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Hepatoprotective and antioxidant activity of watercress extract on acetaminophen-induced hepatotoxicity in rats

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ABSTRACT

Introduction: Acetaminophen (APAP) as an analgesic and antipyretic drug can result to liver damages while using more than 4 g/day. Therefore, APAP toxicity causes the liver to dysfunction. This study aims to investigate the hepatoprotective and antioxidant activity of hydroalcoholic extract of watercress (WC) in APAP-induced hepatotoxicity in rats.

Materials and methods: Randomly, twenty-four Wistar rats were divided into four groups of six each. Groups named as control, APAP, APAP + WC and APAP + S for group 1, 2, 3, and 4, respectively. Group 1 received distilled water 1 ml/kg for 7 days. In group 2, 3, and 4, rats pretreated by receiving distilled water (1 ml/kg), WC extract (500 mg/kg), silymarin extract (mg/kg) for 7 days, respectively. Of note, to induce acute hepatotoxicity in groups 2, 3, and 4, rats posttreated by orally intoxicated with single dose of APAP (2 g/kg) on the sixth day. The animals were sacrificed on the seventh day. Alanine amino transferase (ALT), aspartate amino transferase (AST), ferric reducing ability of plasma (FRAP), protein carbonyl (PCO), total thiol (T-SH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) activities were measured in plasma. It should be noted that the chemical composition of WC extract was identified by GC-MS analysis.

Results: The results have shown that there was a significant increase in AST, ALT, FRAP and PCO content in APAP group in comparison to control. Also, there was a significant reduction in T-SH levels and GPx activity in APAP group compared to control. However, administration of WC extract and silymarin not only causes a significant decrease in AST activity, but they markedly increased T-SH content and GPx activity compared to APAP group. GC-MS analysis showed the major compositions were found to be benzenepropanenitrile (48.30 %), Phytol (10.10 %), α-cadinene (9.50%) and linolenic acid (8.0).

Conclusions: It is concluded that the WC extract reduces APAP-induced toxicity through its hepatoprotective and antioxidant activity in rats.

1. Introduction

Liver has been known as a central place to detoxify and metabolize different drugs and xenobiotics [1]. The excessive or inaccurate use of drugs leads to liver dysfunction [2]. Acetaminophen (N-acetyl-p-aminophenol, APAP) as an analgesic and antipyretic drug can result to liver damages while using more than 4 g/day [3, 4]. In liver, more than 90% of APAP is metabolized to non-toxic sulfate and glucuronide, and the rest, converted to a toxic metabolite called N-acetyl-p-benzoquinone imine (NAPQI) in which the reaction catalyzed by cytochrome P450 under normal conditions [5]. NAPQI, a hepatotoxic reactive molecule, is detoxified to various non-reactive molecules through reaction with glutathione peroxidase. At time of excessive production of NAPQI while using more acetaminophen, it causes a reduction in glutathione

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concentration which leads to hepatotoxicity [2, 5]. However, in time of inadequate glutathione, the production of reactive oxygen species (ROSs) like superoxide, hydrogen peroxide and hydroxyl radicals has been increased by NAPQI. Therefore, oxidative stress was induced by imbalance in the formation and removal of free radicals [6]. Of note, normal hepatocytes against free radicals and other reactants were protected by non-enzymatic and enzymatic defense systems, including glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) [7]. Although mild oxidative stress in cells is easily handled, in a condition with severe oxidative stress, oxidant compounds react with cell components such as lipid, protein, DNA and cell membrane, and lead to pathological complications [8].

Over the past few years, many researchers have focused the use of antioxidant extracts from herbal products as hepatoprotective substances in order to reduce the effects of APAP-induced toxicity [4, 9, 10]. Watercress (WC, or Nasturtium officinale), a green leafy vegetable from the family of Brassicacea, has been used in herbal medicine as remedy for several complications [9]. Study results showed that WC used in the treatment of diabetes, bronchitis, diuresis, influenza, asthma [11]. It was reported that WC had anticancer activity by reducing oxidative stress [12]. Isothiocyanates and carotenoids, the main components of WC, are responsible to regulate antioxidant by decreasing free radicals and therefore prevent the injury [10, 12, 13]. In a current study, we are going to clarify the hepatoprotective effects of WC extract in intoxicated rats with APAP.

