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Storage stability and DPPH scavenging capacity of polyphenols of *Mongolia scotch pine*

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Mongolia scotch pine have recently received great attention for their health benefits, mainly due to their high polyphenols content. Nevertheless, stability of pine polyphenols in liquid solution and DPPH scavenging ability during the process of storage have been only little investigated. In this present study, Mongolia scotch pine cone peer was used as raw material, effect of different storage conditions on polyphenol stability were studied, based on color and DPPH free radical scavenging capacity of polyphenols change. Polyphenols contents were quantified with Folin-Ciocalteu. L*, a* and b* values were detected using Color Companion software and antioxidant ability was evaluated by DPPH scavenging capacity. The results showed that polyphenols decreased 16%, 91.3% and 97.2% at 4 deg C, 25 deg C and 40 deg C respectively during 5weeks storage time and degradation followed first-order kinetics. The Activation energy was 68.98kJ/mol^{-1}. Polyphenols were stable at neutral and alkaline environment. The color analysis revealed that color parameters (L*, a*, b* and TCD) were assumed to have an Arrhenius-type dependence on temperature, model followed a first-order reaction. The DPPH free radical scavenging capacity of phenolics is affected by storage temperature, pH and time, and it reached the highest value at 1th week. This research highlights the importance of considering conditions when preserving polyphenols. In cool, neutral and alkaline environment, the polyphenols were stable for 5 weeks.

Keywords: Mongolia scotch pine cone, Polyphenol, Storage stability, DPPH.

Устойчивость при хранении и способность захвата свободных радикалов ДФПГ полифенолов сосны монгольской обыкновенной

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Сосна монгольская обыкновенная с недавнего времени исследуется всилу своих полезных для здоровья человека свойств, в основном, благодаря высокому содержанию полифенолов. Тем не менее, устойчивость полифенолов сосны в жидком растворе и способность захвата дифенилпикрилгидразила (ДФПГ) в процессе хранения изучены недостаточно. В данном исследовании в качестве сырья использовались шишки сосны монгольской обыкновенной. Анализировалось воздействие различных условий хранения на устойчивость полифенолов на основе изменения их цвета и способности захвата свободных радикалов дифенилпикрилгидразила. Содержание полифенолов измеряли с помощью реагента Folin-Ciocalteu. Значения L*, a* and b* определялись при помощи программного обеспечения Color. Антиоксидантные свойства оценивались на основе способности захвата дифенилпикрилгидразила. Показано, что содержание полифенолов уменьшается на 16%, 91.3% и 97.2% при 4 град. С, 25 град. С и 40 град. С соответственно в течение 5 недель хранения и распад происходит согласно кинетике первого порядка. Энергия активации составила 68,98 кДж/моль$^{-1}$. Полифенолы сохраняли устойчивость в щелочной и кислой средах. Анализ цветности выявил, что параметры L*, a*, b* и полного цветового различия, предположительно, имеют зависимость от температуры, описываемой уравнением Аррениуса, и модель следует реакции первого порядка. На способность захвата свободных радикалов дифенилпикрилгидразила у фенольных смол влияет температура, pH и время хранения, максимальные значения достигаются на первой неделе. Исследование подчеркивает важность учета условий для хранения полифенолов. В прохладной, нейтральной и щелочной среде они устойчивы в течение 5 недель.

Keywords: сосна монгольская обыкновенная, шишка, полифенол, устойчивость при хранении, дифенилпикрилгидразил.

Mongolia Scotch Pine is one of the most important of the pinaceae, belonging to the evergreen trees, strong adaptability, fast growth, widely and mainly distributed in the the Xingan, Jilin Changbai Mountain and the northeast of China [1]. Pine polyphenols contains phenolic compounds in the pinaceae plants, with a series of unique chemical properties and biological activity. Ortho phenolic hydroxyl in polyphenols can easily be oxidized, and have strong ability to capture free radicals, which has antioxidant, hypolipidemic, anti-tumor, bactericidal activity [2–5]. This can be applied to food, medicine, cosmetics, health products and other fields.

