Study on Extract Methodology of Total Flavonoids from Ginger and Hydroxyl Radicals Scavenging Effect

Li Huiduan*, Yu Jianzhong

Department of Chemistry and Life Science, Chuxiong Normal University, Chuxiong, P. R. China

Email address:
lhd08@extc.edu.cn (Li Huiduan)

*Corresponding author

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Abstract: Extract methodology of total flavonoids from ginger and hydroxyl radicals scavenging effect, were researched in this paper. Methanol concentration, solid-liquid ratio, extraction temperature and time were determined as four single-factor in the experiment. The central points of Box-Behnken design were selected according to the experimental results of single-factor experiment, the extract process was further optimized by RSM and BBD. The optimum extract conditions were methanol concentration of 60%, solid-liquid ratio of 3:30 (g:mL), extraction temperature of 60°C and time of 3 h, The maximum experimental extraction ratio was 0.497% by RSM. The experimental extraction ratio matched well with the theoretical value of 0.538% by solving the multiple regression equation. RSM has been proved to be an effective technique for optimization of extraction process and the fitted quadratic model has a predictive effect on target extracts. The scavenging effect of ginger extracts, BHT and L-ascorbic acid on ·OH with the same concentration were sorted by L-ascorbic acid > ginger extract > BHT, and all the three antioxidant regents displayed a significant dose-effect relationship.

Keywords: Extract Methodology, Total Flavonoids, Ginger, Extraction Ratio, Response Surface Methodology, Hydroxyl Radicals Scavenging Effect

1. Introduction

Flavonoids are natural polyphenolic antioxidants with significant effects on anti-peroxidation and free radical scavenging. Flavonoids have significant antioxidant, anti-cancer, anti-inflammatory, bactericidal, anti-virus and regulating body immunity, and etc [1-4]. The Extraction technology of flavonoids reported in the literature includes organic solvent extraction, ultrasonic extraction, microwave extraction, supercritical fluid extraction and Enzyme-assisted extraction [5-6].

There were related reports on extraction of total flavones from ginger in the literature [7-9]. Z. Wang from Central south University reported ethanol extraction of total flavonoids from ginger leaf by Response Surface Methodology and antioxidant activity in vitro, the maximum extraction ratio was 15.42±0.09mg/g under the experimental of ethanol concentration of 70%, extraction temperature of 60-65°C and time of 2 h. A preliminary judgment that the double hydrogen flavonoids were the main composition of total flavonoid from ginger was obtained by UV spot monitoring Ultraviolet spectrum analysis of color reaction, etc [8]. Gao shu-yun from Xuzhou Institute of Technology reported soxhlet extraction of flavonoids from ginger, the optimal experimental conditions were ethanol concentration of 66.4%, solid-liquid ratio of 1.40(g:mL), extraction temperature of 60-65°C and time of 2 h. A preliminary judgment that the double hydrogen flavonoids were the main composition of total flavonoid from ginger was obtained by UV spot monitoring Ultraviolet spectrum analysis of color reaction, etc [8].

Response Surface Methodology (RSM) is a statistical method in order to solve problems containing multiple variables. Multiple quadratic function was obtained by reasonable experimental design to fit the factors and response value, and the optimal process parameters were determined by analyzing regression equation. RSM has advantages of shorten
experimental cycle, high accuracy of the regression equation, Interactions between multiple factors, etc [10]. RSM was demonstrated an effective statistic technique for optimizing complex processes, which has been successfully used to optimize the extraction of total flavonoids from many medicine plants [11-12]. The total flavonoids extraction ratio was greatly influenced by extraction conditions, Box-Behnken Design was performed to predict the optimal extraction conditions [13].

Here, methanol extract methodology of flavonoids from ginger was optimized by RSM. flavonoids content of ginger extracts was further determined by spectrophotometry. Additionally, hydroxyl radicals scavenging effect of ginger extracts was also studied. This research was helpful in providing valuable flavonoids content data for development and utilization of ginger.

