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To Optimize the Brewing Process of Leaf Tea from *Hippophae Rhamnoides* Linn by the Antioxidant Activity and to Explore its Anticoagulant Activity

Yunwen Zhu, Limei Ran and Hongli Zhou

School of Chemistry and Pharmaceutical Engineering, Jlin Institute of Chemical Technology, China

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Corresponding Author:
Hongli Zhou
School of Chemistry and Pharmaceutical Engineering, Jlin Institute of Chemical Technology, China
Email: zhouhongli@jlict.edu.cn

Abstract: To improve the edible value of *Hippophae Rhamnoides* Linn. Leaf (HRLL), investigates the optimal brewing conditions and changes people's brewing habits, and makes better use of resources. The effects of different parameters (brewing times, brewing time, temperature, and solid-liquid ratio) on the brewing process of HRLL tea were optimized by response surface methodology with antioxidant activity as an indicator. The optimal process was as follows: The brewing time was 6 min; the temperature was 80°C and the solid-liquid ratio was 1:49 (g/mL). At this time, the scavenging rate of DPPH free radical of HRLL tea was 83.25% and HRLL tea could significantly prolong the action time of APTT, PT, and TT and reduce the content of FIB. The result shows HRLL tea brewing process has antioxidant properties but also has anticoagulant activity. This study provided a scientific application potential for the value of HRLL.

Keywords: *Hippophae Rhamnoides* Linn. Leaf Tea, Antioxidant, Brewing Process, Anticoagulant

Introduction

In recent years, the medicinal and health properties of tea have been well-known and have been widely explored (Venditti *et al*., 2010). Many studies have also confirmed that tea, antioxidant (Damiani *et al*., 2014; Xu *et al*., 2017), antibacterial (Chan *et al*., 2011), anticoagulant (Patel *et al*., 2012), lowers blood cholesterol (Troup *et al*., 2015), hypotensive and delays aging (Kumar *et al*., 2016). These functions are attributed to the phytochemical composition of tea, including caffeine, theophylline, flavones, phenolic acids and depsides, carbohydrates, alkaloids, minerals, vitamins, and enzymes, as well as numerous flavor compounds and other minor components that affect biological activity in vitro individually or synergistically (Graham *et al*., 1992; Venkata and Indra, 2011).

Hippophae Rhamnosides Linn. (HRL), a deciduous shrub of Elaeagnaceae, is widely distributed in the north and southwest of China at an altitude of 2500-4000 M. Its leaves, berries, seeds and bark are abundant sources of bioactive compounds (Ganju *et al*., 2005). The leaves of *Hippophae Rhamnosides* Linn. (HRLL) are rich in flavonoids, tannins, and triterpenes, which are used in some countries to make extracts, tea, animal feed, pharmaceuticals and cosmetics. In particular, extracts from HRLL exhibited antioxidant, anticoagulant, anti-bacterial, anti-viral, anti-tumor, and immunomodulatory properties (Ganju *et al*., 2005). However, most of HRLL was discarded as waste, and the edible and medicinal values of the existing products have not been fully developed.

There has been an increasing interest in the utilization of HRLL and converting them into tea-type beverage products, yet how brewing conditions (brewing times, brewing time, temperature, solid-liquid ratio) have not attracted enough attention and the medicinal value of HRLL has not been given full play based on diet therapy. Hence, the utilization rate of resources is not high.

Therefore, based on a single factor, response surface methodology with antioxidant activity as an indicator was used to assess the brewing process of HRLL tea. Under optimal brewing conditions, the total phenolic and total flavonoid content and anticoagulant activities were analyzed, which provides a basis for the medicinal and edible value of HRLL.

Materials and Methods

Materials

HRLL tea was collected by Zhongzheng agricultural product co-operation, Longtan District, Jilin, China. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) was purchased from Aladdin (L.A. Southern California. the USA). Ethanol, methanol, gallic acid, and Folin Ciocalteu reagent were from Sigma-Aldrich (St. Louis, MO, USA). Coagulation Kit Purchased from Jiangsu innovos Co., Ltd. All chemical reagents used were analytical grade.