The tea polyphenols were deeply research on the stability of plant polyphenols during storage, temperature and pH value were the main factors to research the stability of catechol in aqueous solution and tea beverage [6–8]; Ortiz [9] demonstrated that catechol in the Green Tea loss increase with the increase of relative humidity of the storage environment; Li [10] studied the catechol's degradation kinetics in dry powder condition of the Green Tea. Pine polyphenols research mainly concentrated in the study of the method of extraction, purification and antioxidant activity, the study on the storage stability neither liquid nor solid has not been reported.

A plurality of ortho phenolic hydroxyl contained in pine polyphenols can easily affected by environmental factors in the storage and application process, once these groups oxidized and degraded, its content, composition, structure and color will change, Besides, its antioxidant activity will be reduced and lose the original function and the value [11]. There are a lot of method to determinate antioxidant activity such as DPPH method, TEAC method, ORAC method, TRAP method and FRAP method [12, 13], and DPPH scavenging activity analysis method has been widely used for screening antioxidants [14, 15]. Therefore, stability of pine polyphenols in liquid solution and DPPH scavenging ability during the process of storage was studied to find out the suitable storage utilization condition, provide the basis for further utilization and effective storage of pine polyphenols.

Materials and methods

1. Materials and instruments

Materials. Mongolia scotch pine cone were from Maoershan forest, Haerbin, Heilongjiang Province. AB-8 macro porous resins were from Tianjin bohong resin technology Co.Ltd.; DPPH was purchased from Sigma Company; other reagents were analytically pure.

Instruments. FW135 high-speed universal grinder, DK-98-IIA electro thermostatic water bath were purchased from Tianjin Taisi Instrument Co.Ltd; RE-52A Rotary evaporator was from Shanghai Biochemical Instrument Co.Ltd; 722UV/vis was purchased from Shanghai Spectrum Instrument Co. Ltd.; DHG-9240 Electric constant heating drying box was purchased from Shanghai Yiheng Scientific Instrument Co. Ltd.; Electronic balance was from Shanghai Precision Scientific Instrument Co. Ltd.; Column chromatography was purchased from Tianjin Haiguang Reagent Company.

2. Experiment method

Preparation of Pine polyphenols extraction.

Mongolia scotch pine cone peer was dried to constant weight in natural light and crushed into powder (40 mesh sieves). The powder was extracted according to the following conditions: ethanol concentration 60%, ratio of solid to liquid 1:30 (g/mL), extraction time 4 h, extraction temperature 60 °C. Solid was abandoned, liquid was add to constant volume after rotary evaporated.

Pretreatment of resins.

Resins were washed by water until stimulated smell disappeared after soaked with 90% ethanol. Immersed in 4% hydrochloric acid solution for 3 hours and washed by water, then immersed in 4% sodium hydroxide solution for 3 hours and washed by water. Once eluted, resins must be immersed in 4% hydrochloric acid solution for 3 hours and washed by water, then immersed in 4% sodium hydroxide solution for 3 hours and washed by water [16].

Purification of polyphenols.

Resins 2 g, polyphenols extract solution (0.15 mg/mL) 50 mL were accurate added to the powder. In the water bath 24 hours for adsorption to balance at room temperature. Filter out the resins and add 50% ethanol solution 50 mL to the resins for desorption at room temperature.

Calculation of polyphenol content [17, 18]

Calculate the content of polyphenol solution with the formula:

$$M = (Y - C) \cdot 500 \cdot 25 / (A \cdot m \cdot 1000),$$

where Y is the absorbance; M is the content of total polyphenol in the product; 500 was the sample volume size; 25 was the measurement of the volume of sample volume.

Storage stability of polyphenol from *Mongolia Scotch pine cone peer*

1. Effect of different pH value on the stability of polyphenols solution.

Get 10 mL polyphenol extract solution and add distilled water to a constant volume (50 mL) in Erlenmeyer flask. 5 mL liquid was removed to test tubes respectively and the pH value was adjusted to 3, 5, 7, 9 and 11. Observe the change of solution during storage (0, 1, 2, 3, 4, 5 week), calculate the polyphenol residual rate and L, a, b value. Do three parallel experiments.

