Identification and bioactivity evaluation of flavan-3-ols in the milk of dairy sheep fed Cynomorium songaricum

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ABSTRACT

Cynomorium songaricum is a traditional medicine and also a food material that is eaten raw or processed as tea or beverages. As a featured plant in semi-desert grasslands, C. songaricum is also eaten by the cattle and sheep in the area. This research study fed dairy sheep C. songaricum to determine the flavan-3-ols in sheep milk. Catechin (Cat), epicatechin (Epi), procyanidin A1 (A1), procyanidin A2 (A2), and procyanidin B1 (B1) were detected in sheep milk with the concentration being Epi > A2 > Cat > B1 > A1 at 24 h after the administration of C. songaricum. Neither A1 nor A2 were detected in the methanol extract of C. songaricum. Cysteine degradation of the plant revealed that in addition to Epi, A2 was the extending unit of the polymeric flavan-3-ols in C. songaricum, indicating that A2 was released digestively from the polymers and enters the milk. Procyanidin B-1 was converted to A1 on incubation in raw but not heated milk, indicating that the A1 in milk is the enzymatically transformed product of B1. Accelerated oxidation showed that the flavan-3-ols, B1, Cat, and Epi significantly protects the unsaturated triacylglycerols in the milk from oxidation. Flavan-3-ols are polyphenols with many health benefits. The present research revealed the antioxidant activities of flavan-3-ols that could be absorbed to sheep milk, adding new evidences for the values of these flavan-3-ols and for the milk.

Key words: Cynomorium songaricum, flavan-3-ol, dairy sheep, milk

INTRODUCTION

Milk is the primary food of infants and animal cubs. Milk is also being consumed in greater amounts by adults worldwide. Milk comprises nutrients (proteins, carbohydrates, fats, vitamins) and diet chemical constituents which penetrate the blood-milk barrier. These compounds could add to the flavor and biofunctions of milk that can influence the health of milk consumers, especially infants and animal cubs, who are more sensitive to food ingredients. For these reasons, the influence of livestock feed on the quality and composition of milk is attracting greater attention. Milk minerals differ between organic and conventional herds (Qin et al., 2021). The mammalian lignan content in livestock milk changes with the type and ratio of livestock diet; these changes also influence the composition of other constituents in milk (Petit and Gagnon, 2009; Brito et al., 2015). The components and fatty acid profiles in the milk of lactating dairy cows are influenced by their feed (Zang et al., 2021). These reported examples demonstrated the significant effects of livestock’s feed on the milk composition.

Cynomorium songaricum is a traditional medicine that is used for its tonic effect and is a food material that is eaten raw or processed as tea or beverages. As a featured plant in semi-desert grasslands, C. songaricum is eaten by the cattle and sheep in these areas. In addition, C. songaricum can be used as supplementary feed to improve the flavor and nutritional value of livestock meat (Chai et al., 2020). Cynomorium songaricum contains triterpenes, sterols, and flavonoids, of which flavan-3-ols abound in milk. Flavan-3-ols are polyphenols with many health benefits. The present research revealed the antioxidant activities of flavan-3-ols that could be absorbed to sheep milk, adding new evidences for the values of these flavan-3-ols and for the milk.
plasma on consumption of cocoa, which is rich in polyphenols, including flavan-3-ols (Ellinger et al., 2020). This study was performed to determine the flavan-3-ols in *C. songaricum* on absorption in dairy sheep milk, and their protection on milk triglycerides against oxidation.

**MATERIALS AND METHODS**

**Reagents and Instruments**

*N*-Ethylmaleimide (NEM) and (+)-catechin hydrate (Cat) were from Sigma Aldrich, Inc.; (-)-Epi was from TCI, Inc; procyanidin A1 (A1) was from Chromadex Inc. Procyanidin A2 (A2) and procyanidin B1 (B1) were from Beijing Zhongke Quality Inspection Biotechnology Co. Ltd. Acetonitrile, methanol, and dimethyl sulfoxide (DMSO) of analytically pure were from Fuchen Chemical Reagent Co., Xilong Scientific Co., and Biotopped Technology Co., respectively. The HPLC grade solvents for UHPLC-MS were from Fisher Scientific Company. Formic acid of HPLC grade was from Aladdin Industrial corporation. Agilent 1290 infinity ultrahigh performance liquid chromatography (UHPLC) Agilent 6430 triple quad MS was from Agilent Technologies Sin-78.