2 Materials and Methods

2.1. Materials

Ginger, Rutin, Methanol, Macroporous Resin, NaNO₂, Al(NO₃)₃, NaOH, AlCl₃, FeCl₃, NH₃(aq).

Ginger (purchased in chuxiong’s agricultural market) → dry → crush → Spare.

2.2. Experimental Methods

Methanol extract methodology of total flavonoids from ginger was initially determined by single-factor experiment, Methanol concentration, solid-liquid ratio, extraction temperature and time were determined as four single-factor in the experiment. The central points of Box-Behnken Design were selected according to the experimental results of single-factor experiment, the extract process was further optimized by RSM and BBD. Finally, Research on hydroxyl radicals scavenging effect of ginger extracts was studied. The experimental illustration of optimization of flavonoids extraction by RSM and study on radical scavenging effects were illustrated in Figure 1. Methanol extraction from ginger, Chromogenic reaction of ginger extracts, and obtains of linear equations from Rutin standard curve were performed according to the literature [14-15].

2.3. Optimization of the Extraction Process by RSM

Optimization of flavonoids extraction by RSM was operated as references [16]. Box-Behnken Design combining with quadratic response model of four factors and three levels were performed to optimize the extract methodology. First, the independent variables of four single-factors were determined, the level of variables were coded by -1, 0, 1 based on the results of single-factor experiment (as shown in Table 1). A total of 25 points were designed, including points 16 factorial, 8 star points and 1 central points to ensure the precision of experiment.

<table>
<thead>
<tr>
<th>Code levels of independent variables</th>
<th>A: Methanol concentration (%)</th>
<th>B: Solid-liquid ratio (mL·g⁻¹)</th>
<th>C: Extraction temperature (°C)</th>
<th>D: Extraction time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>60</td>
<td>3:30</td>
<td>50</td>
<td>2.0</td>
</tr>
<tr>
<td>0</td>
<td>65</td>
<td>4:30</td>
<td>60</td>
<td>3.0</td>
</tr>
<tr>
<td>+1</td>
<td>70</td>
<td>5:30</td>
<td>70</td>
<td>4.0</td>
</tr>
</tbody>
</table>

2.4. Study on Hydroxyl Radical Inhibition Activity

Total flavonoids were extracted from ginger under the preferred conditions by RSM. The extracts was centrifuged, purification by macroporous resin, methanol elution (methanol volume fraction 60%), solvent evaporation, freeze-dried to obtain the total flavonoids powder. ginger flavonoids solutions with different concentrations were prepared. Hydroxyl radical scavenging effect was operated as reference [16-18], ginger total flavonoids, Rutin and BHT solutions with different concentrations were added, The absorbance was measured under maximum absorption wavelength of 495nm, The scavenging ratio was calculated as (1):
The scavenging ratio of hydroxyl radical (%) = \[ \frac{A_0 - (A_x - A_{x0})}{A_0} \times 100 \] (1)

\( A_0 \) is the absorbance of control solution, \( A_x \) was the absorbance of *Broccoli* extract; \( A_{x0} \) was background absorbance of the extract without \( \text{H}_2\text{O}_2 \).

3. Results and Discussion

Table 2 shows the Chromogenic reaction of ginger extracts and Rutin solution, the phenomenons of Chromogenic reaction were consistent with each other. It confirms that ginger contained total flavonoids. The absorption spectra of ginger extracts and Rutin determined the maximum absorption wavelength of 495nm, As shown in Figure 2. The linear regression equation was formulated as \( A=3.283C-0.009, \) \( R^2=0.999 \) by the standard curve.

