation (LPO) in the liver was assayed in two different experimental systems: Fe²⁺/ascorbate-stimulated peroxidation (non-enzymatic) and uninduced peroxidation. The level of lipid peroxidation was assayed by measuring thiobarbituric acid reactive substances (TBARS) [2].

GSH level was assayed in the liver homogenate prepared in phosphate buffer (pH = 7.4) using the method of Sedlak and Lindsay [16] with Ellman’s reagent.

Glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) activities were assayed as described by Mohandas et al. [17]. The activity of other enzymes was determined according to the published methods: catalase (CAT) [18], superoxide dismutase (SOD) [19], DT-diaphorase [20], glucose-6-phosphate dehydrogenase (G-6-P-D) [21].

Cytochrome P450 content was assayed with the method of Omura and Sato [22], based on the carbon monoxide difference spectra of dithionite-reduced microsomes.

NADPH — cytochrome P450 reductase activity was measured using cytochrome c as an electron acceptor in the presence of NADPH [22].

Aminopyrine N-demethylation activity was determined by measuring the amount of formaldehyde formed, using the Nash reagent [23].

Aniline hydroxylase activity was assayed by the spectrophotometric determination of p-aminophenol, produced as a result of aniline hydroxylation [24].

The data were expressed as mean ±SD. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons were used.

RESULTS

Non-enzymatic lipid peroxidation stimulated by Fe²⁺/ascorbate was markedly elevated (by 184%) in APAP-treated rats. The extract tested and α-tocopherol reduced TBARS formation by 95% and 82%, respectively (Table 1). Uninduced lipid peroxidation increased by 190% after APAP administration. The treatment with ethyl ether extract and α-tocopherol resulted in a significant decrease in TBARS level as compared to that in rats treated with APAP alone. Ethyl ether extract was more effective (87% reduction) than α-tocopherol (53% reduction). The extract tested given to rats alone produced a 3-fold increase in microsomal lipid peroxidation in both assays (Table 1).

Table 1. Effect of Aquilegia vulgaris extract on microsomal lipid peroxidation and glutathione in the liver of APAP-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lipid peroxidation (nmol TBARS/mg protein)</th>
<th>GSH (μmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe²⁺/ascorbate</td>
<td>Uninduced</td>
</tr>
<tr>
<td>APAP</td>
<td>12.0±0.4a</td>
<td>0.67±0.17a</td>
</tr>
<tr>
<td>APAP + EEt</td>
<td>0.6±0.1b</td>
<td>0.09±0.04b</td>
</tr>
<tr>
<td>APAP + α-toc</td>
<td>2.2±0.3b</td>
<td>0.31±0.04b</td>
</tr>
<tr>
<td>EEt</td>
<td>12.8±2.3a</td>
<td>0.71±0.11a</td>
</tr>
<tr>
<td>Control</td>
<td>4.2±1.9</td>
<td>0.23±0.10</td>
</tr>
</tbody>
</table>

APAP — acetaminophen; EEt — ether ethyl extract; α-toc, α-tocopherol; GSH — reduced glutathione; TBARS — thiobarbituric acid reactive substances.

Results are mean ±SD, n = 8.

Control rats were administered vehicle only.

a Significantly different from control, p ≤ 0.001.
b Significantly different from APAP-treated group, p ≤ 0.001.

The treatment with acetaminophen alone significantly depleted the hepatic GSH content, by 32%, as compared to control rats. The treatment with ethyl ether extract resulted in an 18% increase in GSH level as compared to the respective value in the APAP-treated rats. The effect of α-tocopherol was stronger (a 48% increase). The extract alone did not affect the level of reduced glutathione in the liver (Table 1).

The activity of the enzymes involved in glutathione metabolism in the liver, except GST, was inhibited by acetaminophen treatment. The response of GR was significant (a 22% inhibition) whereas the inhibition of GPx was weaker (11%) and insignificant. Administration of the ethyl ether extract to the rats pretreated with APAP produced a significant increase in GST activity, to the level higher than that in control rats. GR activity in the same group also increased significantly (by 27%). The extract did not affect the GPx activity in the APAP-pretreated rats. The administration α-tocopherol to APAP-pretreated rats resulted in a significant increase (by 37%,) solely in the GR...
activity. The extract alone caused a marked decrease in GPx and GR activity, by 43% and 47%, respectively. The GST activity also decreased after the extract administration, but this alteration was not significant (Table 2).

