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Aqueous Stability of Astilbin: Effects of pH, Temperature, and Solvent

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Supporting Information

ABSTRACT: The influence of temperature, pH value, and solvent on the degradation behavior of astilbin was studied by HPLC. Results showed that the degradation of astilbin was pH and temperature dependent, and the isomerization of astilbin to its three stereoisomers was found. The degradation process followed the first-order kinetics model, and the degradation rate k values increased, whereas half-life ($t_{1/2}$) values declined with the rise of pH and temperature. The stability of astilbin was related to its B-ring substitution. Engeletin with a 4′-hydroxy-substituted B-ring was more stable than astilbin with a 3′,4′-dihydroxy-substituted B-ring. The stability of astilbin differed depending on the solvent and followed the order 50% ethanol > ethanol > methanol > 50% methanol > water. In cultural media, astilbin was less stable than in water, which may be related to the presence of metal ions. The stability results of astilbin were confirmed in the extraction of dihydroflavonols from *Rhizoma Smilacis Glabrae* and may have a guiding function in turtle jelly production.

KEYWORDS: *astilbin, engeletin, degradation kinetics, isomerization*

INTRODUCTION

Astilbin, (2*R*,3*R*)-3,3′,4′,5,7-pentahydroxyflavanon-3-α-L-rhamnopyranoside, is a dihydroflavonol rhamnoside, significant in the treatment of immunologically related diseases.[1−4] It has also shown many other bioactivities, such as antioxidative,[5] antibacterial,[6] and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitory.[7] It is a natural dihydroflavonol found in many herb-, fruit-, and plant-based foods, for example, *Engelhardia roxburghiana*,[8] *Smilax* genera plants,[9] grape,[10] wine, and turtle jelly.[11] Astilbin is the dominant bioactive compound of *Rhizoma Smilacis Glabrae* (*RSG*) and the *China Pharmacopoeia* requests its content to be not below 0.45%.[12] *RSG* is a herbal material commonly used in China for detoxication. It is the main ingredient of turtle jelly, a popular functional food used for hepatoprotection and for improving skin disorders in Hong Kong. Hence, in a turtle jelly sample, astilbin is detectable and may be used as a key bioactive constituent for its quality control and health benefit effect evaluation.

Besides astilbin, engeletin is also a commonly found dihydroflavonol in nature. Engeletin differs from astilbin by having a 4′-hydroxy-substituted B-ring versus astilbin, which has a 3′,4′-dihydroxy-substituted B-ring. The two compounds were always found together, for example, in *RSG*,[13] *E. roxburghiana*,[8] and grape.[14] Both engeletin and astilbin have shown similar bioactivities, such as anti-inflammatory activity,[8] novel immunosuppressive activity,[15] prevention and treatment of diabetic complications,[16] etc.

Although flavonoids have many health-promoting effects, these compounds are usually unstable to light, pH, and thermal treatment. Knowledge about the stability of flavonoids in aqueous solution is important for predicting the duration of their physiological effects in foods and beverages. However, there is little knowledge on the degradation behavior of astilbin under different aqueous conditions. According to the molecular structure of astilbin, it has four stereoisomers depending on the C-2 and C-3 positions. Our previous study showed that astilbin was unstable, and its isomerization and decomposition were found in alkaline medium.[17] In turtle jelly samples, astilbin was not found in some brands, although the label indicated that *RSG* was used.[11] The wrong use of confusable species of *RSG* may have caused these results.[13] However, it also could be the manufacturing process results in the degradation of astilbin, which makes it absent in the final product.

In the present study, the stability of astilbin in aqueous solutions of different pH values, temperatures, and solvents was studied by HPLC analysis. A first-order reaction model was applied to simulate the degradation behavior of astilbin. The degradation kinetics parameters were deduced and used for stability comparison between astilbin and engeletin. Furthermore, the stability results of astilbin were confirmed in the extraction of dihydroflavonols from *RSG* and may have a guiding function in turtle jelly production.

MATERIALS AND METHODS

Chemicals. Neoastilbin, astilbin, neoisoastilbin, isoastilbin, and engeletin (>98%) were purified from *RSG* by silica gel column chromatography and preparative HPLC in our laboratory similar to the procedures described by Chen et al.[18] The products were identified by UV, IR, MS, and NMR. HPLC grade acetonitrile was purchased from RCI Labscan Ltd. (Bangkok, Thailand). Dulbecco's

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modified Eagle's medium (DMEM) and RPMI 1640 media were purchased from Sigma-Aldrich (St. Louis, MO, USA). *RSG* sample was purchased from Kangmei Pharmaceutical Co., Ltd. (Puning city, Guangong province, China). Double-distilled water was used throughout the study. All other reagents and chemicals employed were of analytical reagent grade.