Methods

DPPH Radical Scavenging Ability

The free radical scavenging ability of the sample extract was determined according to the method described by Burits *et al*. (2015) with some modifications. UV-VIS1700 Shimadzu spectrophotometer was used to measure the absorbance at 517 nm with a reference blank (1 mL MeOH in 1.0 mL of DPPH•solution). The results of the DPPH radical scavenging ability were: $A_{DPPH\,(t)}$- A_{sample} (t)/$A_{DPPH\,(t)}$×100, $A_{DPPH(t)}$ is the absorbance of DPPH•at time (t) and Asample (t) is the absorbance of the sample.

Single Factor Experiment

A single-factor experiment was used to optimize brewing technology for HRLL. The influence of extraction parameters on the DPPH free radical scavenging activity of HRLL, namely brewing times, brewing time, temperature, and solid-liquid ratio, were studied. One parameter was varied at a time while other parameters were kept constant.

Brewing Times

One gram of HRLL was brewed in 50 mL of purified water by fixing 80°C and 6 min, then frequency was varied (1, 2, 3, 4, 5) to explore the effects of brewing frequency on the antioxidant activities of HRLL tea.

Brewing Time

One gram of HRLL tea was brewed in 50 mL of purified water by fixing 80°C and once, then time was varied (2, 4, 6, 8, 10,12, 14 min) to explore the effects of brewing time on the antioxidant activities of HRLL tea.

Brewing Temperature

One gram of HRLL tea was brewed in 50 mL of purified water by fixing 6 min and once, then the temperature was varied (60, 70, 80, 90, 100°C) to explore the effects of brewing temperature on the antioxidant activities of HRLL tea.

Brewing Solid-Liquid Ratio

One gram of HRLL tea was brewed in different milliliter volumes (40, 50, 60, 80, and 100) to explore the effects of brewing solid-liquid ratio on the antioxidant activity of HRLL tea. At the same time, the brewing conditions of 6 min, once, and 80°C were fixed.

Response Surface Analysis Experiments

Using Design Expert 8.0.6.1 based on the Box-Behnken design, A (brewing time), B (brewing temperature), and C (solid-liquid ratio) were selected as the independent variables and the response value was the scavenging rate of DPPH (%). The response surface analysis method was used to optimize the brewing conditions of the antioxidant activity of HRLL tea. The experimental design is shown in Table 1.

Total Phenolic Content Analysis (TPC)

TPC of tea was measured according to the methods of Babbar *et al*. (2001). Briefly, gallic acid was used as a standard substance, the absorbance value as the ordinate and the gradient concentration of gallic acid as the abscissa, total phenolic content of HRLL tea was calculated based on the calibration curve ($Y = 0.0874x+0.0492$, $R^2 = 0.9991$). The TPC of the HRLL tea extracts were expressed as gallic acid equivalents (GAE) per milliliter of tea infusion (mg GAE/g dw).

Total Flavonoids Content Analysis (TFC)

The content of total flavonoids in the tea was measured according to the method of Gursoy *et al.* (2009). The rut in was a standard substance, the absorbance value was the ordinate, and the concentration of the rut in solution was the abscissa, the standard curve obtained was $Y = 9.27x-0.001$ ($R^2 = 9998$).

Statistical Analysis

All independent analyses were performed in triplicate. The data obtained were statistically analyzed using SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA), where the results were expressed as mean ± standard deviation.

Anticoagulant Activities in Vitro

Anticoagulant activity of the HRLL tea was evaluated by the classical coagulation assays of APTT, PT, TT, and FIB, heparin and 0.90% NaCl were used as a reference by the method of assay kits. Plasma pretreatment: 0.109 mol/L sodium citrate and sheep blood was mixed at 1:9 (volume ratio), 3000 r/min for 20 min, collected supernatant platelet-poor plasma and packed in sealed in the plastic tube, frozen storage. During the experiment, preheating was carried out at 37°C and the experiment was completed within 2 h.