The SOD activity decreased by 24% in APAP-treated rats. The administration of ethanol ether extract and α-tocopherol to APAP-pretreated rats caused a further decrease in SOD activity, by 15% (insignificant level) and 56% (significant level), respectively. Administration of the extract alone resulted in about a 40% increase in SOD activity (Table 2).

The CAT activity was significantly (by 27%) lower in APAP-treated rats. The administration of the extract or α-tocopherol attenuated the decrease in CAT activity by about 20%. The treatment with the extract alone caused a 28% decrease in CAT activity (Table 2).

DT-diaphorase was the only enzyme whose activity was significantly increased (by 41%) in APAP-treated rats. This elevation was even greater after the administration of the extract and α-tocopherol to APAP-pretreated rats. Ethyl ether extract administered alone did not affect the DT-diaphorase activity (Table 2).

APAP treatment produced a significant decrease (by about 52%) in glucose-6-phosphate dehydrogenase activity. The extract attenuated the decrease in G-6-P-D activity by 26%. α-Tocopherol appeared to be more effective and caused a 36% attenuation. When administered alone, the extract, caused a significant (23%) decrease in G-6-P-D activity (Table 2).
in APAP-pretreated rats. α-Tocopherol also inhibited the reduction of cytochrome P450 but its effectiveness was lower, a 69% inhibition. The administration of the extract tested caused a significant (95%) increase in cytochrome P450 content (Fig. 1).

NADPH-cytochrome P450 reductase, aminopyrine N-demethylase and aniline hydroxylase activities were not affected either by acetaminophen or the extract tested (data not shown).

DISCUSSION

In order to better understand the nature and mechanism of toxic liver injury, some models of chemical-induced lesions were developed. The hepatotoxins used most frequently in these models include: galactosamine, trichloroethylene, carbon tetrachloride or acetaminophen [25]. The initial biochemical and metabolic events of acetaminophen toxicity have been well described and are believed to be due to the metabolic conversion of APAP to a highly reactive intermediate, namely, N-acetyl p-benzoquinone imine (NAPQI) by cytochrome P450-mediated oxidases [26]. This conversion is primarily inactivated by conjugation with reduced glutathione [27]. At high doses, the detoxification pathways become saturated, and the intermediate metabolite accumulates and causes liver damage by a covalent binding to tissue molecules [28]. Another theory states that NAPQI is an oxidizing agent that depletes GSH, a cellular protectant against reactive oxygen species (ROS), thus leading to oxidative stress [29]. Our previous investigation has demonstrated that the ethanol and ethyl acetate extracts as well as isocytisoside obtained from *A. vulgaris* attenuated the effects of APAP- and CCl<sub>4</sub>-induced hepatic injury by restoring the activity of most of the antioxidant enzymes and by inhibiting microsomal lipid peroxidation [13,14]. The present study was undertaken to evaluate the potential protective effect of ethyl ether extract from *Aquilegia vulgaris* on APAP-induced hepatotoxicity in rats. We expected that both the ethyl ether extract, containing many phenolic acids which are strong antioxidants, and the isocytisoside would be able to protect against oxidative stress.

The level of TBARS was used as a marker of redox balance and lipid peroxidation in hepatic cells. In our experiment, the TBARS level was increased in APAP-treated rats, which is consistent with the oxidative stress theory of APAP toxicity. The extract tested demonstrated antioxidant activity in intoxicated rats in non-enzymatic, stimulated lipid peroxidation as well as in unstimulated LPO assay. The inhibition of iron-stimulated LPO noted in the present study might involve a formation of complexes between the iron and the extract components. This would prevent the generation of *OH, and thus inhibit LPO.

