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Studying and enhancing the hypoglycemic effect of Pingyang Yellow Soup and preliminary exploration of its internal mechanisms in a zebrafish model

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Abstract

Yellow tea belongs to specialty tea, and it is loved by consumers because of its unique flavor and taste. Herein, Pingyang Yellow Soup (PYS, a kind of yellow tea) was chosen. In this study, we investigated the effects of PYS on the hypoglycemic effect of the zebrafish model and explored its potential mechanism of action. Results showed that PYS had very strong and definite hypoglycemic biological activity. Meanwhile, different water/material ratios, extraction temperatures, and extraction times had significant influence on the hypoglycemic performance of PYS, and the strength of influence was ranked as extraction temperature > extraction time > water/material ratio. In addition, results showed that the removal rate of tea polyphenols (TPs) in PYS had a strong linear correlation to the degree of decreased hypoglycemic performance, indicating the hypoglycemic performance was closely related to TPs levels. The comprehensive analysis revealed that the nor-sugar rate of PYS had a variety of regression relationships with its components, and the best hypoglycemic performance does not require the highest content levels of each component but depends on what it is, and there exists a relative optimal value, which provides basic information for exploring the internal mechanism of action of hypoglycemic effects and developing functional tea drinks of PYS.

Keywords: Pingyang Yellow Soup; zebrafish; diabetes; hypoglycemic effects.

Practical Application: Our findings provided some basic information for exploring the internal mechanism of action of hypoglycemic effects, promoting hypoglycemic performance, and developing functional PYS drinks.

1 INTRODUCTION

Tea originated in China, and it is a popular drink all over the world because of its unique flavor and significant health benefits. Current studies have demonstrated that tea has many health benefits due to the fact that it contains many kinds of functional substances and nutrients, such as tea polyphenols (TPs), tea polysaccharides, tea pigments, caffeine (CAF), and other beneficial components (Alam et al., 2020; Zahidin et al., 2018). TPs is one of the most important and key functional components in tea. Studies have shown that TPs can inhibit the activity of amylase (Sun et al., 2017; Yang & Kong, 2016), and its main catechin monomer, epigallocatechin gallate (EGCG), can inhibit gluconogenesis in vivo (Collins et al., 2007), inhibit the digestion and absorption of lipids (Xu et al., 2020), promote the oxidative utilization of body fat (Willems, 2018), and thus exhibit strong regulation ability of glycolipid metabolism (Hu et al., 2022; Sun et al., 2021). In addition to phenolic components, other components such as CAF and theaflavin also play a significant role in regulating glucose and lipid metabolism. For example, CAF can promote fat consumption and reduce fat deposition (Frayer & Kim 2020), and theaflavin can reduce lipid deposition and promote fatty acid oxidation (Lin et al., 2007).

Diabetes mellitus is a worldwide disease; the number of patients has increased significantly in recent years, and it has become one of the major diseases threatening human health (Li et al., 2020). The results based on studies of modern model animals (Liu et al., 2022a; Liu et al., 2022c; Zhang et al., 2020) and people (Adu et al., 2022; Mortazavi et al., 2018) showed that tea has great potential in promoting the balance of blood glucose.

Green tea and black tea are the two famous tea drinks consumed all over the world. Besides, there are four other specialty teas in China. Yellow tea is one among them, and it only exists in China. It is deeply loved by Chinese consumers because of its unique flavor, taste, and character. Generally speaking, yellow tea is produced with tea shoots by heating or steaming to de-enzyme, rolling, heaping for yellowing, drying, and other processes, among which heaping for yellowing is the key to the formation of characteristic flavor. Most importantly, yellow tea also has rich nutrition and good health care effects. Pin yang Yellow Soup (PYS) is a kind of yellow tea, and it has a long history of more than 200 years in China. It is also one of the most famous teas in Zhejiang, China. Studies have found that yellow tea also has an obvious hypoglycemic effect (Wu et al., 2022; Zhou et al., 2018), which is related to its functional components.

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However, the current research on yellow tea still has some deficiencies; for example, the determination of the effective hypoglycemic dose of yellow tea, the optimal hypoglycemic performance of yellow tea under what brewing conditions and its internal regulatory mechanism, and the difference between the hypoglycemic effects of yellow tea and different types of diabetes (I and II) need to be further studied.