The pH 3.0 and 5.0 buffer solutions were made of 0.2 M acetic acid and sodium acetate. The pH 7.0, 8.0, 9.0, and 10.0 buffer solutions were made of 0.2 M sodium phosphate and disodium hydrogen phosphate, and the pH was adjusted by 0.1 M sodium hydroxide.

HPLC Analysis. HPLC analyses were performed on an Agilent 1260 HPLC system equipped with a photodiode array detector (DAD), degasser, and autosampler. An Agilent Zorbax SB C18 column (250 mm × 4.6 mm i.d., 5 μm; Agilent, Santa Clara, CA, USA) was used. The mobile phase was acetonitrile (A) and 0.1% acetic acid aqueous solution (B) with a gradient program as follows: 0−15 min, 16−20% A; 15−40 min, 20−40% A. The flow rate was 1 mL/min. Monitoring was performed at 291 nm, and the injection volume was 10 μL.

Aqueous Stability Tests. The stability of astilbin in different pH solutions was studied at room temperature (18 °C). A 1.0 mL aliquot of the astilbin solution (2 mg/mL in 50% methanol) was mixed with 9 mL of buffer at different pH values. At different time intervals, the remaining astilbin was measured according to the above-mentioned HPLC method.

The stability of astilbin at different temperatures was studied in 0.2 M phosphate buffer at pH 7.0. A 1.0 mL aliquot of the astilbin solution (2 mg/mL in 50% methanol) was mixed with 9 mL of buffer, followed by incubation at different temperatures (4, 18, 25, 35, 45, and 55 °C). At different time intervals, the remaining astilbin was measured according to the above-mentioned HPLC method.

In comparison to astilbin, the effects of pH and temperature on the stability of engeletin were also studied. The experimental design and procedures were exactly the same as described for astilbin.

The stability of astilbin in different solvents (water, methanol, ethanol, 50% methanol, and 50% ethanol) was studied at 85 °C in a water bath. Ten milligrams of astilbin was dissolved in 50 mL of each solvent and then refluxed for 7 h. The remaining astilbin was measured according to the above-mentioned HPLC method.

The stability of astilbin in different culture media was studied at 25 °C. A 1.0 mL aliquot of the astilbin solution (2 mg/mL in 50% methanol) was mixed with 9 mL of water or different culture media (high-glucose DMEM, low-glucose DMEM, and RPMI 1640). After incubation of 8 h, the remaining astilbin was measured according to the above-mentioned HPLC method.

Extraction of Dihydroflavonols from *RSG*. Dried *RSG* was finely homogenized, and 1.0 g of sample was added to 50.0 mL of different extraction solvent (water, 0.1 M $NaHCO_3$ aqueous solution, 60% ethanol) and refluxed for 1 h. The mixture was filtered by a 0.22 μm pore size filter and then used for HPLC analysis.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD) of triplicates. Statistical calculations, plotting, and curve fitting were performed by Origin 8.0 (Origin Lab Co., Northampton, MA, USA).

RESULTS AND DISCUSSION

Effect of pH Values on the Degradation Kinetics of Astilbin. The stability of astilbin in different pH solutions (3.0, 5.0, 7.0, 8.0, 9.0, and 10.0) at 18 °C was studied. As shown in Figure 1B, astilbin was stable in acidic solution. Its peak area in the HPLC chromatogram was almost unchanged at pH 3.0 and 5.0 during 48 h of storage. However, after a pH increase it became unstable. In alkaline solution, the remaining astilbin decreased over storage time, and the higher the pH value, the faster the degradation.