### Table 2. Chromogenic reaction of flavonoids extract from ginger and rutin solution.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>4%NaOH(aq)</th>
<th>NH(_3)·H(_2)O(aq)</th>
<th>AlCl(_3) (aq)</th>
<th>Fe Cl(_3) (aq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginger extracts</td>
<td>Bright-yellow</td>
<td>yellow</td>
<td>Light-yellow</td>
<td>Dark-green</td>
</tr>
<tr>
<td>Rutin</td>
<td>Bright-yellow</td>
<td>yellow</td>
<td>Light-yellow</td>
<td>Dark-green</td>
</tr>
</tbody>
</table>

![Figure 2. The absorption spectrum of diagram of ginger extracts and Rutin solution; the standard curve of Rutin solution.](image)

3.1. Result of Single-Factor Experiment

Influences of each single factor on extraction ratio were shown in Figure 3. The maximum extraction ratio of 0.338% was achieved as the methanol concentration of 65% for ginger, which was attributed to that flavonoid glycosides with moderately polar was rich-contained in ginger. With increasing methanol concentration the polarity of the dissolution system was reduced and increased the dissolution of fat-soluble impurities. The presences of impurities were not conductive to post-separation and purification of total flavonoids. The optimum methanol concentration and the central points of Box-Benhken Design was determined as 65%. Extraction ratio was significantly increased with the increasing of solid-liquid ratio, when the solid-liquid ratio was higher than 4:30 (g:mL), extraction ratio began to decrease. The maximum extraction ratio of 0.303 was obtained under the solid-liquid ratio of 4:30 (g:mL). Lower than 4:30 (g:mL), the concentration gradient of solid-liquid phase was two small, which was not conducive to the dissolution of total flavonoids. Higher than 4:30 (g:mL), resulted in a waste of materials; The preferred solid-liquid ratio was 4:30 (g:mL), which was selected as the central points of Box-Benhken design. The optimum extraction temperature was 60°C with corresponding extraction ratio of 0.468%, The higher temperature would lead to methanol evaporation and oxidation degeneration of flavonoids. The lower temperature decreases the dissolution rate of flavonoids. The optimum extraction time was 3.0 h with the responding extraction ratio of 0.346% from ginger. Lower than 2.0h, the dissolution balances were not achieved, higher than 2.0h, the dissolution of other fat-soluble impurities complicated the post-separation and purification of total flavonoids.

According to the results of single factor experiments, methanol concentration of 60~70%; solid-liquid ratio of 3.0:30~5.0:30 (g:mL), extraction temperature of 50~70°C and extraction time of 2.0 ~ 4.0 h were determined as the factors and scope for response surface analysis.
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Figure 3. Effect of methanol concentration, solid-liquid ratio, temperature and time on the extraction ratio of total flavonoids.

3.2. Response Surface Optimization of Extraction Process

3.2.1. Multiple Regression Model and Analysis of Variance (ANOVA)

Extract methodology of total flavonoids from ginger was further optimized by RSM. According to the single-factor experimental results of figure 3, methanol concentration of 60–70%; solid-liquid ratio of 3.0:30–5.0:30 (g:mL), extraction temperature of 50–70°C and time of 2.0 ~ 4.0 h were selected as the actual levels of factors to maximize extraction ratio by Box-Behnken Design, as listed in table 1. A total of 25 experiments were designed, including 16 factorial experiments, 8 star experiments and 1 central experiments to estimate the absolute error.

Table 3. Experimental design and results for extraction ratio by using Box-Behnken design and RSM.

<table>
<thead>
<tr>
<th>Std</th>
<th>Run</th>
<th>A: methanol concentration (%)</th>
<th>B: Extraction time (h)</th>
<th>C: Extraction temperature (°C)</th>
<th>D: Solid-liquid ratio (g:mL)</th>
<th>Response 1 extraction ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>65</td>
<td>3</td>
<td>50</td>
<td>3:30</td>
<td>0.310</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>60</td>
<td>4</td>
<td>60</td>
<td>4:30</td>
<td>0.359</td>
</tr>
<tr>
<td>19</td>
<td>3</td>
<td>60</td>
<td>3</td>
<td>70</td>
<td>4:30</td>
<td>0.412</td>
</tr>
<tr>
<td>18</td>
<td>4</td>
<td>70</td>
<td>3</td>
<td>50</td>
<td>4:30</td>
<td>0.412</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>70</td>
<td>3</td>
<td>70</td>
<td>4:30</td>
<td>0.445</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>65</td>
<td>3</td>
<td>70</td>
<td>5:30</td>
<td>0.238</td>
</tr>
<tr>
<td>13</td>
<td>7</td>
<td>65</td>
<td>2</td>
<td>70</td>
<td>5:30</td>
<td>0.282</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>65</td>
<td>4</td>
<td>50</td>
<td>5:30</td>
<td>0.426</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>60</td>
<td>3</td>
<td>60</td>
<td>5:30</td>
<td>0.220</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>65</td>
<td>3</td>
<td>60</td>
<td>5:30</td>
<td>0.226</td>
</tr>
<tr>
<td>22</td>
<td>11</td>
<td>65</td>
<td>4</td>
<td>60</td>
<td>3:30</td>
<td>0.471</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>65</td>
<td>3</td>
<td>70</td>
<td>3:30</td>
<td>0.452</td>
</tr>
</tbody>
</table>