Therefore, PYS was selected, and in this study, the effects of PYS on the hypoglycemic effect in zebrafish with type I and II diabetes were investigated and studied. Most importantly, the internal mechanism of hypoglycemic performance was explored by combining the basic research on functional substances with big data mining technology. Finally, the research findings will help provide some basic information for yellow tea hypoglycemic theory and technical guidance for the development of yellow tea functional drinks, as well as health gospel for special diabetic groups.

2 MATERIALS AND METHODS

2.1 Experimental materials

Main instruments: Thermostatic Water Bath (Shanghai Jinghong Experimental Equipment Co., Ltd.); Automatic Microplate Reader [Thermo Fisher Technology (China) Co., Ltd.]; Hand-held High Speed Homogenizer (MY-20, Shanghai Jingxin Industrial Development Co., Ltd.); Inverted Fluorescence Microscope (Nikon SMZ25); Automatic Intelligent Biochemical (Mold) Incubator (Tianjin Hongnuo Instrument Co., Ltd.); Precision Electronic Balance [AL-204, Mettler-Toledo International Trade (Shanghai) Co., Ltd.]; High-Speed Centrifuge (3K1S, Sigma Centrifuge Company, Germany).

Preparation of reagents and stock solution: the stock solution of 10 mg/mL [measured by raw drug (milligram) to volume (milliliter) ratio] PYS (Pingyang Tianrun Tea Co., Ltd.) was prepared by water/material ratio of 50, the extraction temperature of 80℃ for 30 min, and the times of extraction were two. TPs and its monomer were produced and provided by the Zhejiang Key Laboratory of Tea Resource Transboundary Application Technology. The CAF (0.1 mg/mL standard solution) was provided by the National Tea Quality Inspection Center. Glucose was obtained from the Sinopharm Chemical Reagent Co. Ltd.; alloxan (purity ≥ 98.0%) was provided by the Hefei Pomei Biological Technology Co., Ltd.; poly-vinylpolypyrrolidone (PVPP) was provided by the Shanghai Aladdin Biochemical Technology Co., Ltd.; and a glucose detection (glucose oxidase method) kit was provided by the Nanjing Jiancheng Bioengineering Institute.

Experimental animals: zebrafish (3 months old, type AB). Zebrafish bred were produced in a natural mating manner. Each spawning box was filled with 3:2 male and female fish and then placed in a 28.5℃ incubator to spawn. The photocycle in the incubator was set to 14 h light and 10 h darkness. The embryos were collected the next morning, and the dead eggs and feces were removed. After cleaning, the embryos were incubated in the incubator at a constant temperature with incubation water (60 μg/mL sea salt water), and the liquid was changed once a day. After 5 days of incubation, the healthy and well-developed zebrafish larvae were selected for experimental study.

2.2 EXPERIMENTAL METHODS

2.2.1 Study on the hypoglycemic effect of yellow tea

2.2.1.1 Analysis of the hypoglycemic effect of yellow tea on zebrafish with insulin deficiency and diabetes mellitus

Based on previous studies (Liu et al., 2021; Liu et al., 2022b), healthy zebrafish larvae were selected and chosen to establish a diabetes model induced by 22 mg/mL glucose and 0.02 mmol/L alloxan for 24 h. Then, diabetic zebrafish were randomly divided into seven treatment groups, with 60 fish in each group, named as MC, T25-T575, and respectively treated with 50 mL of 22 mg/mL glucose and 0, 25, 50, 75, 125, 200, 425, and 575 μg/mL PYS for 24 h. Meanwhile, the other 60 healthy zebrafish larvae were synchronously incubated with 50 mL of 0 mg/mL glucose for 48 h (the liquid was changed once a day), named as the NC control group. At the end of the experiment, samples were taken for the determination of glucose content, and deaths were recorded for the calculation of mortality statistics and the recommendation of an optimal dosage.

2.2.1.2 Analysis of the hypoglycemic effect of yellow tea in zebrafish based on non-insulin-dependent diabetes mellitus

Based on previous studies (Liu et al., 2021; Liu et al., 2022b), healthy zebrafish were randomly divided into 5 groups, with 60 fish in each group, named as NC1, NC2, T25, T50, and T75, and respectively treated with 50 mL of 0 mg/mL glucose, 22 mg/mL glucose, and 0, 25, 50, and 75 μg/mL PYS for 24 h. After the experiment, samples were taken and chosen for the detection of glucose content.