Figure 1A clearly shows that some new peaks appeared in the HPLC chromatogram of astilbin after storage in alkaline solution. The most obvious is peak 2, which was identified as

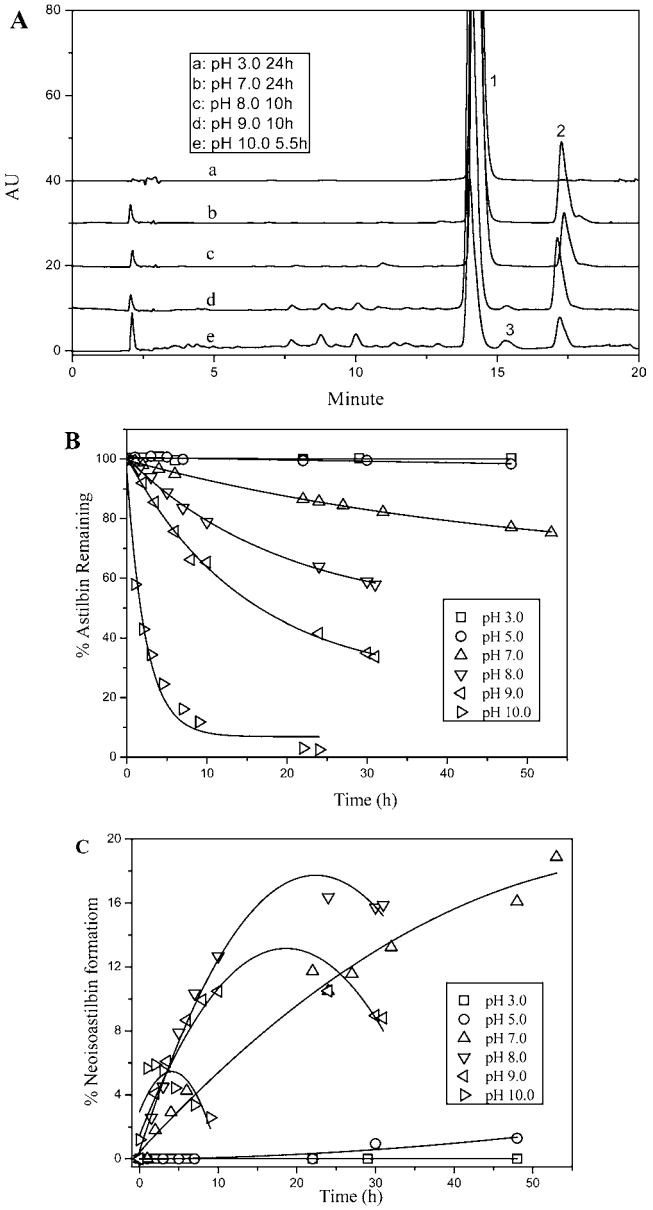
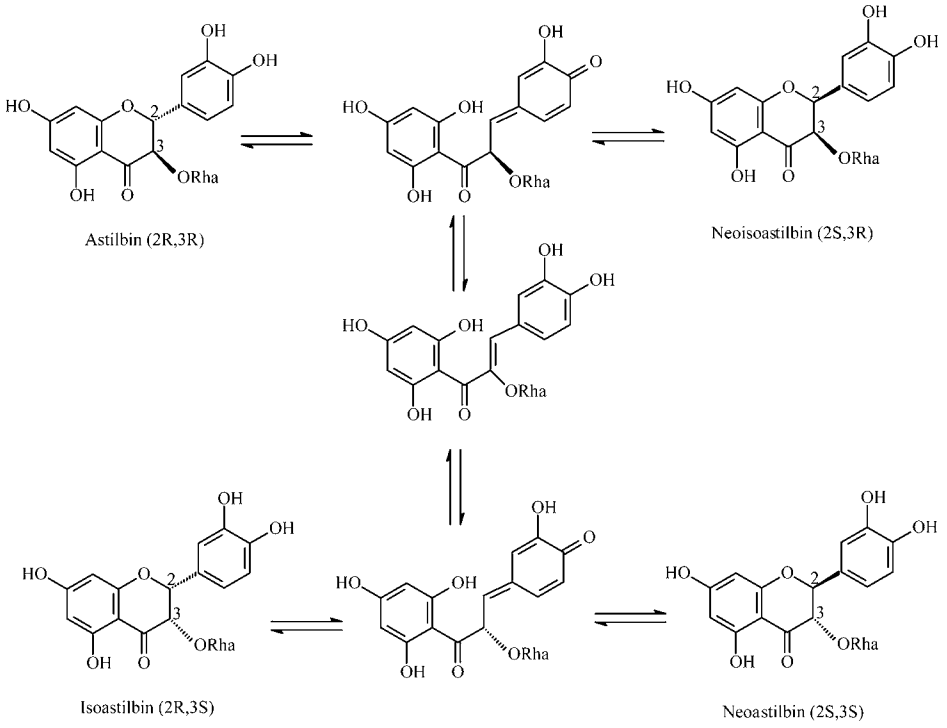