2. Effect of different temperatures on the stability of polyphenols solution.

The samples were placed in 4 °C refrigerator, room temperature away from light, 40 °C constant temperature box. Observe the change of solution during storage (0, 1, 2, 3, 4, 5 week), calculate the polyphenol residual rate and L, a, b value. Do three parallel experiments.

Degradation kinetic parameters during storage [10, 19, 20].

According to the rate equation: $\ln \frac{x}{x_0} = -kt$,

Where x_0 is the initial mass concentration (mg/mL); x is the mass concentration when reaction time is t (mg/mL); t is reaction time (week); k is the reaction rate constant (week^{-1}).

The activation energy was determined according to the Arrhenius equation:

$$\ln k = \ln A - (\frac{E_a}{R})(\frac{1}{T}),$$

where k is the reaction rate constant (week^{-1}), A is the frequency factor, E_a is the activation energy (kJmol^{-1}), R is the molar gas constant (8.3145 J/mol·K), T is the temperature (K). Using the formula $t_{1/2} = 0.693/k$ can find out the half-life, k is first-order reaction rate constant.

DPPH free radical scavenging capacity of polyphenols during storage [21].

The sample solution were added with 50 μL of different concentration gradient in the test tubes respectively, anhydrous methanol at 1 mL, 1 mL, 200 μmol (79 μg/mL) of DPPH methanol solution, were mixed with samples as the experimental group, evaded the light place at room temperature. Starting from 0min up to 60 min, reaction solution absorption values were measured every 10 min at 517 nm, the control group using anhydrous methanol solution instead of samples, other operating above, anhydrous methanol was used in the blank group. Determination of the reaction index was absorption values, the median inhibitory rate (IC_{50}) evaluates the samples on DPPH free radical scavenging capacity.

DPPH radical scavenging rate expressed as: clearance = $(A_1 - A_2)/A_1 \times 100\%$. A_1 is the absorption value of control group; A_2 is the absorption value of experimental group with sample solution. The antioxidant BHT standard as a reference, prepare four concentration gradient standard solution (182.926, 365.853, 548.780, 731.7073 μg/mL) to measure the capability of DPPH radical scavenging. The polyphenol extract was diluted to 16.6 μg/mL, 33.2 μg/mL, 49.8 μg/mL, 66.4 μg/mL, 83 μg/mL with 50% ethanol, then determined DPPH free radical scavenging and IC_{50} in different concentration gradient to get the correlation between different storage conditions and DPPH free radical scavenging capacity of Mongolia scotch pine cone polyphenols.

Statistical analyses.

All experimental data were determined for 3 times, the results as mean ± standard deviation (± *s*, *n* = 3), Excel 2003 is used to analyze the data.

Results and analyses

1. Gallic acid standard curve

There is a good linear relationship between the absorbance and the content of Gallic acid, regression equation of the standard curve is: $y = 0.0126x + 0.0123$ ($R^2 = 0.9977$).

2. Effect of different pH value on the stability of Mongolia Scotch pine cone peer during storage

Compare the change of polyphenol residual rate in different pH environment, analysis the influence of pH value on the storage stability of polyphenols, as shown in figure 2.

Polyphenols decreased most quickly in the environment of pH 3 after stored for one week, the polyphenol residual rate fell by 53%. With the extension of time, pine polyphenols residual rate continues decrease, Mongolia Scotch pine cone peer polyphenols most unstable in the environment of pH3. The polyphenol residual rate is reduced by 6%, then decreasing trend slowly after stored for one week in the environment of pH 5. Mongolia Scotch pine cone peer polyphenols showed the stable trend of pH value were 7, 9 and 11, the residual rate reductions were 5.6%, 7.2%, 10.4% after stored for five weeks. With the increase of pH value, pine polyphenols residual rate decreased slowly and tend to be stable, the polyphenol stability influence of neutral and alkaline environment smaller than acidic.