2.2.2 Optimization of extraction conditions for PYS based on hypoglycemic function

2.2.2.1 Single-factor experiment

The effects of single factors, such as water/material ratio, extraction temperature, and extraction time, on the hypoglycemic performance of PYS were investigated. The details were as follows: (1) Water/material ratio: PYS was extracted according to the water/material ratio of 35:1, 40:1, 45:1, 50:1, 55:1, and 60:1, with a fixed extraction temperature of 80℃ and an extraction time of 30 min. (2) Extraction temperature: the extraction temperature of PYS was 50, 60, 70, 80, 90, and 100℃; the fixed water/material ratio was 50:1; and the extraction time was 30 min. (3) Extraction time: PYS was extracted for 10, 20, 30, 40, 50, and 60 min; the water/material ratio was fixed at 50:1; and the extraction temperature was 80℃. All the samples were extracted twice. The final extraction soup was used for functional evaluation.

Healthy zebrafish larvae were selected and chosen to establish a diabetes model induced by 22 mg/mL glucose and

0.02 mmol/L alloxan for 24 h. Then, diabetic zebrafish were randomly divided into several treatment groups, with 60 fish in each group, and respectively treated with 50 mL of 22 mg/mL glucose, 22 mg/mL glucose, and 50 μg/mL extraction soups with different water ratios, extraction temperatures, or extraction times for 24 h, respectively. Meanwhile, the other 60 healthy zebrafish larvae were synchronously incubated with 50 mL of 0 mg/mL glucose for 48 h (the liquid was changed once a day), named as the NC control group. At the end of the experiment, samples were taken for the determination of glucose content.

2.2.2.2 Optimization of PYS extraction conditions based on Box–Behnken response surface experiment design

On the basis of a single-factor experiment, the effects of water/material ratio, extraction time, and extraction temperature of PYS on hypoglycemic performance in zebrafish with non-insulin-dependent diabetes mellitus (NIDDM) were studied through a three-factor and three-level experiment design using the response surface method under the Box–Behnken experiment design principle (Table 1). Design Expert 11 was used for statistical analysis in experimental design, data processing, and model building.

Based on previous studies, healthy zebrafish were randomly divided into 19 groups, with 60 fish in each group. They were given 50 mL of 0 mg/mL glucose, 22 mg/mL glucose, 22 mg/mL glucose, and 25 μg/mL of the above different PYS solutions and cultured for 24 h, respectively. At the end of the experiment, samples were taken for the determination of glucose level and calculation of the nor-sugar rate, and the specific experimental design is shown in Table 2.

Table 1. Factors and levels of response surface test design.

Factors	Levels		
	-1	0	1
Water/material ratio	45	52.5	60
Extraction temperature/°C	60	75	90
Extraction time/min	20	35	50

2.2.3 Basic research on hypoglycemic functional substances of PYS

2.2.3.1 Analysis of the role of TPs in lowering glucose in PYS

TPs is the general name of polyphenols in tea, including flavanols, anthocyanins, flavonoids, and phenolic acids, which account for 20%–35% of tea. It is one of the main functional components of tea. Therefore, the effect and contribution of TPs on the hypoglycemic effect of PYS were investigated and studied. PVPP is a kind of material that can be used for directing and removing phenols. PVPP was chosen for directing the removal of TPs from PYS solutions. Meanwhile, the changes in TPs contents in PYS solutions by high-performance liquid chromatography (HPLC) according to the National Tea Standardization Technical Committee (2018) and the changes in hypoglycemic performance were evaluated by functional evaluation based on zebrafish with NIDDM.

The detailed experimental process is as follows. First, the stock solutions of 9.5238 mg/mL PYS were extracted according to the optimized extraction methods (water/material ratio of 52.5, extraction temperature of 76℃, extraction time of 30 min, and times of extraction of two). Then, the stock solution of PYS was divided into 6 groups of 30 mL each, and 0, 10, 20, 40, 80, and 180 mg of PVPP were added, respectively. After shaking and stirring at room temperature for 30 min, the supernatant was collected and centrifuged at 3,500 r/s for 20 min. They were used for the detection of TP contents and for the evaluation of hypoglycemic performance. Finally, the hypoglycemic function of the above samples of supernatant was evaluated according to the above experiment on zebrafish with NIDDM.