Figure 1. (A) HPLC chromatogram of astilbin under different pH conditions at 18 °C. (B) Time course for loss of astilbin in solutions of different pH values. (C) Time course for neoisoastilbin formation in solutions of different pH values. Results are shown as percentage of remaining astilbin and formed neoisoastilbin to initial astilbin concentration as determined by HPLC peak area. Peaks: 1, astilbin; 2, neoisoastilbin; 3, taxifolin.

neoisoastilbin (2*S*,3*R*), one stereoisomer of astilbin (Scheme 1), by comparing the retention time with standard marker. In Figure 1C, the formation of neoisoastilbin at pH 7.0 stably increased over time. The sum of remaining astilbin and formed neoisoastilbin represented 94.1% of the original astilbin after 48 h of storage. However, at higher pH, the concentration of neoisoastilbin quickly increased along with storage time and then decreased. The higher the pH value, the shorter time it reached the maximum, and the maximum also decreased. At pH 9.0 and 10.0, the sum of remaining astilbin and formed neoisoastilbin represented 75.7 and 14.4% of the original astilbin at 10 and 9 h, respectively. These values decreased to 52.0 and 2.5% at 24 h, respectively. The results clearly showed that alkaline medium could accelerate the isomerization of

Scheme 1. Proposed Route of the Rearrangement of Astilbin and Its Stereoisomers



astilbin. On the other hand, the decomposition of astilbin and its isomer also became faster with the rise of pH value. As shown in Figure 1A, many small peaks were found in the chromatogram of astilbin after storage in pH 9.0 and 10.0. These peaks represented the degradation products of astilbin and neoisoastilbin. Maini et al. have investigated the decomposition products of UVA-irradiated flavonols (galangin, kaempferol, quercetin, and myricetin) by HPLC-MS/MS analysis. The results showed that the major decomposition products were depside, 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, etc.[19] Peak 3 was identified as taxifolin, the aglycon of astilbin. The results show that in alkaline medium the hydrolysis of the rhamnose moiety in astilbin occurs.

Effect of Temperature Values on the Degradation Kinetics of Astilbin. The stability of astilbin at different temperatures (4, 18, 25, 35, 45, and 55 °C) at pH 7.0 was investigated. As shown in Figure 2B, the degradation rate of astilbin increased with the rise of temperature. The HPLC chromatogram (Figure 2A) shows that the main degradation product of astilbin was neoisoastilbin. In Figure 2C, the formation of neoisoastilbin stably increased with time at low temperatures (4, 18, and 25 °C). However, with the temperature rise, the concentration of neoisoastilbin quickly increased and then decreased gradually. At 55 °C, neoisoastilbin reached its maximum within 1 h and then decreased. These results indicated that the isomerization of astilbin was an endothermic reaction, and its rate increased with the rise of temperature. Furthermore, the decomposition of astilbin and its enantiomer was also accelerated by the temperature. The sum of remaining astilbin and neoisoastilbin represented 91.4% of the original astilbin after 10 h of storage at 25 °C. However, this value was only 24.8% after 8.5 h of storage at 55 °C.

At 45 and 55 °C, more isomerization products of astilbin were found. Peaks 1 and 4 in Figure 2A were identified as neoastilbin (2*S*,3*S*) and isoastilbin (2*R*,3*S*), another two stereoisomers of astilbin (Scheme 1), by comparing the retention time with standard markers, respectively. However, the formation of these two isomers seemed very difficult. They were not found in the chromatogram of astilbin when the storage temperature was below 25 °C. The maximum amounts of neoastilbin at 45 and 55 °C were 0.91 and 1.92% of initial astilbin, respectively. The amount of isoastilbin was even less. A proposed route of the isomerization reaction is shown in Scheme 1. In alkaline medium, hydroxyl ion caused the ring-opening of the C-ring at positions 1 and 2 in astilbin (2*R*,3*R*) and formed a quinone methide. The recyclization of quinone methide caused the formation of neoisoastilbin (2*S*,3*R*). The intermediate chalcone formed through the migration of the double bond in quinone methide caused the formation of the other two isomers. Because the formation of neoastilbin (2*S*,3*S*) and isoastilbin (2*R*,3*S*) needs more process than neoisoastilbin (2*S*,3*R*), they were difficult to form.