APTT: Take 20 μL of the extract under the optimal conditions and add to 80 μL in plasma. The mixed solution was incubated for 1 min at 37°C in a coagulation apparatus, an additional 100 μL of APTT detection reagent was added, and the second mixed solution was incubated for 1 min at 37°C in a coagulation apparatus. Finally, 25 mmol/L $CaCl_2$ solutions were added to 100 μL, the clotting time was recorded.

PT: Take 20 μL of the extract under the optimal conditions and add to 80 μL in plasma. The mixed solution was incubated for the 30s at 37°C in a coagulation apparatus and 200 μL of PT reagent was added. After incubation for another 30 s at 37°C, the clotting time was recorded.

TT: Take 20 μL of the extract under the optimal conditions and add to 80 μL in plasma. The mixed solution was incubated a 37°C for 30 s, added 0.1 mL of TT reagent and the clotting time was recorded.

FIB: The standard curve for FIB according to the reagent instructions was: lgT = -0.821lgC+3.3413. Then, take 20 μL of the extract under the optimal conditions, add to 80 μL in plasma, and add imidazole diluted in good (20 mmol/L) 0.9 mL. The mixed solution was incubated at 37°C for 30 s, 0.1 mL of the above-diluted plasma was added to 0.05 mL of thrombin and the clotting time was recorded.

In a word, the flow chart of the experimental methods is shown in Fig. 1.

Table 1: Experimental factors and coding level design

	Independent variable		
Level	A/min	B/°C	C/(g/mL)
1	4	70	1:40
0	6	80	1:50
-1	8	90	1:60

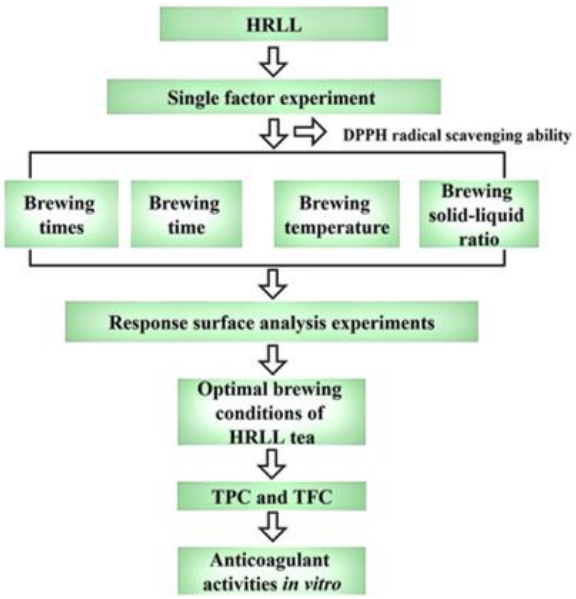

Fig. 1: The flow chart of the experiment methods

Results and Discussion

Brewing Times

The effect of brewing times on the scavenging rate of DPPH is shown in Fig. 2. The scavenging rate from the first two brewing was significantly higher than that of the latter few times. After the first brewing, there were still more antioxidant-active ingredients in the tea that had not been leached. In the second brewing, although the dissolution of the antioxidant active ingredients decreased, still a large amount was leached. When the dissolution of antioxidant components decreased significantly, especially exceeding 5 times, the antioxidant active ingredients would be basic equilibrium. The results showed that the antioxidant activity was consistent with the color of the tea, which indicated that the antioxidant components were mainly water-soluble. Considering the quality of the tea and the color of the beverage, it is better to choose the number of brewing times from 1 to 2.

Brewing Time

The effect of brewing time on the scavenging rate is shown in Fig. 3. The samples all showed an increasing trend brewing for 2~6 min and a decreasing trend after 6 min. After 6~8 min, the effective ingredients were not completely dissolved or were oxidized, resulting in a decrease in the scavenging rate (Yang *et al*., 2007). Interestingly, the scavenging rate started to increase again at 8 for two min and might be due to the dissolution of other substances (Guo *et al*., 2017). However, as time went by, the amount of dissolution gradually becomes saturated for two min, and the scavenging rate begins to decrease after 12 min. Therefore, the brewing times of 4, 6, and 8 min were selected for the response surface test.