*Cynomorium songaricum* was collected in May 2020 in Qinggelegacha (105.44934°N, 40.147734°E), Alxa, Inner Mongolia Autonomous Region, China. Peanut skin purchased from Cunyutang was from Haozhou Huirentang Pharmaceutical Technology Co., Ltd. The plant material was pulverized with an electronic pulverizer (Xuman 800Y-304) to pass through an 80-mesh sieve.

**Preparation of C. songaricum Extracts and Quantification of the Chemical Constituents**

The powder of the stems of *C. songaricum* (1 g) was extracted with 5 mL of CH$_3$OH under sonication for 30 min. The mixture was filtered, and 1 mL of the filtrate was concentrated to dryness to obtain an extract. The extract was dissolved in 200 μL of DMSO and microfiltered with 0.2-μm disposable syringe filter (PTFE, 13 mm in diameter, Xiboshi). The same type of membrane was used for the different extractions throughout this research. The chemical constituents were quantified with UHPLC-MS.

**Ewe Maintenance and Milk Sampling**

The experiments were approved by the animal experimental ethics review committee of Inner Mongolia University (IMU-sheep-2021–030) and carried out in accordance with the guidelines about animal welfare and handling. The experiments on dairy sheep were carried out in Ulanqab at Mengtianran dairy sheep experimental base of the State Key Laboratory of Reproductive Regulation and Breeding of Grassland Livestock, China. The ewes used in this research were produced through transferring the DairyMeade embryos from New Zealand to the local breed as recipients. The ewes were provided with TMR of the reported ingredients and nutritional composition (Zhuang et al., 2021) twice daily for 2 mo. There were no flavan-3-ols, A2, Epi, Cat, B1, and A1 detectable in the TMR (Supplemental Figure S1; https://doi.org/10.7910/DVN/JJKX0W).

Three ewes at 74.3 ± 4 DIM and 69.2 ± 2 kg of BW were randomly picked out from a flock of 40 dairy sheep ewes at 2 yr old. The 3 ewes were fed separately in 1,792 × 790 × 1,615 cm metabolic cages 2,000 cm apart from each other in the same environment. After 12 h, the milk was collected from each ewe as blank control (0 h), and then a suspension of 250 g of *C. songaricum* powder in 1,200 mL of water was gavaged to each ewe, and milking was taken every 12 h in the routine milking time. The ewes were provided with clean water at all times. To avoid possible influence on the ewes’ physiology by longer fasting time, 36 h was set as the end point. The amount of *C. songaricum* powder and the volume of the water suspension given to the ewes; decided based on a preliminary experiment to observe the amount and volume of feed that ewes ate. No side effects or adverse events occurred in the intervention group.

The milk (10 mL) was mixed with 3-fold volume of a CH$_3$CN solution containing 1% formic acid, vortexed for 1 min, and centrifuged at 4°C by 12,225 × g for 5 min. The supernatant was collected and pretreated by passing through an Oasis HLB 1-cc extraction cartridge (Waters, wat094225) column and eluted with 1 mL of CH$_3$OH. The cheese-like lower phase after centrifugation was re-extracted with 3-fold volume of the CH$_3$CN solution and treated in the same procedure. The combined eluate was evaporated with a rotating vacuum evaporator. The obtained residue was redissolved in 300 μL of methanol and microfiltered. The filtrates (milk extracts) were subjected to UHPLC-MS analysis of flavan-3-ols.

**Incubation of B1 in Raw and Heated Sheep Milk**

The milk collected from the ewes before administration of *C. songaricum* was divided to 2 portions. One portion was heated at 98°C for 10 min as heated milk, and the other portion was used as control raw milk without heating. Mixtures of 200 μL of raw or heated
milk and 10 μM of B1 were incubated at 37°C. At time points of 10, 20, 30, 60, 120 min, the mixture was mixed with 600 μL of acetonitrile containing 1% formic acid. After shaking and mixing for 1 min, the mixture was centrifuged at 12,225 × g for 5 min at room temperature. The supernatant was moved to a new tube and evaporated using a vacuum centrifugal concentrator (Eppendorf, Concentrator Plus) to dryness. The residue was dissolved in 200 μL of CH₃OH, microfiltered, and analyzed with UHPLC-MS for procyanidins.