As mentioned earlier in the text, the ethyl ether extract from *A. vulgaris* contains a lot of phenolic acids. Their hydroxyl groups are capable of donating hydrogen atoms at the initial stage of LPO, which leads to the inactivation of hydroxyperoxides formed from PUFAs. Thus, the propagation chain is broken. It should be emphasized that the ability of the extract to inhibit microsomal lipid peroxidation was similar to that demonstrated by α-tocopherol, the model antioxidant.

On the other hand, the extract alone caused about a 3-fold increase in the TBARS level in both the LPO assays, thus demonstrating its prooxidant activity. The possible explanation of that phenomenon can be a conversion of some compounds present in the extract into prooxidant metabolites. Examples of such conversions were reported by other authors: for ubiquinols [30] and some flavonoids [1].

The role of glutathione as a protective agent against oxidative organ damage has been subject to extensive studies [31]. The exposed sulfhydryl groups bind to a variety of electrophilic radicals and metabolites that may cause cell damage [32]. Detoxification of xenobiotics or their metabolites is one of the major functions of glutathione. These electrophilic compounds form conjugates with GSH, either spontaneously or enzymatically, in reactions catalyzed by glutathione S-transferase. Additionally, the endogenously produced hydrogen peroxide and organic peroxides are reduced by GSH in the presence of glutathione peroxidase [33]. As it could be expected, acetaminophen treatment remarkably depleted hepatic GSH stores in the rats. This may be related to a direct conjugation of GSH with acetaminophen metabolite, NAPQI, and/or
to APAP-induced lipid peroxidation [29]. Our results are consistent with other reports concerning APAP-induced depletion of GSH [27,28,34–36].

Ethyl ether extract partially restored the GSH level in the APAP-treated rats; however, its protective effect was weaker than that of α-tocopherol, the model antioxidant. It can be concluded that due to its antioxidant activity, the extract tested causes detoxification of the toxic metabolite of APAP as well as decreases the formation of toxic metabolites of the drug. In this way, it decreases the demand of hepatocytes for GSH which conjugates with that metabolite.

The *A. vulgaris* extract itself did not affect the GSH level. However, in APAP-treated rats, the effect of the extract on restoring glutathione was remarkable. There are many reports that the protective compound or preparation has no capability to increase the GSH level itself, but when administered together with a toxin, it can substantially reduce GSH depletion. This refers e.g. to lipoic acid and cisplatin [37], and lycopene and T-2 toxin [38]. These findings might be partially explained by the regulatory mechanisms of GSH synthesis [39]. Glutamyl cysteine synthetase is down-regulated by cellular GSH levels, and a decrease in GSH concentration caused by the toxin treatment provides conditions for its enhanced synthesis that can be additionally stimulated by the protective substances.

It is known that antioxidant enzymes can be inactivated by lipid peroxides and ROS [40]. SOD is inhibited by hydrogen peroxide, GPx and CAT through an excess of superoxide radical [41]. It was also shown that severe oxidative stress might result in the inhibition of microsomal GST [42]. In our experiment, the activities of antioxidant enzymes (SOD, CAT, GR, GPx) were decreased in the rats treated with APAP. It can be suggested that the free radicals generated by APAP inhibit antioxidant enzymes, which is consistent with the theory of oxidative stress as a mechanism of APAP toxicity. Similar results were reported by other authors who observed a decreased activity of antioxidant enzymes in the liver of rats treated with APAP [27,36,43–46]. The extract tested restored the activity of CAT, GR and GST in APAP-treated rats, which at least in part, enhanced the activity of the antioxidant defense system impaired by APAP. SOD was the only enzyme whose activity was induced by the extract alone. This effect can be considered beneficial since SOD plays an important role in catalyzing dismutation of superoxide radical to hydrogen peroxide and molecular oxygen, thereby preventing the Haber-Weiss reaction that generates $\cdot$OH. In contrast, CAT, GR, GPx and GST activities were decreased (GST insignificantly) by the extract treatment alone. As mentioned before, this reduction of antioxidant enzyme activity could be due to the prooxidative properties of some constituents of the extract or their metabolites.