The detailed process is as follows. Healthy zebrafish were randomly divided into 8 groups, with 60 fish in each group. They were given 50 mL of 0 mg/mL glucose, 22 mg/mL glucose,

Table 2. Response surface, central composite design, and the results.

No.	*A:* **water/material ratios**	*B*:**extraction temperatures/**℃	*C:* **extraction times/min**	*R1:* **nor-sugar rates%**
1	52.5	75	35	59.22
2	60	75	50	50.93
3	52.5	75	35	59.13
4	60	90	35	23.09
5	45	75	50	34.74
6	52.5	90	20	41.34
7	60	75	20	31.28
8	52.5	60	50	33.42
9	52.5	75	35	59.82
10	52.5	90	50	29.35
11	60	60	35	18.45
12	45	90	35	31.93
13	45	60	35	11.69
14	52.5	75	35	58.42
15	52.5	60	20	16.17
16	52.5	75	35	59.03
17	45	75	20	46.07

22 mg/mL glucose, and 25 μg/mL of the above different supernatant samples and cultured for 24 h, respectively. At the end of the experiment, samples were taken for the determination of glucose level and the calculation of the nor-sugar rate.

3.2.3.2 Analysis of the effect of phenolic components on the hypoglycemic function of PYS

According to the experimental results, TPs play an important and key role in the hypoglycemic effect of PYS. However, TPs are not a single component but consist of many components. Therefore, it is necessary to further study the mechanism behind the action of hypoglycemic performance. First, five groups with significantly different hypoglycemic performance were selected and chosen according to the results of the nor-sugar rate from the above response surface method. The experimental groups were numbered as follows: test number 1 (59.22%), test number 4 (23.09%), test number 6 (41.34%), test number 12 (31.93%), and test number 13 (11.69%) (Table 2). Next, the contents of the phenolic components of PYS from the above five groups were detected by HPLC, according to the National Tea Standardization Technical Committee (2018). Finally, the correlation analysis between the content level of the contained substance and its corresponding hypoglycemic effect was carried out to explore and reveal the key factors with strong potential hypoglycemic value.

2.3 Detection method

The glucose level was detected as follows. According to the solution of 100 μL phosphoric acid buffer salt solution for 5 fish, homogenate for 1 min with a high-speed hand-held homogenizer until the tissue of the fish is fully decomposed, and then centrifuge at 4℃ at 2,500 r/s for 10 min. Then, an upper sample of 5 μL was taken, and a glucose detection (glucose oxidase method) kit was used to determine the glucose concentration. The determination procedure and method are carried out in accordance with the steps specified in the kit instructions.

2.4 Statistical analysis

All experimental data were preliminarily processed by Excel 2016; Graphpad Prism 8.0 was used for mapping; and SPSS Statistics 24.0 was used for one-way analysis of variance, dimension-reduced analysis, and multiple linear regression. All data were presented as mean±standard deviation. Duncan analysis was used for the significance of differences and multiple comparisons. $P < 0.05$ indicated significant differences between groups.

3 RESULTS AND DISCUSSION

3.1 Study on the hypoglycemic effect of PYS

3.1.1 Evaluation and analysis of the hypoglycemic effect of PYS in zebrafish with IDDM

The tolerance of zebrafish to PYS is shown in Figure 1A. Figure 1A showed that the mortality rate of 125 μg/mL PYS incubated for 24 h was 34.67%, and that of 300 μg/mL PYS for 24 h was 58.67%. The results showed that the lethal effect of PYS on zebrafish increased significantly with the increase in dosage.

Figure 1B shows the hypoglycemic results of PYS treatment for 24 h. Compared with the NC group, the glucose concentration of zebrafish in the MC group was increased by 3.42 times ($p < 0.05$). Compared to the MC group, the levels of glucose in the T-25, T-50, T-75, T-125, T-200, and T-300 groups decreased by 31.56% ($p < 0.05$), 29.65% ($p < 0.05$), 14.64% ($p < 0.05$), 13.38% ($p < 0.05$), 10.78% ($p < 0.05$), and 8.95% ($p > 0.05$), respectively.

The results showed that PYS had an obvious hypoglycemic effect on IDDM zebrafish within the observed dose concentration range. Meanwhile, the strength of the hypoglycemic effect was related to the dose concentration but the health side effects may be related to the high concentration of PYS (Figure 1A).