**UHPLC-MS Analysis of the Flavan-3-ols**

Stocking solutions of each standard compound was prepared at 10 mg/mL in DMSO. The calibration solutions for quantification of the chemical constituents in the plant extract were prepared by serial dilutions of the stock solution with CH₃OH. The calibration solutions for quantification of the compounds in the milk were prepared by adding 10 μL of the solution to 90 μL of milk that was taken from the ewes before administration of *C. songaricum*. The milk with standard compounds was then treated in the same way as for milk sampling described above.

An Agilent 1290 Infinity UHPLC-6430 triple quadrupole mass spectrometer with Agilent Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm) was used for quantification of the chemical constituents. The sample solution was injected to the instrument at 2 μL with an autosampler. The mobile phase was set at 0.3 mL/min with a program containing varying percent of methanol in 0.1% aqueous formic acid and programmed as: 0 to 2 min, 22 to 25%; 2 to 9 min, 25 to 50%; 9 to 9.01 min, 50 to 93%; 9.01 to 12 min, 93%; 12 to 12.01 min, 93 to 100%, 12.01 to 14 min, 100%. Mass detector was set at capillary 3 kV, gas temperature 325°C, gas flow 12 L/min, and nebulizer 40 psi. Detection using multiple reaction monitoring (MRM) in negative ionization mode was optimized, and the resulting parameters are shown in Supplemental Table S1 (https://doi.org/10.7910/DVN/JJKX0W).

**Degradation of Polymeric Flavan-3-ols with Cysteine**

The degradation was performed using reported method (Meng and Ma, 2013) with minor modification. The powder (0.5 g) of *C. songaricum* or peanut skin and cysteine hydrochloride (0.5 g) was reacted in water (5 mL) at 60°C for 48 h and then centrifuged at 12,225 × g for 5 min. The supernatant was microfiltered and analyzed by UHPLC-MS using the Agilent C18 column, and mobile phase A (0.1% HCOOH in H₂O) and B (CH₃OH) in the program of 0 min 10% B, 4 min 100% B, and 6 min 100% B. The injection volume was 2 μL and the flow rate was 0.3 mL/min. The MS detection was performed using MRM with capillary 4 kV, gas temperature 350°C, gas flow 11 L/min, and nebulizer 45 psi. Compound was quantified in negative ionization mode with the precursor ion, product ion, fragmentor, and collision energy being 694, 573, 125, and 15, respectively.

**Measurement of Glutathione Level**

Procyanidin B-1 was added to blank milk at a final concentration of 10 μM. The mixture was incubated at 37°C for 2 h and the glutathione levels were quantified as the stable NEM derivative. To the sample was added 200 μL of a water solution 10 mM NEM, and 3 times the volume of CH₃CN containing 1% formic acid. The mixture was vortexed for 1 min and then centrifuged at 12,225 × g for 5 min at room temperature. The supernatant was passed through an Oasis HLB 1cc extraction cartridge (Waters, wat 094225), microfiltered, and subjected to UHPLC-MS analysis using a 5C18-AR-II column (2.1 × 50 mm, 1.8 μm, Nacalai Tesque) and a mobile phase (0.1% formic acid as A, and methanol as B) in the programs of 0 min 10% B, 5 min 50% B, 6 to 8 min 100% B, and post time 3 min. Glutathione and glutathione-NEM were detected by ESI-MS (-) and ESI-MS (+), respectively. The MRM mode was used for quantification, where the precursor-product pairs were 306.2→128.3 and 433→304.2 for glutathione and glutathione-NEM, respectively. The standard solutions for calibration were prepared by mixing 10 mM NEM with different concentrations of glutathione.