Brewing Temperature

The effect of temperature on the scavenging rate is shown in Fig. 4. The antioxidant capacity of HRLL tea slowly increased with temperature, reaching the highest activity at 70ºC. The reason might be that when the brewing temperature was 50ºC, the lower temperature affected the dissolution of the effective ingredients. When the water temperature reaches 70ºC, it might accelerate the dissolution of the active ingredients, thus improving the scavenging rate of tea. If the brewing temperature is too high, some of the effective ingredients may be destroyed, the scavenging rate will be decreased. Therefore, the brewing temperatures of 60, 70, and 80ºC were selected for the response surface test.

Brewing Solid-Liquid Ratio

The effect of the solid-liquid ratio on the scavenging rate is shown in Fig. 5. The antioxidant capacity gradually

increased with the solid-liquid ratio from 1:30 to 1:50 (g/mL). When the solid-liquid ratio was 1:40 (g/mL), the content of antioxidant active ingredients might be less dissolved due to too little solvent. When the solid-liquid ratio exceeded 1:50 (g/mL), the amount of solvent increased and the content of active ingredients reached the maximum and the scavenging rate gradually decreased with the increase of the solid-liquid ratio from 1:50 to 1:80 (g/mL). Therefore, the brewing solid-liquid ratios of 1:40, 1:50, 1:60 (g/mL) were selected for response surface test.

Brewing Process Optimization

Based on the above results of the single-factor experiment, A brewing temperature, B brewing time, and C solid-liquid ratio of HRLL tea were selected as independent variables. According to the Box-Behnken design, 17 experimental sites were designed and the response value was the scavenging rate (%). The results are shown in Table 2 and the results of the variance analysis are shown in Table 3.

Through the response surface software, the regression equation was obtained: the scavenging rate/% = +83.49-0.58 × A-0.11 × B-0.37 × C-0.41 × A × B +0.33 × A × C-0.017 × B × C-2.58 A^2-1.70 B^2-3.01 C^2. According to the regression equation, the three-dimensional response surface analysis diagram composed of various factors (A, B, C) was obtained.