2. Materials and methods

2.1. Drugs and chemicals

Some materials were purchased from Sigma Chemical Co (St Louis, MO, USA), including acetaminophen, (5, 5′-dithiols-(2-nitrobenzoic acid)) (DTNB) and ethylenediaminetetraacetic acid (EDTA). And also remaining components tested were standard laboratory materials, provided primarily from Merck (Germany), including trichloroacetic acid (TCA), 2, 4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride and 2,4,6-tris (2-pyridyl) -s-triazine (TPTZ). It should be noted that all chemicals and reagents used in the experiment, had analytical grade.

2.2. Preparation of WC extract

In December 2017, stems and leaves of WC were collected from the Sisakht region located in Yasuj, Iran. The plant was recognized by the botanist, where a voucher sample (Herbarium No. HYU30230) was deposited. Plant was cleaned, dust and soil removed by using deionized water. And then mentioned parts were cut off using scissors. To prepare WC extract, 70% ethanol was used at room temperature (25 ± 2 °C) for 48 h. Subsequently, hydroalcoholic extract was filtered, and plant deposit was totally vaporized under reduced pressure by using a rotary at 60 °C. Finally, to do the experiment, the concentrated extract was dried and stored at -20 °C.

2.3. Animals

Male Wistar rats (8 weeks old, weight 200–250 g) were obtained from the animal house of Yasuj University of Medical Sciences. The Ethics Committee approved this study (Code number: IR.YUMS.REC.1397.114). The rats were kept under constant temperature and humidity (23 ± 2 °C, 50 ± 5%) on a controlled light–dark cycle with free access to food and water. The animals were kept in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH US publication no. 85–23 revised 1985).

2.4. Study design

Randomly, twenty-four Wistar rats were divided into four groups of six each. Group 1 received distilled water 1 ml/kg for 7 days as a control group. In group 2, 3, and 4, rats pretreated by receiving distilled water (1 ml/kg), WC extract (500 mg/kg) [14] silymarin (100 mg/kg) [15] for 7 days, respectively. Of note, to induce acute hepatotoxicity, in group 2, 3, and 4, rats posttreated by orally intoxicated with single dose of APAP (2 g/kg) on the sixth day [16].

Blood samples were taken from the animals by cardiac puncture, collected in heparin-containing tubes, centrifuged at 3000×g for 10 minutes. For biochemical analysis, the plasma was separated and stored at -20 °C. In order to evaluate antioxidant enzyme markers in liver, the rats were sacrificed on day 7. Liver tissue sectioned to small pieces and homogenized (10% w/v) by IKA Werke Ultra-Turrax T25 basic homogenizer in PBS (pH 7.4 and 50 mM), centrifuged at 10000×g for 10 min at 4 °C, and finally stored in -20 °C.

2.5. Biochemical analysis

To evaluate biochemical markers in liver injury, alanine aminotransferase) ALT (and aspartate aminotransferase (AST) were measured in plasma samples using enzymatic activity assay kits (Pars Azmoon Co., Tehran, Iran).

2.6. Determination of oxidative stress markers

2.6.1. Determination of ferric reducing ability of plasma (FRAP)

In this experiment, the scale of antioxidant plasma capacity in the reduction of ferric (Fe^{+3}) ions was measured according to Benzie and Strain [17]. The blue-colored Fe-TPTZ (Tripyridyl-s-triazine) complex is formed by reducing Fe^{+3} into ferrous (Fe^{+2}) ions in acidic pH. Color intensity was measured by spectrophotometric at 593 nm. In comparison to standard solution of $FeSO_4.7H_2O$ (0–1500 μmol/L), the plasma level of FRAP was reported.