Table 3. Experimental design and results for extraction ratio by using Box-Behnken design and RSM.
The Box-Behnken design and results of extraction ratio for ginger was shown in Table 3. The experimental extraction ratio ranged from 0.220% to 0.497%. The optimal experimental conditions were methanol concentration of 60%; solid-liquid ratio of 3:30 (g:mL), extraction temperature of 60°C and extraction time of 3.0h with corresponding maximum extraction ratio of 0.497%. The experimental data was analyzed by using Design-Expert 8.0, the response variable and the four independent variables were related by the following multiple regression equation of (2) and (3):

\[
\text{Extract ratio} = +0.31-0.012*A+9.167E-003*B-0.018*C-0.046*D-0.018*AB-0.024*AC+0.050*AD+0.011*B^2+0.035*C^2+0.035*D^2+0.011* D^2
\]  
(2)

\[
\text{Extract ratio} = +4.10308-0.13328* \text{methanol concentration}+0.28592* \text{extraction time}+8.49167E-003* \text{extraction temperature}+0.032583* \text{solid liquid ratio}-3.70000E-003* \text{methanol concentration}* \text{extraction time}-4.75000E-004* \text{methanol concentration}^2-3.30000E-003* \text{extraction time}^2-3.30000E-003* \text{extraction temperature}^2+9.95000E-003* \text{methanol concentration}* \text{solid liquid ratio}+1.15000E-003* \text{extraction solid-liquid ratio of 3:30 (g:mL), extraction temperature of experimental conditions were methanol concentration of 60%; solid-liquid ratio ranged from 0.220% to 0.497%. The optimal temperature was shown in Table 3. The experimental extraction (g:mL), extraction temperature of 60°C and extraction time of 3.0h with corresponding maximum extraction ratio of 0.497%. The experimental data was analyzed by using Design-Expert 8.0, the response variable and the four independent variables were related by the following multiple regression equation of (2) and (3):

\[
\text{Extract ratio} = +0.31-0.012*A+9.167E-003*B-0.018*C-0.046*D-0.018*AB-0.024*AC+0.050*AD+0.011*B^2+0.035*C^2+0.035*D^2+0.011* D^2
\]  
(2)

The analysis of variance (ANOVA) for the above multiple regression equation was listed in table 4. the analysis result indicated that the response variable value of extraction ratio and the four test factor were not a simple linear relationship. The "Model F-value" of 1.86 implies the model is not significant relative to the noise. There is a 16.28% chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms that have a "signal (response) to noise (deviation)" and the model could not be used to navigate the design space. The P value of 0.1628 for the model were higher than 0.05, indicated that the model was not significant. The value of coefficient of the variance (C. V. =18.30%) also indicated that the model need to be further optimized. The adequate precision value of 5.491 was higher than the desirable value of 4.00, which indicated an adequate "signal (response) to noise (deviation)" and the model could be used to navigate the design space. The P value of 0.1628 for the model were higher than 0.05, indicated that the model was not significant. The value of coefficient of the variance (C. V. =18.30%) also indicated that the model have a predictable effect for the extraction process of total flavonoids.