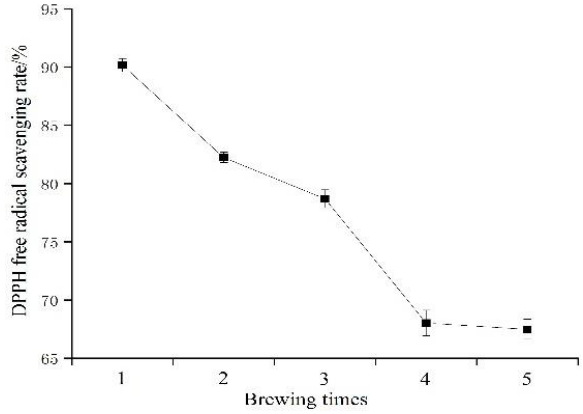

Fig. 2: The effect of brewing times on the scavenging rate

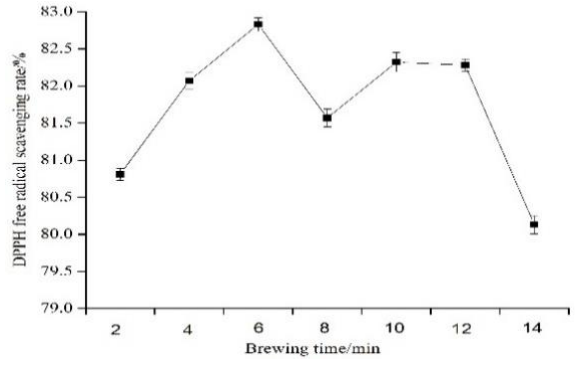

Fig. 3: The effect of brewing time on the scavenging rate

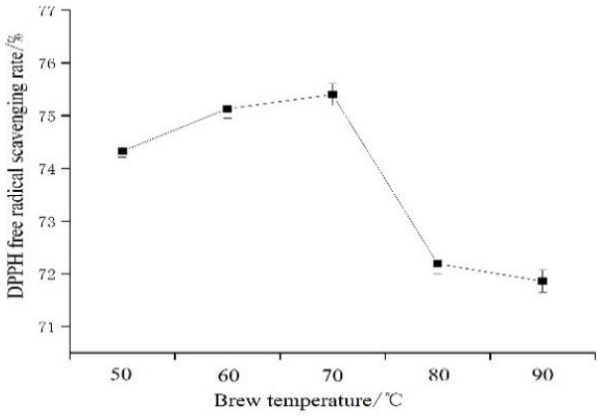

Fig. 4: The effect of brewing temperature on the scavenging rate

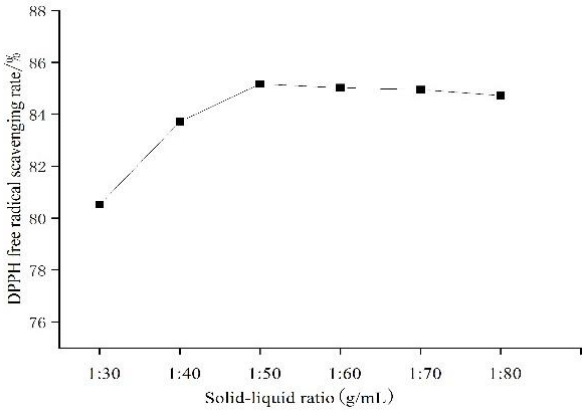

Fig. 5: The effect of solid-liquid ratio on the scavenging rate

Table 2: Experimental results of response surface analysis

No.	A (°C)	B (min)	C (g/mL)	Scavenging rate (%)
1	80	6	50	83.12
2	80	4	60	78.49
3	90	4	50	79.36
4	70	8	50	79.86
5	80	8	60	78.42
6	70	6	40	79.35
7	80	6	50	83.56
8	80	6	50	83.97
9	70	4	50	79.45
10	80	6	50	83.12
11	90	6	60	77.09
12	90	6	40	77.26
13	70	6	60	77.85
14	80	4	40	79.08
15	80	6	50	83.66
16	90	8	50	78.12
17	80	8	40	79.08

Table 3: Analysis of variance for the fitted quadratic polynomial model

Source	SS	DF	MS	F	*P*(prob>F)
Model	92.250	9	10.250	94.130	<0.0001**
A	2.740	1	2.740	25.140	0.0015**
B	0.100	1	0.100	0.930	0.3670
C	1.070	1	1.070	9.790	0.0166*
AB	0.680	1	0.680	6.250	0.0410*
AC	0.440	1	0.440	4.060	0.0837
BC	1.225	1	1.225	0.011	0.9185
A^2	28.120	1	28.120	258.240	<0.0001**
B^2	12.230	1	12.230	112.310	<0.0001**
C^2	38.260	1	38.260	351.330	<0.0001**
Residual	0.760	7	0.110		
Lock of Fit	0.220	3	0.075	0.560	0.0096**
Error	0.540	4	0.130		
Cor total	93.010	16			
R^2=0.9981	R^2Adj = 0.9813				

Note: **very significant difference(p<0.01); *significant difference (0.01<p<0.05); p>0.05, no obviously different

As shown in Fig. 6, it can be seen from the graph of the 3D plots rose slowly when the brewing temperature was between 70-90ºC, which the highest point of the 3D plots the temperature of 78.84ºC and the time was 5.96 min, and the contour plots were close to an elliptical, illustrating the obvious interaction of antioxidant capacity between brewing time and temperature. According to Fig. 7, when the brewing time was between 5-7 min, the highest point of the 3D plots was that the material-to-liquid ratio was 1:49.33 g/mL, the time was 5.96 min and the contour plots was elliptical, which indicated that the interaction between solid-liquid ratio and brewing time had an extremely significant effect on the antioxidant activity. As shown in Fig. 8, the highest point of the 3D plots was the temperature of 78.84ºC, the material-to-liquid ratio was 1:49.33 g/mL, the contour plots were close to an elliptical and the 3D plots rose significantly, indicating that the interaction effect of solid-liquid ratio and temperature had a significant effect on the antioxidant activity.