2.6.2. Determination of protein carbonyl (PCO)

The total protein levels of plasma and liver tissue homogenate were measured by the biuret reaction [18]. The method proposed by Kholari *et al*, was applied to measure an index of protein oxidation [19]. To treat liver tissue homogenates and plasma samples, DNPH (10 mmol/L) in HCl (2 mol/L) was used in darkness for 1 hour at room temperature. The precipitate was treated with 50% TCA, rinsed with a solution of ethanol and ethyl acetate (1:1 v/v) and then dissolved in guanidine hydrochloride (6 mol/L) for 15 minutes at 37 °C. A molar absorption coefficient of 2.2×10^4 $M^{-1}cm^{-1}$ was used to measure PCO content in μmol/g and μmol/mg level in tissue and plasma, respectively.

2.6.3. Determination of total thiol (T-SH)

According to previously described method, the level of T-SH group was evaluated with slight modifications [20]. To do the experiment, the sample (25 μl) was added to a microtube containing Tris-EDTA buffer (150 μl), absolute methanol (790 μl) and 10 mM DTNB (10 μl). It was gently mixed by pipette and then incubated for 15 minutes at the room temperature. Finally, the optical density was read at 412 nm versus DTNB blank and blank tube. A molar absorption coefficient of 13,600 M^{-1} cm^{-1} was considered to express the T-SH.

2.7. Determination of antioxidant enzymes activity

According to the instructions, GPx, SOD and CAT activity were measured in the liver tissue homogenate using commercial colorimetric assay kits (Zell Bio GmbH, Ulm, Germany).

2.8. Chromatographic conditions

GC-MS analysis was carried out on a Hewlett-Packard 5973 connected with a mass detector HP6890 using a DB-1 column (55 m × 0.25 mm, film thickness 0.25 μm). The oven temperature was adjusted from

40 °C (1 min) to 250 °C (30 min) at 3 °C min-1. The transferor gas was helium with the flow rate of 1.0 ml min-1. The mass spectrometer (Hewlett-Packard 5973, USA) was activating in EI mode at 70 eV.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 17 (SPSS Inc., Chicago, IL, USA). Data was presented as mean ± standard error of the mean (SEM). For post hoc analysis, multiple comparisons were analyzed using one-way ANOVA and Tukey test. A P value less than 0.05 was considered as statistically significant.

3. Results

3.1. Biochemical parameters

To evaluate Liver injury, the level of liver enzymes was determined in plasma. As shown in Table 1, the plasma levels of AST and ALT were significantly higher in the APAP groups compared to control rats ($p < 0.05$). Administration of WC extract and silymarin at dose of 500 mg/kg and 100 mg/kg, respectively, caused a significant decrease in AST level compared to APAP group ($P \leq 0.05$). However, they had no effect on the ALT activity compared to APAP group.

3.2. Oxidative stress markers

The results showed not only markedly increased in FRAP levels, but slightly increased in protein carbonyl levels in APAP group compared to control. However, WC extract had no effect on the FRAP content compared to APAP group (Table 2). Plasma PCO was decreased by 31.6% in WC + APAP in comparison to APAP group. Our findings showed a significant reduction in T-SH content in APAP group compared to control. Of note, a significant increase in T-SH level in WC + APAP and S + APAP groups was seen, compared to APAP group ($P \leq 0.05$).

3.3. Antioxidant enzymes activity

Antioxidant enzymes activity was measured in groups treated compared to control. The GPx activity had a significant decrease in the APAP group compared to control ($P < 0.05$). However, its activity indicated a significant increase in the APAP group receiving WC extract (500 mg/kg) (WC + APAP) compared to the APAP group ($P < 0.05$) (Fig. 1 A). More importantly, CAT and SOD activities were not significantly changed in the rats treated with WC + APAP and S + APAP groups compared to APAP group (Fig. 1 B-C).

3.4. Separation and identification of the hydroalcoholic extract of WC.

The WC extract was analyzed by HPLC in the previous study [21]. The chemical composition obtained from WC extract, presented in Table 3. However, eleven components have been identified with their percentage composition, represented 99.0 % of total WC. Of which the major ones, were found to be benzenepropanenitrile (48.30 %), Phytol (10.10 %), α-cadinene (9.50%) and linolenic acid (8.0).