Therefore, in order to scientifically evaluate the hypoglycemic effect of PYS, it is necessary to control the administration dosage reasonably. As shown in Figure 1C, there was an obvious dose–effect relationship between zebrafish mortality (%) and the administration dosage of PYS (μg/mL), and the regression equation was (Equation 1):

$$y = 0.2007x - 1.146 \quad R^2 = 0.8976 \tag{1}$$

The calculation and analysis showed that the LC_{50} (the concentration lethal to half of any given species over a certain time) value was 254.84 μg/mL. If 10%–25% LC_{50} were recommended as the administration dosage, the dosage of PYS should be controlled within 25.48–63.71 μg/mL.

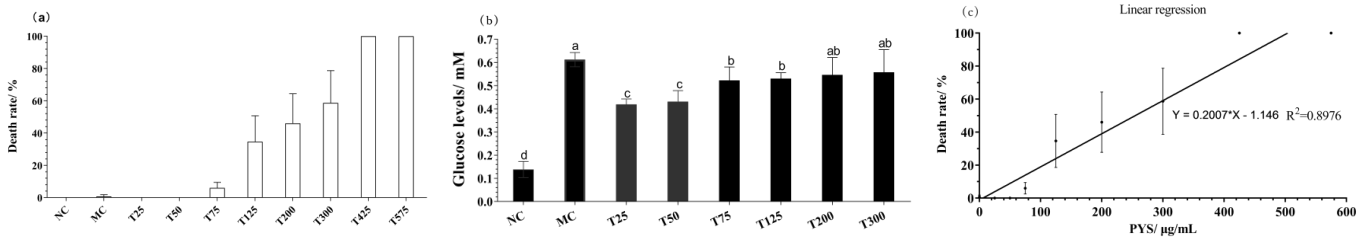

Figure 1. Effects of Pingyang Yellow Soup (PYS) on the hypoglycemic effects in zebrafish with insulin deficiency and diabetes mellitus (IDDM). (A) The relationship between Pingyang Yellow Soup (PYS) concentration and zebrafish death rate; (B) the influence of different concentrations of PYS on hypoglycemic effect; (C) the dose–effect relationship between PYS concentration and zebrafish death rate. Lowercase letters with different superscripts in the figure indicate significant differences were found between groups ($p < 0.05$), the same as below.

3.1.2 Evaluation and analysis of the hypoglycemic effect of PYS in zebrafish with NIDDM

The hypoglycemic effect of PYS on NIDDM zebrafish is shown in Figure 2. As shown in Figure 2, compared with the NC2 group, the glucose levels of zebrafish in the T-25, T-50, and T-75 groups decreased by 53.84%, 41.14%, and 38.01%, respectively, and all the differences reached significant levels ($p < 0.05$). The results showed that 25–75 μg/mL of administration dosage also had an obvious hypoglycemic effect on NIDDM zebrafish.

3.2 Optimization of extraction conditions for PYS based on hypoglycemic function

3.2.1 Analysis of a single-factor experiment

The effects of different extraction conditions of water/material ratio, extraction temperature, and extraction time on the hypoglycemic performance of PYS are shown in Figure 3. As can be seen from Figure 3A, compared with the MC group, the glucose values of zebrafish in the T35:1, T40:1, T45:1, T50:1, T55:1, and T60:1 groups decreased by 23.41, 25.01, 24.39, 33.05, 39.99, and 27.13%, respectively, and all the differences reached significant levels ($p < 0.05$). The glucose levels of zebrafish in the T50℃, T60℃, T70℃, T80℃, T90℃, and T100℃ groups decreased by 26.07, 28.16, 30.88, 37.55, 27.97, and 22.42%, respectively, and the differences were all significant ($p < 0.05$) (Figure 3B). The glucose levels of zebrafish in the T10 min, T20 min, T30 min, T40 min, T50 min, and T60 min groups decreased by 6.76, 20.46, 34.54, 36.03, 39.37, and 16.78%, respectively. All the other groups except the T10 min group had significant differences ($p < 0.05$) (Figure 3C). The results showed that different water/material ratios, extraction temperatures, and extraction times had different effects on the hypoglycemic effect of PYS.