It could be seen from Table 3 that when the above regression equation was used to describe the relationship between the various factors and the response value, the linear relationship between the dependent variable and the overall independent variable was significant, the significance *P* value of the model was less than 0.01, the quadratic regression variance model was extremely significant and the lack of fit term of the equation was significant (*P*<0.01), indicating that the equation fitting was good and the error was small. The models could be used to determine the best brewing conditions with different brewing times, water temperatures, and solid-liquid ratios.

The optimal brewing conditions of HRLL tea by response surface method were: solid-liquid ratio of 1: 49.33 (g/mL), a temperature of 78.84°C, time of 5.96 min, the scavenging rate was 83.53±1.75%. Combined with the actual situation, the brewing conditions were amended as follows: Solid-liquid ratio of 1:49 (g/mL), a temperature of 80°C, time of 6 min. Repeat three experiments under these optimized conditions, the experimental value of the scavenging rate was 83.25±1.75%, which was close to the value of the response surface regression equation of 83.53±1.82%.

Under each optimal brewing process, Pu-er tea as a famous tea in China, and Vine tea as a medicinal tea, had their scavenging rate on DPPH free radical, 60.37 and 86.49%. The former is the solid-liquid ratio of 1:30 g/mL, the temperature of 100°C, and a single brewing time of 120 min (Ding *et al*., 2018). The latter is the solid-liquid ratio of 1:56 g/mL, the temperature of 80°C, and a single brewing time of 5.8 min (Wang *et al*., 2014).

Compared with the brewing process of Pu-er tea, the advantages of HRLL tea were treasured for the characteristics of relatively low temperature and relatively short time, but high antioxidant activity; this is similar to the brewing conditions and antioxidant activity of medicinal Vine tea. Therefore, HRLL tea has a high antioxidant activity and a certain anticoagulant effect.

TPC and TFC of the Brewing Process

The contents of total phenolics and total flavonoids under the optimal brewing process were 18.45±0.72 (%) and 2.51±0.25 (%), respectively.

Tea polyphenols, mainly flavonoids, are well-known for their antioxidant properties, which are mainly attributable to the combination of aromatic rings and hydroxyl groups. These rings combined with hydroxyl groups can neutralize lipid free radicals. (Michalak, 2006). There is a significant positive relationship between antioxidant activity and TPC and TFC (Samadi and Fard, 2020). This indicates that polyphenols and flavonoids from HRLL tea extract could be the major contributors to antioxidant activity.

Anticoagulant Action of HRLL Tea

The effects of HRLL tea extract on coagulation parameters from APTT, TT, PT, and FIB are shown in Table 4.