Table 1
Effect of WC extract on plasma biochemical parameters in APAP intoxicated rats.

Groups	AST (U/L)	ALT (U/L)
Control	110.83 ± 12.76	29.85 ± 3.78
APAP	226.62 ± 13.92[a]	74.31 ± 7.90[a]
WC + APAP	156.04 ± 21.18[b]	65.33 ± 11.84[a]
S + APAP	128.13 ± 18.23[b]	63.35 ± 8.78[a]

ALT: alanine aminotransferase; AST: aspartate aminotransferase. Each value represents the mean ± SEM. APAP: acetaminophen; WC: 500 mg/kg of hydroalcoholic extract of watercress; S: 100 mg/kg of silymarin.
[a]statistically significant compared to control group, P-value ≤ 0.05.
[b]statistically significant compared to APAP group, P-value ≤ 0.05.

Table 2
Effects of WC extract on oxidative stress markers in APAP intoxicated rats.

Groups	FRAP (μmol/L)	PCO (μmol/mg protein)	T-SH (μmol/mg protein)	PCO (μmol/g tissue)
Control	1039.41 ± 57.04	1.39 ± 0.07	3.29 ± 0.10	3.04 ± 0.08
APAP	2284.83 ± 123.46[a]	1.74 ± 0.22	2.27 ± 0.27[a]	3.15 ± 0.04
WC + APAP	2418.16 ± 184.23[a]	1.19 ± 0.10	3.10 ± 0.17[b]	2.98 ± 0.02
S + APAP	1639.00 ± 142.00[a,b]	1.37 ± 0.17	3.27 ± 0.19[b]	3.04 ± 0.09

FRAP: ferric reducing antioxidant power; PCO: protein carbonyl; T-SH: total thiol. PCO content in μmol/g and μmol/mg level in tissue and plasma, respectively. Each value represents the mean ± SEM. APAP: acetaminophen; WC: 500 mg/kg WC extract; S: 100 mg/kg silymarin.
[a]statistically significant compared to control group, P-value ≤ 0.05.
[b]statistically significant compared to APAP group, P-value ≤ 0.05.

4. Discussion

Oxidative stress is the most prevalent cause of drug-induced hepatotoxicity [5, 22]. The present study investigated the hepatoprotective effect of WC extract as an antioxidant on liver injury induced by APAP. In various studies, APAP-induced hepatotoxicity is a reliable model to evaluate hepatoprotective agents, administered orally or intraperitoneally [5, 23, 24].

In current study, the aminotransferase enzymes were investigated to evaluate the biochemical properties of the liver function after exposure to antioxidant component. ALT and AST as hepatic leakage enzymes after hepatocellular injury, released into peripheral blood [25]. Results showed significant elevation in the levels of ALT and AST in the APAP group compared to control. This increase may be due to APAP-induced liver damage which leads to a reduction in cell integrity and leakage as a consequence of this treatment, causes enzymes release into the bloodstream [15]. Karami *et al.* reported hepatoprotective effect of WC against gamma-radiation-induced hepatotoxicity in mice [26].

In previous studies, hepatoprotective activity of Clerodendron inerme, Adansonia digitata and Piper retrofractum extract was confirmed against APAP-induced hepatic injury in rats [15, 27, 28]. For the first time, the protective effects of WC extract were evaluated in rats with APAP-induced hepatotoxicity. Of note, the level of AST enzyme was significantly reduced in WC + APAP group in which WC extract orally administrated, and also this group represented hepatocytes regeneration and repair. However, it had no significant effect on ALT level.