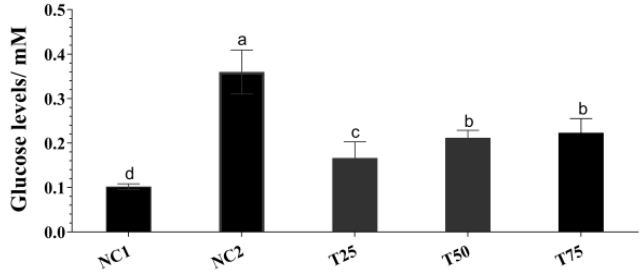

Figure 2. Effects of Pingyang Yellow Soup (PYS) on the hypoglycemic effects in zebrafish with non-insulin-dependent diabetes mellitus (NIDDM).

3.2.2 Optimization of extraction conditions for PYS based on the Box–Behnken response surface method

3.2.2.1 Box–Behnken test design and results

As can be seen from Table 2, a total of 17 treatment groups were obtained through the Box–Behnken test design. After the functional evaluation, the values of the nor-sugar rate among the 17 treatment groups ranged from 11.69 to 59.82%.

3.2.2.2 Model establishment and significance analysis

By analyzing the variance analysis on the statistical test model and quadratic multinomial regression fitting through the usage of the Design-Expert 11 software, the quadratic polynomial regression equation between water/material ratio (A), extraction temperature (B), extraction time (C), and nor-sugar rate (R1) can be obtained, and they are $R1 = -1366.0146 + 25.5179\,A + 19.51354\,B + 0.427918\,C - 0.03466\,AB + 0.068839\,AC - 0.032494\,BC - 241321\,A^2 - 0.107821\,B^2 - 0.021312\,C^2$. For detailed data, see Table 3.

As can also be seen from Table 3, the extraction temperature had a markedly significant ($p < 0.01$) influence on the nor-sugar rate, the extraction time had a significant ($p < 0.05$) influence, but the water/material ratio had no significant influence. In terms of interaction terms, they all showed markedly significant effects on the nor-sugar rate ($p < 0.01$). In the aspects of secondary items, they also had markedly significant effects on the nor-sugar rate ($p < 0.01$).

The comprehensive results showed that the model fitted well with the experimental values and could better reflect the relationship between the hypoglycemic effect of PYS and the different extraction conditions of water/material ratio, extraction temperature, and extraction time. Therefore, the obtained regression equation helps speculate and obtain the optimal extraction conditions based on the hypoglycemic function.

3.2.2.3 Response surface analysis

The controlled factors were set to the "0" level. The Design-Expert 11.0 software was used to analyze the interaction between the other two factors. The analysis of the response surface and contour map were used to reveal the interaction effect between factors, and the results are shown in Figure 4.

The size of the steepness and contour ellipticity values exhibited on the response surface can effectively judge the degree of interaction between factors. The steeper the surface, the larger

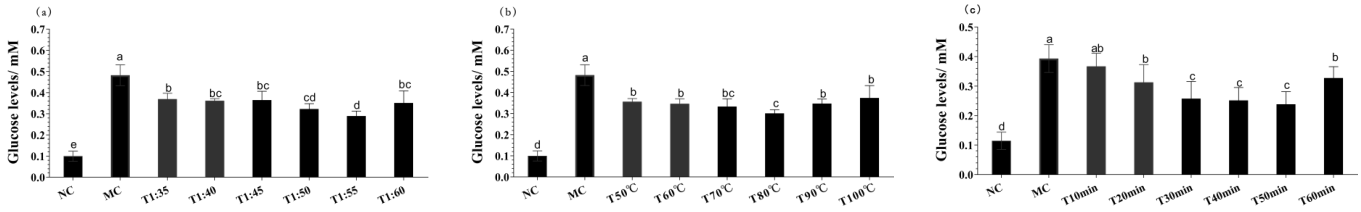

Figure 3. Effects of Pingyang Yellow Soup (PYS) extracted from different conditions on the hypoglycemic effects in zebrafish with non-insulin-dependent diabetes mellitus (NIDDM). (A–C) Evaluation of the hypoglycemic effect of Pingyang Yellow Soup (PYS) under different extraction conditions of water/material ratio, extraction temperature, and extraction time, respectively.