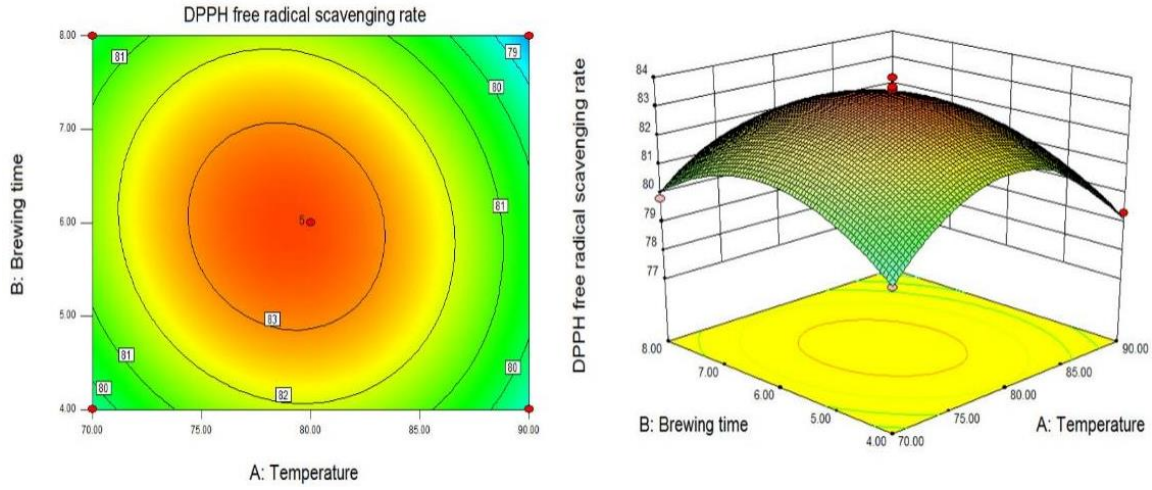

Fig. 6: Antioxidant capacity of the interaction between brewing time and temperature

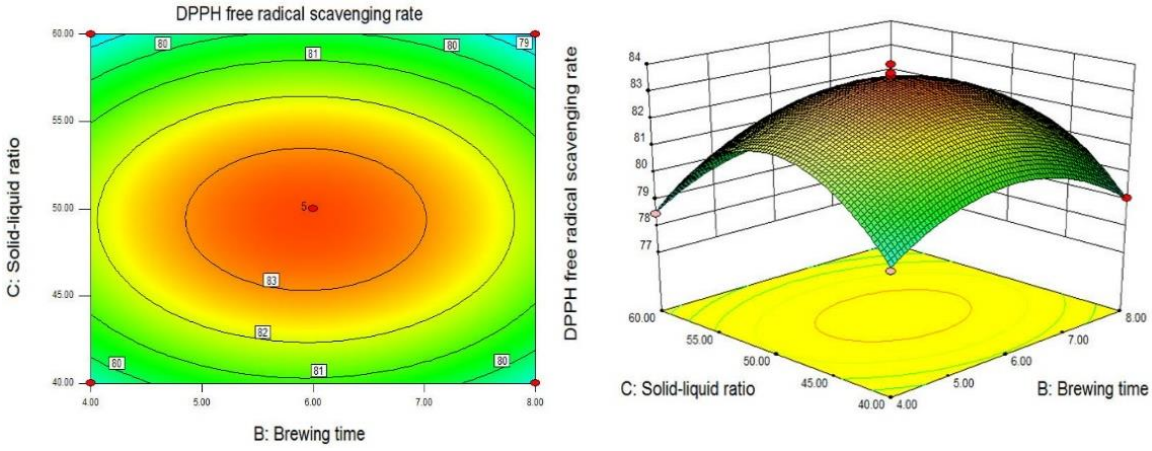

Fig. 7: Antioxidant capacity of the interaction between solid-liquid ratio and brewing time