Excessive generation of NAPQI in APAP-induced hepatotoxicity, can covalently binds to lipids, proteins and DNA molecules. Additionally, NAPQI led to a lower glutathione concentration in liver and caused depletion of this antioxidant compound [24]. Proteins are critical targets of oxidative stress products, and more vulnerable due to enzymatic activities in the cell. Among oxidative changes in proteins, PCO formation is probably the first protein adduct generated due to free radical reaction with proteins [29]. Furthermore, it has been demonstrated that PCO level in homogenized liver and renal tissue significantly elevated in the rats orally treated with 3 g/kg APAP compared to control group [24]. It is also reported that oral administration of 3 g/kg APAP significantly increased PCO formation in homogenized liver tissue [4]. Results showed that PCO level elevated in the intoxicated rats with 2 g/kg of APAP compared to the control group. Nevertheless, this increase was not statistically significant. This was mainly due to using lower doses of APAP. Although WC extract (500 mg/kg) reduced PCO level in homogenized liver tissue and plasma, the difference was not statistically significant. However, this reduction suggests that WC extract may play as an antioxidant

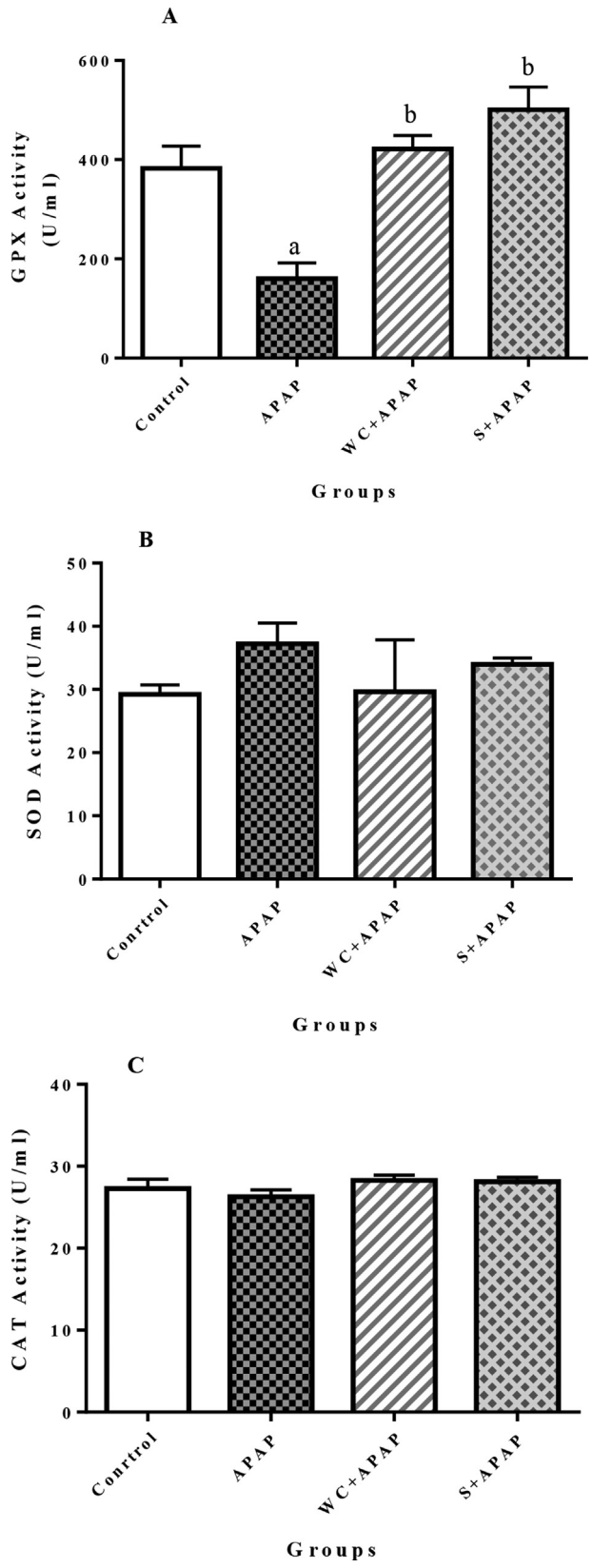

Fig. 1. Effect of WC extract on GPx (A), SOD (B) and CAT (C) activity in the liver tissue of APAP intoxicated rats. APAP: acetaminophen; WC: 500 mg/kg of hydroalcoholic extract of watercress; S: 100 mg/kg of silymarin. [a]statistically significant compared to control group, P-value $\leq$ 0.05. [b]statistically significant compared to APAP group, P-value $\leq$ 0.05.