**Accelerated Oxidation and Quantification of the Triglycerides in the Milk**

The pooled milk from the 3 ewes collected before feeding with *C. songaricum* was divided into control, B1, A1, A2, Cat, and Epi groups, with each group containing 3 tubes and each tube containing 1 mL of the milk. To each tube was added 10 μL of a 10 mM stock solution of the respective flavan-3-ol, and to the control group was added 10 μL of DMSO. Milk with or without flavan-3-ols were heated at 60°C for 7 d, during which, fresh air was pumped in for 5 min every 24 h. After the process, to each tube was added 1 mL of CHCl₃, 1 mL of CH₃OH, and 0.8 mL of water. The mixtures were shaken for 30 min, then 1 mL of CHCl₃ was added, then shaken for an additional 15 min. After centrifugation at 12,250 × g for 30 min, 1 mL of the lower organic layer was moved into a new centrifuge tube and dried, and the residue was dissolved with 1
mL of acetonitrile-isopropanol 1:1, microfiltered, and subjected to UHPLC-MS analyses of triglycerides using the Agilent C18 column, at temperature 30°C, injection volume 2 μL, with the mobile phase flow rate of 0.4 mL/min at gradient elution conditions: acetonitrile as B, isopropanol as A, 0 min 100% B; 3 min 50% B; 6.5 min 30% B; 7 min 100% B, 3 min post time. Atmospheric pressure chemical ionization (APCI) in positive mode was used for detection with scanning range of 100 to 1,200 m/z, scanning time 500, fragmentor 10 V, acceleration voltage 7 Pro, gas temperature 350°C, evaporation temperature 350°C, gas flow rate 6 L/min, nebulizer 60 psi, capillary voltage 3,500 V, and corona current 4 μA. The accelerated oxidation experiment was repeated and quantification results of all the 6 tubes (3 × 2) were statistically analyzed.

**Statistical Analysis**

The quantification data were statistically analyzed with SAS/STAT (version 9.4; SAS Institute Inc.), and the statistical significance was assessed by 2-tailed, unpaired Student’s t-test. All the experimental units or data points in each experimental group were included in the analysis.

**RESULTS AND DISCUSSION**

**Contents of the Chemical Constituents in *C. songaricum* Extract**

The plant was extracted with methanol and the contents of the chemical constituents in the extract were quantified with UHPLC-MS. Among these chemical constituents, Cat was of the highest content in the methanol extract, followed by Epi and B1 (Table 1). No A1 or A2 were detected, indicating that the concentrations of A1 and A2 in the extract were below the detection limits (41.74 and 500 nM, respectively).

### Table 1. Comparison of the flavan-3-ol contents in *Cynomorium songaricum* extract and in dairy sheep milk (n = 3, mean ± SD)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Content (nM) in milk¹</th>
<th>Amount in milk (nmol)</th>
<th>Content (nmol/g) in plant extract</th>
<th>Amount in plant feed (nmol)</th>
<th>Ratio²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>12.61 ± 5.62</td>
<td>8.45 ± 3.77</td>
<td>1,058.5 ± 2.15</td>
<td>264,625 ± 537.5</td>
<td>3.19 × 10⁻⁵</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>70.47 ± 75.39</td>
<td>47.21 ± 50.51</td>
<td>263.0 ± 5.99</td>
<td>65,740 ± 1,497.5</td>
<td>7.18 × 10⁻⁴</td>
</tr>
<tr>
<td>Procyanidin A1</td>
<td>5.02 ± 4.02</td>
<td>3.36 ± 2.69</td>
<td>NQ</td>
<td>NQ</td>
<td>NA³</td>
</tr>
<tr>
<td>Procyanidin A2</td>
<td>48.98 ± 34.55</td>
<td>32.82 ± 23.15</td>
<td>NQ</td>
<td>NQ</td>
<td>NA³</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>10.52 ± 6.36</td>
<td>7.05 ± 4.36</td>
<td>101.2 ± 1.54</td>
<td>25,300 ± 385</td>
<td>2.79 × 10⁻⁴</td>
</tr>
</tbody>
</table>

¹The milk was collected at 24 h after administration of the *C. songaricum*.
²The ratio of the amount of each flavan-3-ol in the milk taken at 24 h to that in *C. songaricum* fed to the sheep with the latter amount being calculated by multiplying the concentration in the plant extract and the grams of plant fed to the sheep.
³NQ = not quantified as the concentration was below the quantification limit.
⁴NA = not applicable as there was no value for the denominator.