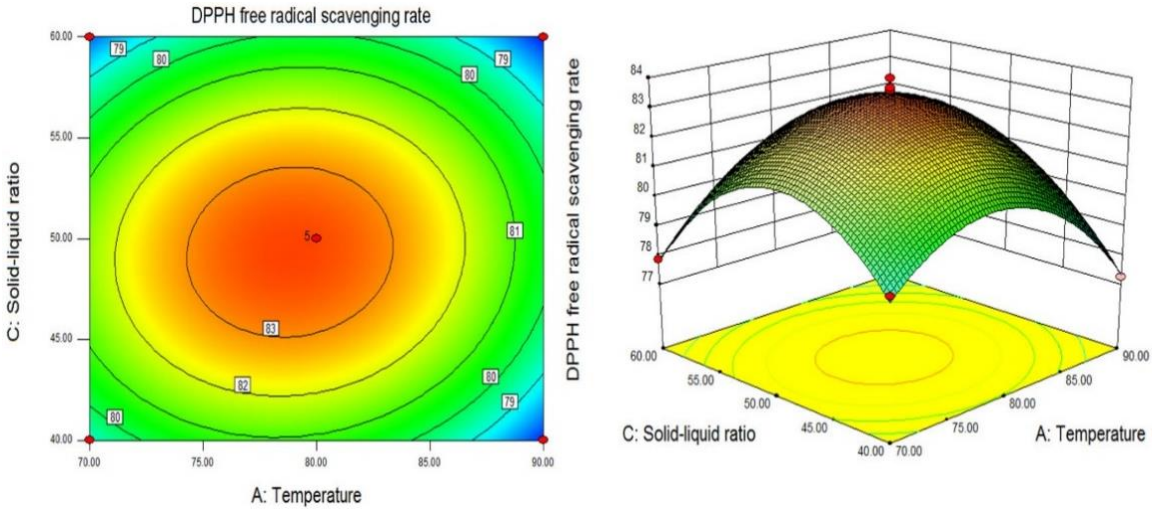

Fig. 8: Antioxidant capacity of the interaction between solid-liquid ratio and temperature

Table 4: Anticoagulation activity of the extract under the optimal conditions

Group	APTT (s)	PT (s)	TT(s)	FIB (g/L)
12.5 mg/mL	40.90±2.04**	25.80±1.29*	31.60±1.58*	2.42±0.17*
2.5 mg/mL	30.40±1.5200	19.80±0.990	16.50±0.820	2.65±0.230
0.90% NaCl	26.48±1.2200	14.09±0.700	16.56±0.830	3.06±0.250
Heparin 2 μg/mL	55.62±2.7800	38.79±1.940	33.06±1.650	1.94±0.070

Note: *significant difference (p<0.05)

Compared with normal saline, HRLL tea extract could significantly prolong the action time of APTT, PT, and TT and significantly reduce the content of FIB. Among them, APTT was a very significant difference. With the increase in concentration, the anticoagulation activity also showed an upward trend.

Therefore, the optimal brewing conditions based on the antioxidant activity and anticoagulation, HRLL tea also has a certain role in preventing thrombosis. External antioxidation is involved in the coagulation mechanism, antioxidants facilitate the release of Reactive Oxygen Species (ROS) from vascular endothelial cells (Snow *et al*., 2014).

Tea contains active substances such as polyphenols, flavonoids, etc. There were reported that phenolics can play an antioxidant role (Frei and Higdon, 2003), flavonoids play an anticoagulant role (Liu *et al*., 2015; 2018), antioxidation was also involved in the coagulation mechanism and the content of polyphenols and flavonoids varies under different cooking conditions (Zhang, 2019). It was speculated that different brewing conditions would affect the phenolic and flavonoid contents of HRLL tea and then affect its biological activity. Usually, tea is consumed with neither attention to brewing temperature nor to the amount of water, brewing time, and brewing times. To improve the dietetic value and medicinal value, the brewing process of HRLL tea is important.

Conclusion

In this study, the best brewing process on the Box-Behnken analysis of response surface design was as follows: Solid-liquid ratio of 1:49 (g/mL), a temperature of 80°C, and a time of 6 min. At this time, the contents of total phenolics and total flavonoids were 18.45±0.72 (%) and 2.51±0.25 (%), the scavenging rate of DPPH free radical reached 83.25% and it has significant anticoagulant activity.

The screening of the brewing process greatly improves the value and utilization rate of HRLL, which enables people to change their practice of brewing, improving the value of food therapy. This study provides a scientific basis for the development of new products and improves the economic value of HRLL.

Acknowledgment

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Author's Contributions

Yunwen Zhu: The experiment was carried out and completed. Revised and proofread the thesis.

Limei Ran: The experiment was carried out and completed.

Hongli Zhou: Guided the design route and provided experimental guidance for this manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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Nomenclature

HRL : *Hippophae Rhamnoides* Linn
HRLL : *Hippophae Rhamnoides* Linn. Leaf
APTT : Activated Partial Thromboplastin Time
PT : The Prothrombin Time
TT : The Thrombin Time
FIB : Transformation of Fifibrinogen Into fifi-Brin
RSM : Response Surface Methodology
DPPH: 1,1-diphenyl-2-picrylhydrazyl