3. Effect of different temperatures on the stability of polyphenols solution

Effect of different temperatures on the stability of Mongolia Scotch pine cone peer during storage.

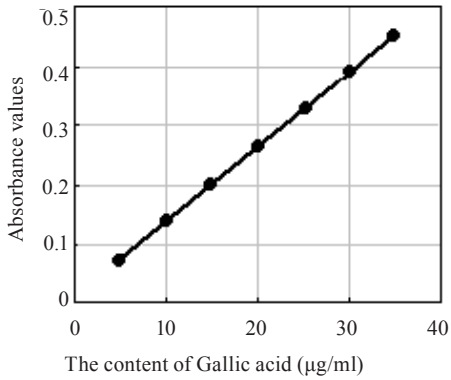

Fig. 1. Gallic acid standard curve

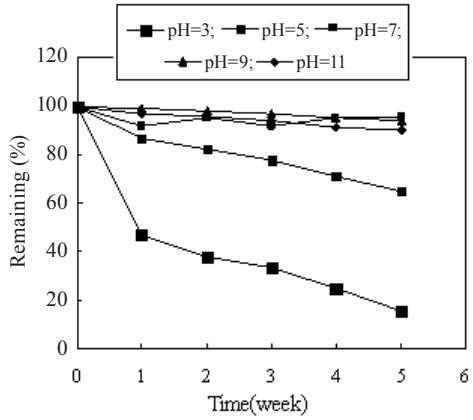

Fig. 2. Effect of different pH value on the stability of Mongolia Scotch pine cone peer during storage

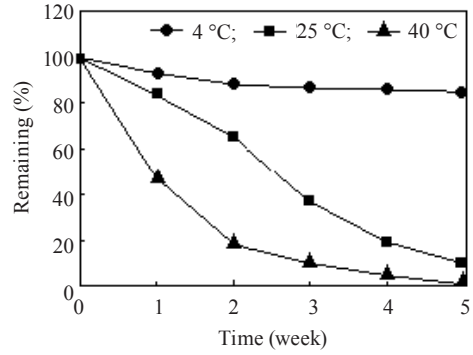

Fig. 3. Effect of different temperature on the stability of Mongolia Scotch pine cone peer during storage

The polyphenols solution was stored at 4 °C, 25 °C and 40 °C for five weeks, the results are shown in figure 3.

The influence of temperature on stability of polyphenols was large, with the increase of storage temperature, polyphenols residue rate changed obviously. Polyphenols decreased from 0.083 mg/ml to 0.0697 mg/ml and the residual rate is reduced by 16% after stored five weeks at 4 °C. Polyphenol content sustained decreased during storage for five weeks at 25 °C, polyphenol content decreased from 0.083 mg/ml to 0.0072 mg/ml and the residual rate is reduced by 91.3%.

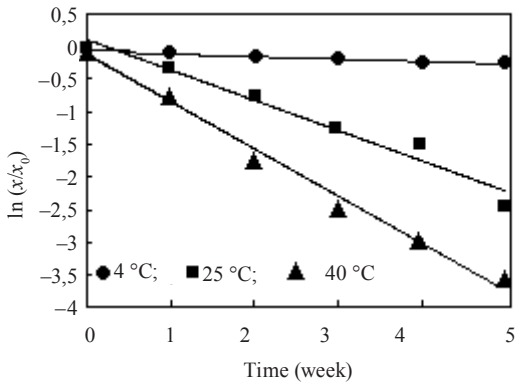

Fig. 4. The degradation of polyphenols at different temperature (4, 25, 40 °C) during storage

Table 1

The thermal degradation kinetic parameters of Mongolia scotch pine cone peer polyphenols

T, °C	R^2	K, week^{-1}	$t_{1/2}$, week	E_a, KJ mol^{-1}
4	0.8958	0.0399	17.37	29.95
25	0.9374	0.4164	1.67	
40	0.9888	0.7583	0.91	