The result of analysis of variance (ANOVA) showed that significant levels of the four factors were sorted by solid-liquid ratio > extraction temperature > methanol concentration > extraction time. The linear term of solid liquid ratio, the interaction terms of extraction time and solid-liquid ratio, extraction temperature and solid-liquid ratio, were significant for the response variables.

**Table 4. ANOVA for Response Surface Quadratic Model for ginger:**

<table>
<thead>
<tr>
<th>Source of deviation</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F Value</th>
<th>p-value</th>
<th>Prob &gt; F</th>
<th>significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.116965</td>
<td>14</td>
<td>0.008355</td>
<td>1.863</td>
<td>0.1628</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.001776</td>
<td>1</td>
<td>0.001776</td>
<td>0.396</td>
<td>0.5432</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.001008</td>
<td>1</td>
<td>0.001008</td>
<td>0.225</td>
<td>0.6455</td>
<td></td>
<td>s</td>
</tr>
<tr>
<td>C</td>
<td>0.003816</td>
<td>1</td>
<td>0.003816</td>
<td>0.851</td>
<td>0.3780</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.025576</td>
<td>1</td>
<td>0.025576</td>
<td>5.704</td>
<td>0.0381</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 3.2.2. RSM Analysis and Research on the Optimum Extract Process

The multiple regression models could be vividly reflected by the 3D response surface and Contour lines plots, as listed in figure 4-9. The 3D response surface plots reflected the effects of multiple independent variables on the response value, the sensitivity of response value to different factors could also be analyzed. The corresponding surfaces were more steeper, indicated its extremely significant impact on response value. Contour lines plots with oval indicated strong interaction between independent variables, the closer the curve to the center, the greater significant impact of the variable on the response value.

![Figure 4. RSM analyses for interactive effects of methanol concentration and extraction time.](image-url)

![Figure 5. RSM analyses for interactive effects of methanol concentration and extraction temperature.](image-url)
The interaction effect of methanol concentration and extraction time on the extraction ratio was illustrated in the 3D response surface plots of figure 4, the corresponding surface of methanol concentration was more steeper than that of extraction time, indicated its more significant impact on responding value. Circle-like Contour lines plot indicated the weak interaction effect of the two terms on responding values with the p value of 0.5927. the maximum extraction ratios of 0.538% was achieved as methanol concentration of 60.46% and extraction time of 2.57h. The interaction terms of methanol concentration and extraction temperature on the responding values were displayed in the 3D response surface plots of figure 5, the impact of the latter term on extraction ratio was more significant than that of the former. Contour lines plots was approaching to circle-like, indicated weak impact of above two interaction terms on the responding value with P =0.4943. The maximum extraction ratios of 0.538% was obtained as methanol concentration of 60.46% and extraction temperature of 69.91°C.

The impact of solid-liquid ratio on the responding value was more significance than that of methanol, as shown in figure 6. Contour lines plots also indicated the weak interaction effects of the above two terms on the response values P = 0.1681. The maximum extraction ratios of 0.538% was obtained as methanol concentration of 60.46% and solid-liquid ratio 3.03:30 (g:mL). Figure 7 showed that impact of the interaction terms of extraction time and temperature on the responding value was not significant with the responding value with p=0.7384. Circle-like Contour lines plots also indicated weak interaction effect of above two terms on the response values the maximum extraction ratios of 0.538% was achieved as extraction time of 2.57 h and temperature of 69.91°C.

The significant impact of interaction terms of extraction time and solid-liquid ratio on extraction ratio was illustrated in the 3D response surface and Contour lines plots of figure 8. the linear of solid-liquid ratio has a more significance effect on the responding value from the more steeper surface. Contour lines plots was approaching to oval, also indicated strong impact of above two interaction terms on the responding value with P = 0.04. Both the linear and interaction effects of extraction temperature and solid-liquid ratio on the extraction ratio were significance with p value of 0.0165, as listed in the 3D response surface and Contour lines plots of figure 9. The maximum extraction ratio of 0.538% was achieved under extraction temperature 69.91°C and solid-liquid ratio of 3.03:30 (g:mL).
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Figure 8. RSM analyses for interactive effects of solid-liquid ratio and extraction time.