**Flavan-3-ols in the Milk of Dairy Sheep Fed with *C. songaricum***

By UHPLC-MS analysis (Supplemental Figure S2; https://doi.org/10.7910/DVN/JJKX0W) and comparison with standard compounds, the flavan-3-ols, Cat, Epi, A1, A2, and B1 (Figure 1) were identified in the milk of dairy sheep that were fed with the powder of *C. songaricum*. The flavan-3-ols were quantified by UHPLC-MS in MRM mode with the parameters listed in Supplemental Table S1. The quantification method showed good linearity, accuracy and sensitivity (Supplemental Table S2; https://doi.org/10.7910/DVN/JJKX0W). By quantification of the compounds in the milk collected at different time points, the dynamic profiles of the flavan-3-ols were obtained. From the concentration vs. time curves (Supplemental Figure S3; https://doi.org/10.7910/DVN/JJKX0W), it was found that the concentrations of these flavan-3-ols peaked at 24 h in the milk after administration of *C. songaricum*.

**Absorption of Epicatechin and Procyanidin A2 in Milk.** Epicatechin and procyanidin A2 were absorbed in the milk in large amounts, as they are the extending units of the polymeric flavan-3-ols in *C. songaricum*. Among the monomeric flavan-3-ols that entered the milk at 24 h after administration of *C. songaricum*, Epi had higher concentration than Cat (70.47 vs. 12.61 nM), whereas Cat was of much higher concentration than Epi (1,058.5 vs. 263.0 nmols/g) in the plant extract, resulting in higher (amount in milk)/(amount in plant feed) value for Epi than that for Cat (7.18 × 10⁻⁴ vs. 3.19 × 10⁻⁵). Epicatechin was known to be the major extent unit of polymeric flavan-3-ols in *C. songaricum* (Ma et al., 1999), the high content of Epi in the sheep milk was thus deduced to be produced by digestion of polymeric flavan-3-ols to release Epi that entered the milk.

In the dairy sheep milk at 24 h after administration of *C. songaricum*, a large amount of A2 was detected,
with its content being 48.98 nM, which was the highest concentration among all the C. songaricum flavan-3-ol dimers quantified. However, there was little A2 in the plant extract (Table 1). Thus, it was deduced that either A2 was another extend unit of the polymeric flavan-3-ols or it was biotransformed from other compounds. Cynomorium songaricum was then subjected to cysteine degradation and compared with that of peanut skin which was reported to have A2 as the major extend unit of its polymeric flavan-3-ols (Zhang et al., 2021). As shown in Supplemental Figure S4 (https://doi.org/10.7910/DVN/JJKX0W), by MRM scan targeting at procyanidin A-cysteine conjugates (precursor ion: 694, product ion: 573) in UHPLC-MS, the cysteine degradation products of C. songaricum displayed an UHPLC-MS chromatogram very similar to that of peanut skin with A2-cysteine conjugate as the major peak. The result indicated that C. songaricum contains A2 as another extending unit in its flavan-3-ol polymers as illustrated in Figure 1, and the large amount of A2 in C. songaricum-fed dairy sheep milk also came from digestive degradation of the flavan-3-ol polymers in C. songaricum.

**Procyanidin A1 Was Enzymatically Transformed from B1 in the Milk.** Both A1 and A2 were detected in the milk in large amounts, whereas they were not detectable in the plant extract (Table 1). As one of the extending units of polymeric flavan-3-ols in C. songaricum, A2 was deduced to be generated by digestion and subsequent absorption to the milk. In addition, other factors may improve the concentration of procyanidin A in the milk. It was reported that procyanidin B could convert to procyanidin A by oxidative agents, such as 1,1-diphenyl-2-picrylhydrazyl (Kondo et al., 2000), and by enzymes, such as laccase (Osman and Wong, 2007). Some enzymes in vivo may also be able to transform procyanidin B to A. To verify this speculation, B1 was incubated in raw and heated milk followed by UHPLC-MS analysis, and A1 was detected in the raw milk in a preliminary experiment. Confirmation experiments were carried out, and the contents of A1 and B1 were quantified by UHPLC-MS. As shown in Figure
2, A1 generated and its amount was increasing within 1 h on incubating B1 in the raw milk. In contrary, A1 was undetectable on incubating B1 in heated milk, in which the enzyme was denatured by heating. The results indicated that A1 could come from the enzymatic transformation of B1 in the milk.