Table 2

Parameters of kinetic model of color changes of polyphenols at different storage temperatures

Color value	Initial data	Storage temperature, °C	K, week^{-1}	R^2	Activation energy E_a, kJ/mol
L	44.5	4	0.0406	0.6419	21.47
		25	0.076	0.7811	
		40	0.1192	0.7769	
a	29.6	4	0.0449	0.9904	30.12
		25	0.1685	0.9411	
		40	0.1893	0.9848	
b	43.3	4	0.1311	0.9867	5.19
		25	0.1552	0.9700	
		40	0.1696	0.9784	
TCD	0	4	5.7955	0.9900	10.53
		25	8.5086	0.9946	
		40	9.7073	0.9976	

Polyphenol content decreases from 0.083 mg/ml to 0.0023 mg/ml, the residual rate was reduced by 97.2% at 40 °C. So the low temperature environment had smaller influence to pine polyphenols, during storage, which appropriate the storage of polyphenols.

Effect of different temperatures on the degradation kinetics of Scotch pine cone scales polyphenols.

According to the changes of polyphenol residual rate, calculate the reaction rate constant and activation energy to determine its degradation kinetics equation, as shown in figure 4.

From Figure 4, stored at different temperatures (4, 25, 40 °C), there was a good linear relationship between — ln (x/x_0) and time, linear regression coefficient (R^2) were greater than 0.89 which indicated polyphenols degradation followed first-order kinetics.

According to Arrhenius (Arrhenius) equation, logarithmic on both sides at the same time, thermal degradation half-life $t_{1/2}$ and activation energy E_a (kJ/mol) of Mongolia scotch pine cone peer polyphenols were educed by plotted the inverse of first-order reaction rate constant (ln$k$) with the inverse of storage temperature (T), which on the basis of the relationship of different temperature (T) and the degradation rate constant (K), as shown in table1.

According to the table 1, different temperatures had effect on the reaction rate constant (k) and half-life $t_{1/2.}$ With the increase of temperature, the K value increased and the half-life decreased, which indicated that Mongolia Scotch pine cone peer was easier degradation in high temperature environment. The activation energy was from 0 to 400 KJ·mol^{-1}, the activation energy smaller, the reaction more easily occurred. The reaction rate was giant when E_a > 400KJ·mol-1 and tiny when E_a < 42 KJ·mol^{-1}. The activation energy was 29.95 KJ·mol^{-1} measured from experiment showed degraded reaction was easy occurred of Mongolia scotch pine cone peer polyphenols.

4. Color changes of Mongolia Scotch pine cone peer polyphenols during storage

The degradation rate constants of color parameters (*L**, *a**, *b** and *TCD*) of Mongolia Scotch pine cone peer polyphenols were analyzed during storage.

*L**, *a**, *b** decreased with the increase of the storage time, with the increase of temperature, decreased faster. Pine polyphenols were oxidized and appeared browning. Linear regression analysis showed that the degradation rate constants of color parameters (*L**, *a**, *b** and *TCD*) were assumed to have an Arrhenius-type dependence on temperature, the model followed a first-order reaction. The Activation energy and the change rate constant of *L**, *a**, *b**, *TCD* were showed in the table 2.

The activation energy is one of the most important parameters for the kinetic study of the reaction, which reflects the amount of energy absorbed from the external environment when a chemical reaction to be occurred. According to the theory of reaction kinetics, the activation energy of the reaction lower, the reaction response faster, conversely, shown as slow reaction [22].

According to the table 2, the activation energy of *a** could be much larger than the activation energy of *b**.The rate constant of *a** and *b** crossed at point with the decrease of temperature (1/T increased) form figure 6, which indicated that the degradation rate of *a** and *b** were the same value at this point. When the temperature is higher than the point, the degradation rate constant of *a** was larger than *b**, and when the temperature is lower than the point, the *b** degradation rate constant of *a** was smaller than *b**. So the activation energy could not be simply relied on to determine the speed of different reaction rates.