Glutathione S-transferases, particularly those which belong to the α class, play an important role as the antioxidant enzymes (they express GPx activity towards organic hydroperoxides but not towards $\text{H}_2\text{O}_2$) in addition to their well established role in the detoxification of electrophilic xenobiotics by catalyzing their conjugation to GSH [47,48].

The GST activity was not reduced by APAP, but it was significantly increased in rats treated with the extract and α-tocopherol, to a level higher than that in the control group. This can be considered a very beneficial effect that enhances the antioxidant status of the organism.

DT-diaphorase catalyses the conversion of quinones to hydroquinones in two-electron reduction with oxidation of NADPH. This pathway is non-toxic, unlike the one-electron reduction by NADPH-cytochrome P-450 reductase which results in the formation of a semiquinone free radical. Semiquinones are readily autooxidizable, which leads to the oxidation of NADPH and oxidative stress. It is known that DT-diaphorase is induced by agents that cause oxidative stress through redox cycling (e.g. quinones, me-nadione) [49]. In our study, the DT-diaphorase activity was increased in APAP-treated rats. This may have been due to oxidative stress induced in the hepatocytes by the toxic APAP metabolite, NAPQI, and ROS [50]. This elevation was even greater after the extract administration to APAP-pretreated rats. It was found that some antioxidants were the inducers of this enzyme [20]. It is likely that the antioxidants present in the extract were responsible for the slight increase in the DT-diaphorase activity. However,
the extract alone did not affect the activity of the enzyme.

G-6-P-D serves an important function in acquiring tolerance against oxidative stress since its activity is a factor limiting the rate of NADPH synthesis within the pentose phosphate pathway. NADPH is a coenzyme involved in the reduction of GSSG to GSH which is catalyzed by glutathione reductase [51]. In our experiment, the activity of G-6-P-D was decreased by APAP administration, which seems to be compatible with GSH depletion. In the APAP-treated rats, the extract tested did not change significantly the activity of G-6-P-D. However, the administration of the extract alone brought about a significant decrease in this activity.

Generally, the effect of α-tocopherol (a positive control) on the antioxidant enzyme activity in APAP-treated rats was similar to that demonstrated by the extract tested. The mechanism by which APAP induces liver injury involves its biotransformation by the liver microsomal cytochrome P450, especially CYP2E1, to form NAPQI [52]. Hence, we attempted to examine whether the extract tested could decrease the activation of APAP by suppressing some phase I drug-metabolizing enzymes. We determined total cytochrome P450 content, NADPH-cytochrome P450 reductase as well as the activity of two monoxygenases. The activity of aniline hydroxylase is known to be mainly CYP2E1-dependent [53]. Recent studies have shown that mainly the CYP2E1 accounted for the formation of NAPQI, while the contribution of other isoforms of cytochrome P450, such as CYP1A2 and CYP3A, appeared to be negligible [52]. Aminopyrine is used as a non-specific substrate for measuring the hepatic metabolic capacity of the cytochrome P450 system. It was found that a number of monoxygenases were involved in aminopyrine metabolism but with a slight to moderate weight of catalysis carried by CYP1A2 [54].

In our study, the cytochrome P450 content was significantly decreased in the rats intoxicated with APAP. The free radicals which are generated during APAP biotransformation are thought to be responsible for the inactivation of the enzyme [29,49]. In the rats receiving APAP pretreatment, the extract tested restored the level of cytochrome P450 by 100%. The efficiency of the extract alone was also very high. It is plausible that the components of the extract not only protect cytochrome P450 against free radical insult but they may also stimulate the synthesis of this enzyme under physiological conditions.

The activities of NADPH-cytochrome P-450 reductase, aminopyrine N-demethylase and aniline hydroxylase, were not affected by acetaminophen or the extract tested. It can be concluded that the extract had no influence on the production of the toxic metabolites of APAP.

Our results demonstrated that the ethyl ether extract from Aquilegia vulgaris attenuated the effects of APAP-induced hepatotoxicity by restoring the activity of some antioxidant enzymes and by inhibiting microsomal lipid peroxidation. On the other hand, when administered alone, the extract produced disturbances in the antioxidant defense system, by decreasing the activity of some antioxidant enzymes and increasing lipid peroxidation in the liver.

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