Gaffield et al. have studied the isomerization of astilbin under different conditions.[20] All four stereoisomers were found in the final reaction solution. However, the isomeric ratio was dependent on the reaction condition, and the reaction mechanisms were different. Heating astilbin with D_2O/pyridine at 75 °C resulted in chalcone formation, whereas isomerization of astilbin in ethanolic NaOAc at room temperature yielded selective epimerization at C-2 and C-3. Taxifolin, the aglycon of astilbin, also has four stereoisomers like astilbin. Elsinghorst et al. have investigated the thermal and enzymatic conversion of taxifolin to alphitonin.[21] Their results that during thermal treatment the conversion of (+)-(2*R*,3*R*)-taxifolin resulted in the temporary enrichment of (+)-(2*S*,3*R*)-epitaxifolin are in accordance with our results that neoisoastilbin (2*S*,3*R*) was more easily converted from astilbin than the other two isomers.

Kinetics Comparation between Astilbin and Engeletin. A first-order reaction model has been applied for the description of degradation of astilbin under various conditions. The model is expressed as

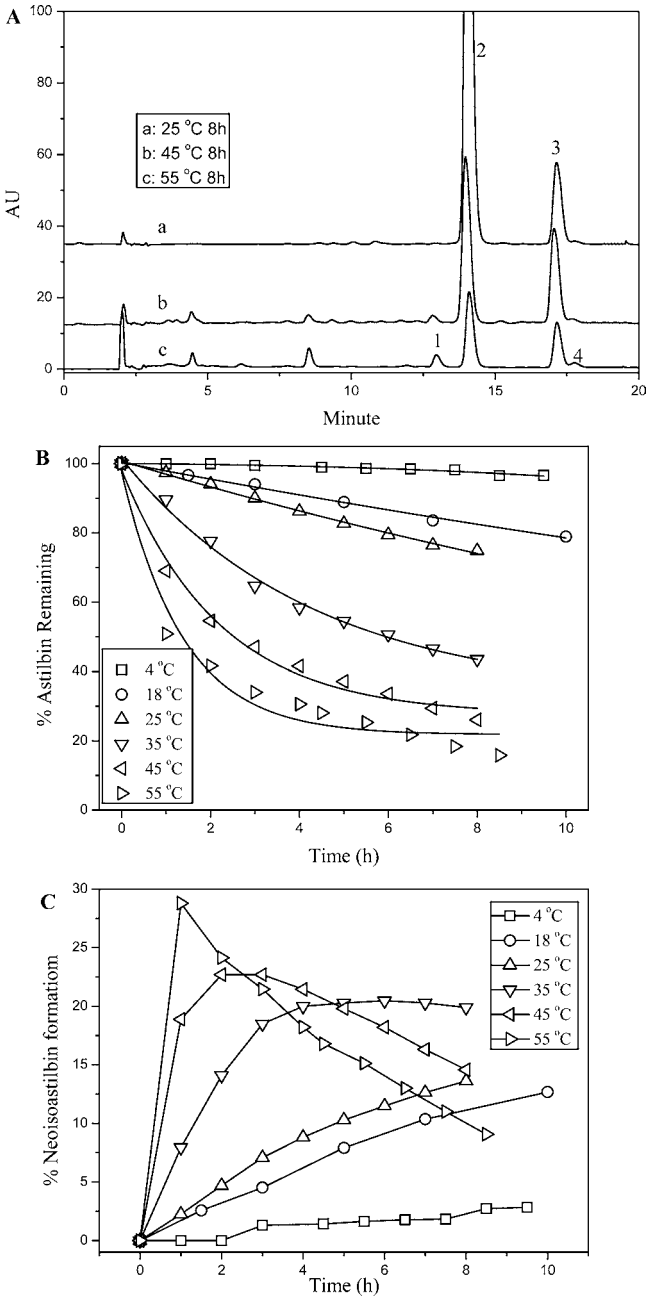


Figure 2. (A) HPLC chromatogram of astilbin in phosphate buffer at pH 7.0 under different incubation temperatures. (B) Time course for loss of astilbin at different temperatures. (C) Time course for neoisoastilbin formation at different temperatures. Results are shown as percentage of remaining astilbin and formed neoisoastilbin to initial astilbin concentration as determined by HPLC peak area. Peaks: 1, neoastilbin; 2, astilbin; 3, neoisoastilbin; 4, isoastilbin.