Flavan-3-ols Protect the Polyunsaturated Triglycerides and Glutathione from Oxidation in Milk

Catechin, Epi, B1, A1, and A2 were detected in the milk of dairy sheep that were fed with *C. songaricum*. We then investigated if these flavan-3-ols could influence the stability of milk triglycerides. Milk with or without flavan-3-ols was heated for 7 d and fresh air was pumped in every 24 h. The triglycerides were extracted from milk with CHCl₃-CH₃OH and analyzed with UHPLC-APCI MS in positive ionization mode. In (+) APCIMS, the triglycerides displayed pseudomolecule ions at [M+H]+ or [M+NH₄]+. Characteristic product ions were generated by losing the fatty acids at C1 or C3 of the glycerol (Mottram et al., 1997; Peng et al., 2021). The total ion chromatograms of triglycerides of different milk samples after accelerated oxidation are shown in Supplemental Figure S5 (https://doi.org/10.7910/DVN/JJKX0W), and a representative MS spectrum is illustrated in Supplemental Figure S6 (https://doi.org/10.7910/DVN/JJKX0W). On these chromatograms separated with the octadecyl silane column, the triglycerides were eluted out in the order of their equivalent carbon numbers (ECN), in agreement with that reported in literature (Zhang et al., 2012; He et al., 2015). The MS spectra (Supplemental Figure S6) indicated that the chromatograph peak at 4.28 min contained 4 triglycerides, SPC (18:0/16:0/10:0), SMLa (18:0/14:0/12:0), OMM (18:1/14:0/14:0), and OPLa (18:1/16:0/12:0) that had the same ECN, 44.

Highly unsaturated triglyceride has less ECN than more saturated ones. As highly unsaturated triglycerides were more vulnerable to oxidation, their amounts decreased more rapidly than the saturated ones after accelerated oxidation. By comparing the amounts of triglycerides having fewer ECN with those having more ECN, it is possible to assess the oxidation degrees. As the triglycerides containing 3 SFA with ECN of 44 were eluted out at 4.28 min, the area sum of the chromatography peaks with retention times less than 4.28 was compared with that with retention times equal to and larger than 4.28. On this basis, the amount ratios of the triglycerides having ECN less than 44 to that having ECN equal to and more than 44 were obtained and expressed as ratio (R). After accelerated oxidation, the milk samples containing Cat, Epi, or B1 showed significantly larger R values than the blank milk, indicating that Cat, Epi, and B1 protected the unsaturated triglycerides in the milk from oxidation (Figure 3A, Supplemental Figure S5). The results are consistent with the phenomena observed for the antioxidant quer- cetin in preventing the oxidation of fatty acids (Liu et al., 2021).

Further experiment was carried out to see if these flavan-3-ols could stabilize the level of the endogenous antioxidant glutathione in the milk. Because glutathione is quickly oxidized, it was quantified as the NEM.
derivative using the method reported (Hamad et al., 2019). After heat treatment, the content of glutathione in the B1-containing milk was higher than that in the blank milk (Figure 3B), indicating that B1 could slow down the oxidation of glutathione and the latter may play an important role in preventing the milk triglycerides from oxidation.

Flavan-3-ols have been reported to have many health benefits, including antioxidant, anti-inflammatory, cytoprotective, and antimicrobial activity (Fraga and Oteiza, 2011; Rahman et al., 2017; Nawrot-Hadzik et al., 2021). The present research revealed the antioxidant activities of flavan-3-ols that could be absorbed to sheep milk, adding new evidences for the values of these flavan-3-ols and for the milk.

CONCLUSIONS

Dairy sheep that consumed C. songaricum, which contains high levels of flavan-3-ol polymers, had increased levels of monomeric and dimeric flavan-3-ols in the sheep milk. Epicatechin and A2 in the milk were derived from the absorption of polymeric flavan-3-ols during digestion. Procyanidin A in the milk can also come from enzymatic conversion of the corresponding procyanidin B. The higher levels of these flavan-3-ols increased the antioxidant effects of the milk.

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