Figure 9. RSM analyses for interactive effects of solid liquid ratio and extraction temperature.

The optimum values of the selected variables were obtained by solving the multiple regression equation. The values obtained were A=60.46%, B=2.57 h., C=69.91°C and D=3.03:30 (g:mL), with the corresponding extraction ratio of 0.538% for ginger, calculated by Design-Expert 8.0 software.

Table 5. The obtained experimental data of extraction ratio under preffer experimental conditions.

<table>
<thead>
<tr>
<th>Run</th>
<th>Optimal extraction condition determined by BBD and RSM</th>
<th>absorbance</th>
<th>extraction ratio (%)</th>
<th>Average value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A=60</td>
<td>0.779</td>
<td>0.498</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>B=3, C=60</td>
<td>0.775</td>
<td>0.496</td>
<td>0.499</td>
</tr>
<tr>
<td>3</td>
<td>D=3:30</td>
<td>0.786</td>
<td>0.503</td>
<td></td>
</tr>
</tbody>
</table>

In the experiment, three triplicate experiments were performed under preferred extract conditions of methanol concentration of 60%, extraction time of 3h, extraction temperature of 80°C and solid liquid ratio of 3:30 (g:mL), the average maximum value of extraction ratio was 0.499% for ginger. The obtained experimental data were listed in table 5, the experimental extraction ratio and calculated values of response variable were not well consistent with each other, which indicated that the model have a predictable effect for the extraction methodology of flavonoid from ginger, the experimental extraction value and the calculated values by the Design-Expert 8.0 software were not approaching, indicated that the model need to be further optimized.

3.3. Study on Hydroxyl Radical Scavenging Activity

The flavonoids compounds had a scavenging effect on hydroxyl radical, superoxide radicals and DPPH radicals as the o-dihydrox from the structural benzene ring[14-16]. Hydroxyl radical scavenging effects of ginger extracts, BHT and L-ascorbic acid with different concentrations were measured and listed in Figure 10. With the concentration increasing, the scavenging ratio for hydroxyl radicals increased, which showed a significant degree of dose-effect relationship. Hydroxyl radical scavenging effects of the three antioxidant with the same concentration were sorted by L-ascorbic acid >ginger extract> BHT. The experimental result of antioxidant was consistent with that reported in the literature [7]. The lower antioxidant activity of ginger extracts contrast to L-ascorbic acid could be attributed to the following two reasons: firstly, o-dihydrox from benzene rings were partly methylated, leading to the reduction of scavenging activity on hydroxyl radical [16-18]. Secondly,
the lack of necessary separation and identification for extracts, and the presence of impurities also affected their scavenging effects.

Figure 10. scavenging activity comparison of ginger extracts, BHT and L-ascorbic acid.

4. Conclusions

Extract methodology of total flavonoids from ginger and hydroxyl radicals scavenging effect, were researched in this paper. Methanol concentration, solid-liquid ratio, extraction temperature and time were determined as four single-factor in the experiment. The central points of Box-Benhnken Design were selected according to the experimental results of single-factor experiment, the extract process was further optimized by RSM and BBD. The optimum extract conditions were methanol concentration of 60%, solid-liquid ratio of 3:30 (g/mL), extraction temperature of 60°C and time of 3 h with maximum experimental extraction ratio of 0.497% by RSM. The theoretical value was 0.538% by solving the multiple regression equation, which indicated that the fitted quadratic model has a predictive effect on target extracts. The above experimetal and theoretical value were not approaching, also indicated that the model need to be further optimized.

The scavenging effect of ginger extracts, BHT and L-ascorbic acid on ·OH with the same concentration were sorted by L-ascorbic acid > ginger extract > BHT, and all the three antioxidant reagents displayed a significant dose-effect relationship. The isolation, purification and structure identification of ginger extracts, relationship between antioxidant activity and structure of flavonoids, and related research works are underway.

Acknowledgements

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References