$$\ln(C_t/C_0) = -k \times t$$

where C_0 is the initial astilbin concentration, C_t is the astilbin content after treatment time t (h) at a given condition, and k is the rate constant (h^{-1}). The half-life ($t_{1/2}$) is the time needed for 50% degradation of astilbin. $t_{1/2}$ is calculated by the following equation:

$$t_{1/2} = -\ln 0.5 \times k^{-1}$$

The first-order reaction model has been found to be appropriate to simulate the degradation behavior of many natural compounds, for example, anthocyanins,[22,23] catechin,[24] and chlorogenic acid,=.[25] Our results showed that the first-order reaction model was appropriate to simulate the degradation behavior of astilbin. The simulation equations and calculated k and $t_{1/2}$ values are shown in Figure S1 and Table S1 (in the Supporting Information). The deduced parameters showed that the degradation rate k values of astilbin increased and that $t_{1/2}$ values decreased along with the rise of pH and temperature.

Maini et al. revealed that the number of substituents on the B-ring could influence the stability of a flavonol in aqueous solution.[19] In the present study, the stabilities of astilbin and engeletin were compared. The difference between these two compounds is that engeletin has just one 4′-hydroxy group, whereas astilbin has a 3′,4′-dihydroxy group in its B-ring. The results shown in Figure 3 revealed that, similar to astilbin, the stability of engeletin was also pH and temperature dependent. With the pH and temperature rise, the k values of engeletin increased; thus, it degraded more rapidly. However, it could be observed that at the same conditions, astilbin has higher k values than engeletin, which indicate that the degradation of astilbin was faster than that of engeletin. Under treatments at pH 10.0 and 18 °C, the k value of astilbin (0.2666) was about 2.5 times that of engeletin (0.1051). The $t_{1/2}$ values of astilbin and engeletin in pH 7.0 at 45 °C were 3.70 and 9.08 h, respectively. These results that engeletin has higher stability than astilbin are in accordance with the conclusion of Maini et al. that flavonol stability decreases with increasing B-ring substitution.[19]

Degradation of Astilbin in Different Solvents. Figure 4A shows the remaining astilbin and formed neoisoastilbin in different solvents after 7 h of refluxing at 85 °C. In methanol and ethanol, only the isomerization of astilbin to neoisoastilbin was found, because the remaining astilbin and formed neoisoastilbin represented nearly 100% of the initial astilbin. However, in distilled water, besides isomerization, the decomposition of astilbin was also found. Furthermore, the isomerization rate was faster than those in methanol and ethanol. Although the boiling points of methanol and ethanol are 64.7 and 78.4 °C, respectively, Figure 4A clearly shows that the isomerization of astilbin in methanol was faster than that in ethanol. The boiling point of the mixture is lower than that of either component. However, the degradation of astilbin in 50% methanol and 50% ethanol was totally different. In 50% ethanol, almost no isomerization and decomposition of astilbin were found. On the contrary, the degradation of astilbin in 50% methanol was faster than that in methanol and similar to that in water. These results showed that besides temperature, the isomerization and decomposition of astilbin were solvent dependent. The stability of astilbin in different solvents followed the order 50% ethanol > ethanol > methanol > 50% methanol > water.

The degradation of astilbin in different cultural media was studied at 25 °C after 8 h of incubation. As shown in Figure 4B, astilbin was unstable in different cultural media even at 25 °C, and the stability followed the order RPMI 1640 > high-glucose DMEM > low-glucose DMEM. Besides isomerization, the decomposition of astilbin in DMEM was notable. After 8 h of incubation, the sum of remaining astilbin and formed neoisoastilbin represented only 63.9% of initial astilbin in low-glucose DMEM. However, the sum value in double-distilled water was nearly 100%. Astilbin in cultural media was more unstable than in water. One possible explanation is that