5. DPPH free radical scavenging capacity of polyphenols during storage

According to the changes between polyphenol and DPPH responded at 0–60 min, the diagram of the DPPH free radical scavenging capacity of polyphenols was plotted, as shown in figure 7.

As you can see from figure 7, in 60 min, the stable free radical of DPPH in methanol solution almost unchanged (top line), so the solvent does not influence the experiment. The scavenging DPPH free radical reaction kinetics model of polyphenol extracts was characterized by the dual dependence between concentration and reaction time. The DPPH free radical scavenging ability was increased with polyphenol content increased, in the first 10 min, the absorption value decreased rapidly, then gradually flatten, reactions to the steady state required about 20 min and absorption value was stable at 30 min, meanwhile maximum protons had provided by polyphenols for reacting with DPPH free radical, so the clearance rate could be calculated by absorbance value at 30 min as the reaction end point.

According to the method of 1.2.7, the value of IC_{50} of BHT was 478 μg/mL, related results was shown in figure 8.

There had a good linear correlation between different concentrations of Mongolia Scotch pine cone polyphenols and DPPH radical scavenging rate, so as the reference compound BHT. Compared with BHT, Mongolia Scotch pine cone polyphenols had larger slope. By scavenging 50% DPPH radical, the content of polyphenols accounted for only 12.97% of the content of BHT. The polyphenol extract was diluted to 16.6 μg/mL, 33.2 μ g/mL, 49.8 μg/mL, 66.4 μg/mL and 83 μg/mL with 50% ethanol, then determined DPPH free radical scavenging and IC_{50} in different concentration gradient to get the correlation between different storage conditions and DPPH free radical scavenging capacity of Mongolia scotch pine cone polyphenols, as shown in figure 9.

When the pH value was adjusted to 3, 5, 7, 9 and 11, the change of DPPH free radical scavenging capacity of Mongolia Scotch pine cone polyphenols were showed in the figure 9. The capacity of DPPH free radical scavenging of polyphenols at alkaline environment was better than at acid. With the decrease of pH value, the capacity of DPPH free radical scavenging decreased at acid environment, while the capacity increased with the decrease of pH value at alkaline environment. With the increase of storage time, the capacity of DPPH free radical scavenging decreased when pH value were 3 and 5. While when pH value were 7, 9 and 11, the values of IC_{50} were decreased first and then increased with the increase of storage time, the capacity of DPPH free radical scavenging first increased and then decreased, reached the highest value at the 1th week.

The results showed that the change of IC_{50} at 4, 25 and 40 °C respectively. With the increase of storage time, the capacity of DPPH free radical scavenging first increased and then decreased and the capacity order was 4 °C >25 °C >40 °C (figure 10). The capacity of DPPH free radical scavenging reached the highest value at the 1th week.

Conclusions

Mongolia Scotch pine cone peer was researched as raw material to study the stability, color and DPPH free radical scavenging capacity of polyphenols during storage, we can draw the following conclusions:

1. Polyphenols were more stability at neutral and alkaline environment, the stability order is pH7 > pH9 > pH11. With the decrease of pH value, the capacity of DPPH free radical scavenging decreased.

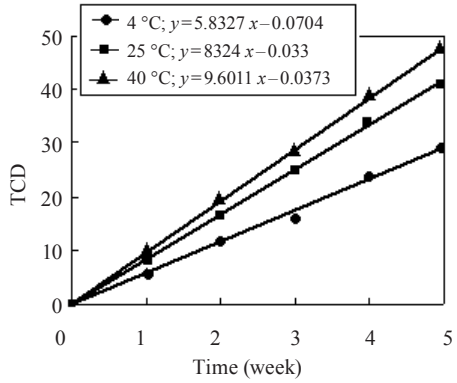

Fig. 5. Thermal degradation of TCD as a function of time and temperature

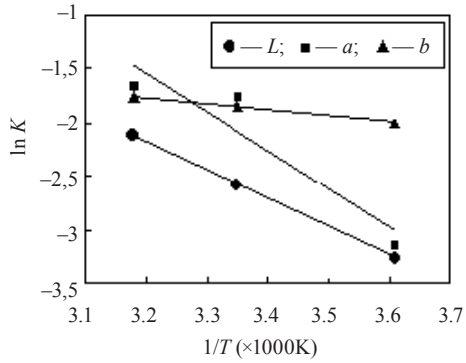

Fig. 6. The relationship between reaction rate constant (k) and color values under different temperatures (T) during storage