Table 3
GC-MS components of the hydroalcoholic extract of WC.

NO	Compound	Molecular Formula	Percentage (%)
1	Benzenepropanenitrile	C_9H_9N	48.3
2	Phytol	$C_{20}H_{40}O$	10.1
3	α-Cadinene	$C_{15}H_{24}$	9.5
4	Linolenic acid	$C_{18}H_{30}O_2$	8.0
5	Palmitic acid	$C_{16}H_{32}O_2$	7.1
6	3-Methyl-4-isopropylphenol	$C_{10}H_{14}O$	6.0
7	Durenol	$C_{10}H_{14}O$	2.5
8	Methyl ester group	-	2.4
9	Methyl 11,14,17-icosatrienoate	$C_{21}H_{36}O_2$	2.0
10	Myristicin	$C_{11}H_{12}O_3$	1.6
11	Thymol	$C_{10}H_{14}O$	1.5
Total			**99.0**

component by inhibiting the oxidation process in proteins.

All thiol groups are related to proteins in the plasma, play not only as sensitive indicators of oxidative stress but defense mechanism against ROS [26]. T-SH consists of two main parts: protein thiol groups (P-SH) and glothathione (GSH). Due to the negligible amount of GSH, there is a slight difference between T-SH and P-SH levels [30]. Current results are consistent with report that showed that T-SH level of hepatic tissue significantly decreased in APAP intoxicated rats compared to control group [31]. More importantly, NAQPI production as an active metabolite of APAP causes a reduction in thiol-associated proteins like GSH. Therefore, it leads to a decrease in the number of thiol groups in our experiment. Furthermore, T-SH content was increased in WC + APAP group in compared to APAP group. Altogether, administration of WC extract (500 mg/kg) increased T-SH content which was related to its high antioxidant properties as a free radical scavenger.

Antioxidant enzymes including SOD, GPx and CAT play as free radical scavengers [32]. In a research by Aycan *et al.* in the rats with APAP-induced hepatotoxicity, a decrease in GPx activity was seen [5]. Interestingly, it was reported that APAP administration significantly reduced SOD, GPx and CAT activities [33]. In this study, APAP administration showed a reduction in GPx activity compared to control group. Of note, treatment with WC increased enzyme activity in WC + APAP group. However, there was no significant difference in SOD and CAT activities in liver tissue among groups. In accordance with our results, it has been revealed that WC supplementation had no effect on these enzymes in healthy adults [12]. Free radicals, including superoxide anion, radical hydroxyl and H_2O_2 [34], are the major substrates for SOD and GPx antioxidant enzymes [5, 35]. After APAP overdose, GPx activity was reduced probably because of increasing in the formation of free radicals. The mechanism by which WC extract effects on antioxidant enzymes activity have not been fully understood. It is probably due to an increase in GPx activity by reducing hydroxyl radical formation.

5. Conclusion

In summary, it was for the first time that the effects of WC extract were evaluated on APAP-induced hepatotoxicity. The results showed that WC extract reduced oxidative stress by increasing T-SH content as well as enhancing GPx activity. It should be noted that the hepatoprotective effects of WC extract is probably because of its antioxidant activity as a free radical scavenger. More investigations are needed to suggest WC extract as an efficient supplementation to treat APAP-induced hepatotoxicity.

Declarations

Author contribution statement

Nahid Azarmehr: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Parisa Afshar: Performed the experiments.

Mona Moradi, Kazem Abbaszadeh-Goudarzi: Analyzed and interpreted the data; Wrote the paper.

Heibatollah Sadeghi: Conceived and designed the experiments.

Hossein Sadeghi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Behnam Alipoor: Conceived and designed the experiments; Analyzed and interpreted the data.

Bahman Khalvati: Contributed reagents, materials, analysis tools or data.

Zahra Barmoudeh: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Amir Hossein Doustimotlagh: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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