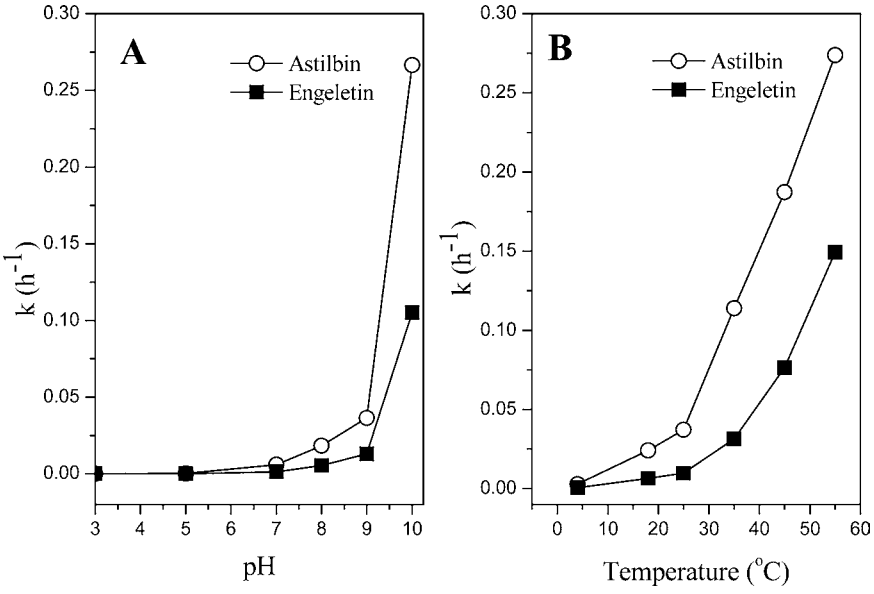


Figure 3. Correlation between the *k* values of astilbin and engeletin on various values of pH (A) and temperature (B).

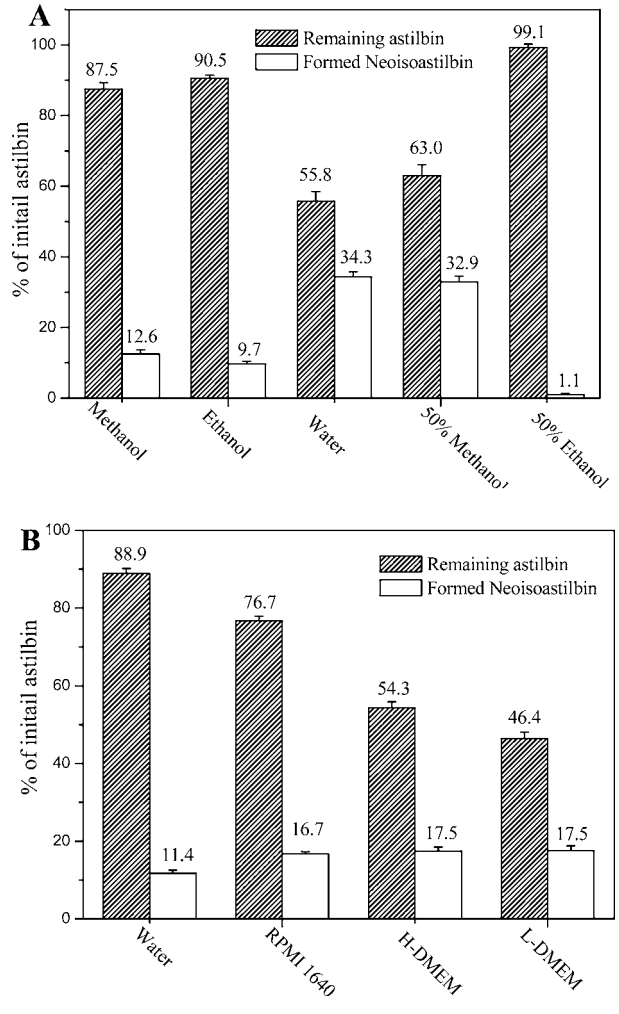


Figure 4. (A) Remaining astilbin and formed neoisoastilbin in different solvents after for 7 h of refluxing at 85 °C. (B) Remaining astilbin and formed neoisoastilbin in different cultural media after incubation at 25 °C for 8 h.

cultural media contain many metal ions, such as Fe^{2+}, Ca^{2+}, and Mg^{2+}, which may chelate to the 3′,4′-B-ring hydroxyl groups of astilbin and accelerate its oxidation. According to the media formulations of Sigma-Aldrich, the contents of calcium chloride and magnesium sulfate in DMEM are 0.2 and 0.09676 g/L, respectively, which are almost twice that of RPMI 1640. Furthermore, Fe^{2+} is absent in RPMI 1640 medium, but contained in DMEM with a content of 0.1 mg/L (ferric nitrate·$9H_2O$). The results revealed that the decomposition of astilbin in RPMI 1640 medium was slower than that in DMEM. Maini et al. also found that metal ions in solution could enhance the rate of flavonol decomposition in DMEM relative to DPBS.[19] Our results suggest that when the bioactivity of astilbin is studied by using a cell model, the isomerization and decomposition of astilbin in the cultural medium should be concerned.