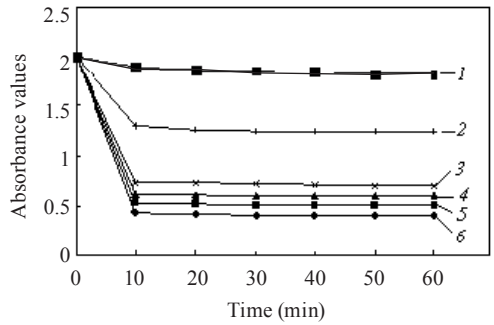

Fig. 7. The DPPH free radical scavenging capacity of different contents of polyphenols:
1 — 79 μg/mL; 2 — 16.6 μg/mL; 3 — 33.2 μg/mL;
4 — 49.8 μg/mL; 5 — 66.4 μg/mL; 6 — 83 μg/mL

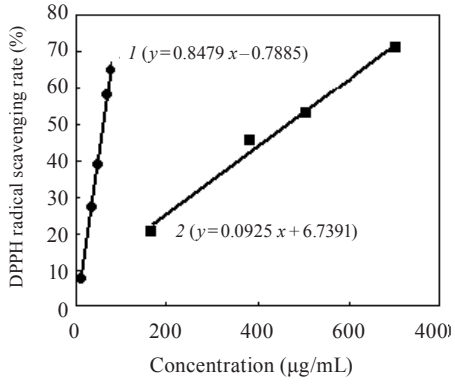

Fig. 8. The relativity of DPPH radical scavenging rate of Mongolia Scotch pine cone polyphenols (1) and the reference compound BHT (2)

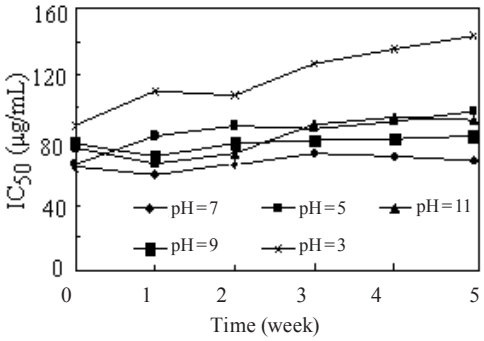

Fig. 9. Effect of different pH value on DPPH free radical scavenging capacity of Mongolia Scotch pine cone peer during storage

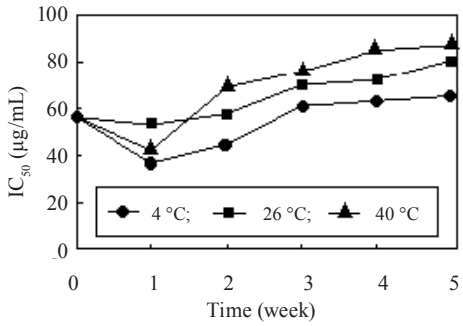

Fig. 10. Effect of different temperature on DPPH free radical scavenging capacity of Mongolia Scotch pine cone peer during storage

2. Mongolia Scotch pine cone peer polyphenols degradation followed first-order kinetics. With the increasing temperature, the *K* value increased and the half-life decreased, E_a was 68.98 kJ/mol^{-1}, which indicated that Mongolia Scotch pine cone peer was suitable for preservation in low temperature environment.

3. The color parameters (*L**, *a**, *b**) of phenolics were affected by storage temperature, pH and time.

4. The DPPH free radical scavenging capacity of phenolics was affected by storage temperature, pH and time. DPPH free radical scavenging capacity of phenolics reached the highest value at the 1th week.

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