Effect of Different Solvents on Astilbin Extraction from *RSG*. Dihydroflavonols such as astilbin, isoastilbin, neoastilbin, neoisoastilbin, and engeletin are the main bioactive constituents in *RSG*,[13] which is the main ingredient of turtle jelly. Besides *RSG*, *Mesona chinensis* Benth. is also used in turtle jelly, which contains high amounts of polysaccharides that could synergistically interact with starch to facilitate the formation of jelly. The polysaccharides are always extracted by using sodium bicarbonate or sodium carbonate.[26]

However, the use of sodium bicarbonate or sodium carbonate will cause the decoction to become alkaline. On the basis of our present study, we suspect that this process may cause the decomposition of dihydroflavonols from *RSG*. To prove it, *RSG* was extracted by different solvents and the constituents in the extract were analyzed by HPLC. As shown in Figure 5, peaks 1−5 in the chromatogram were neoastilbin, astilbin, neoisoastilbin, isoastilbin, and engeletin, respectively. None of theses peaks was found in the extract of *RSG* refluxed with 0.1 M $NaHCO_3$ for 1 h, which means that all of these dihydroflavonols were decomposed. Our previous study showed that astilbin was not found in some turtle jelly samples, although the label indicated that *RSG* was used.[11] On the basis of the constituent comparison between *RSG* and its confusable species, we proposed that the manufacturers may have used

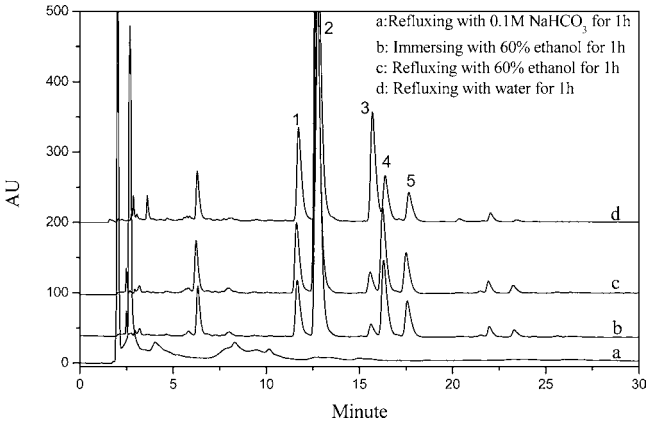


Figure 5. HPLC chromatogram of *RSG* sample extracted with different solvents and methods: extraction with (a) refluxing with 0.1 M $NaHCO_3$ for 1 h; (b) immersing with 60% ethanol for 1 h; (c) refluxing with 60% ethanol for 1 h; (d) refluxing with water for 1 h. Peaks: 1, neoastilbin; 2, astilbin; 3, neoisoastilbin; 4, isoastilbin; 5, engeletin.

confusable species of *RSG* in their product. The HPLC fingerprint of *RSG* was developed for raw material identification.[13] On the basis of our present study, we conclude that the absence of astilbin in turtle jelly may also result from improper processing. Thus, the manufacturers can preferably keep weak acidic conditions during decoction of the herbs. Furthermore, to prolong the shelf life, turtle jelly should be weakly acidic, for example, at pH around 5.

We supposed that immersing *RSG* with 60% ethanol at room temperature (~18 °C) would not cause the isomerization of astilbin. In the chromatogram of the *RSG* sample that was extracted by refluxing with water (Figure 5d), the areas of peaks 1 (neoastilbin) and 3 (neoisoastilbin) increased notably compared to the *RSG* sample that was immersed with 60% ethanol (Figure 5b), whereas the areas of peaks 4 (astilbin) and 7 (isoastilbin) decreased significantly. However, these changes were minor between the *RSG* samples that were extracted by refluxing and immersing with 60% ethanol (Figure 5b,c). The results showed that compared to 60% ethanol, the isomerization reaction between astilbin and its three stereoisomers was more significant when water was used as the extraction solvent by refluxing. The results are in accordance with previous findings that the isomerization reaction was temperature and solvent dependent.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1 and Table S1. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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