Table 3. Variance analysis of the response surface regression model

Sources	Sum of the squares	df	Mean square	F-values	p-values
Model	4,412.59	9	490.29	626.79	< 0.0001**
A-A: water/material ratio	0.0582	1	0.0582	0.0745	0.7928
B-B: extraction temperature/℃	264.2	1	264.2	337.76	< 0.0001**
C-C: extraction time/min	23.02	1	23.02	29.43	0.001**
AB	60.84	1	60.84	77.78	< 0.0001**
AC	239.9	1	239.9	306.7	< 0.0001**
BC	213.81	1	213.81	273.33	< 0.0001**
A^2	775.84	1	775.84	991.84	< 0.0001**
B^2	2,478.05	1	2478.05	3,167.97	< 0.0001**
C^2	96.82	1	96.82	123.78	< 0.0001**
Residual	5.48	7	0.7822		
Lack of fit	4.48	3	1.49	5.98	0.0584
Pure error	0.9981	4	0.2495		
Cor total	4,418.07	16			

$R^2 = 0.9960$, $R^2_{adj} = 0.9909$, $R^2_{pre} = 0.9394$; *$p < 0.05$ indicated significant differences were found between groups; **$p < 0.01$ indicated markedly significant differences between groups.

the contour ellipticity (Jaimez-Ordaz et al., 2021; Lin et al., 2020), which represents the strength of the interaction effect. Figure 4 shows that the strength of the interaction effects of BC on hypoglycemic performance was stronger than that of AC and then stronger than that of AB.

3.2.2.4 Results of optimization extraction conditions for PYS

The regression model was analyzed by the Design-Expert 11 software, and the "numerical procedure" in "optimization" was used for limiting the extraction conditions. The extraction conditions were set as water/material ratio (30–60), extraction temperature (40–90℃), and extraction time (20–50 min). The optimal extraction conditions were a water/material ratio of 52.609, an extraction temperature of 76.499℃, and an extraction time of 36.681 min. Considering the feasibility of the actual operation, the optimal conditions were modified, and they were set as the water/material ratio of 52.5, the extraction temperature of 76℃, and the extraction time of 36 min. After scientific evaluation, the nor-sugar rate of the optimal extraction conditions was better than that of the extraction conditions of a water/material ratio of 52.5, an extraction temperature of 75℃, and an extraction time of 35 min. The hypoglycemic performance had been strengthened under the optimal extraction conditions, suggesting that the model can predict the optimal extraction conditions well.

Our findings showed that different extraction conditions had a significant influence on the biological activity of yellow tea. The optimal combination of water/material ratio, extraction temperature, and extraction time helps improve and promote the biological activity of tea. The likely explanation was that different production and processing conditions influenced the content levels of functional ingredients in yellow tea soup (Balci & Özdemir, 2016; Rubanka et al., 2020; Şahin-Nadeem et al., 2013) and hence the biological activity of tea. Balci and Özdemir (2016) investigated different extraction temperatures (75, 85, and 95°C) and times (3, 5, 10, 15, and 20 min) on functional compounds in green tea, and their findings showed that the total phenolic and flavonoid contents increased with the increase of the extraction temperatures and times. Finally, the antioxidant capacity of the green tea was significantly increased. In addition to traditional extraction conditions, the microwave method has high extraction capability and reduces the processing time (Farahmandfar & Aziminezhad, 2021). Farahmandfar & Aziminezhad's (2021) studies showed that different production processes and brewing methods had a certain degree of influence on the antioxidant activity of tea. The microwave brewing method helps increase the contents of phenolic and flavonoid compounds and antioxidant activity. For this study, the underlying mechanisms of the changes in the hypoglycemic property of yellow tea extracted from different conditions were still unknown, and further study was needed. Therefore, a series of follow-up studies were conducted.

3.3 Basic research on hypoglycemic functional substances of PYS

3.3.1 Analysis of the effect of TPs on hypoglycemic performance in PYS

Figure 5A showed that with the increase in PVPP dosage, the hypoglycemic effect of PYS solution was significantly decreased and exhibited an obvious linear relationship and binomial response curve relationship (Figure 5B). Compared to the NC2 group, the glucose levels of zebrafish in the T-0.00 PVPP, T-0.33 PVPP, T-0.66 PVPP, T-1.33 PVPP, T-2.67 PVPP, and T-6.00 groups decreased by 55.93, 47.25, 37.33, 35.56, 31.45, and 17.95%, respectively, and all the differences reached significant levels ($p < 0.05$).

When the total amount of TPs in the PYS solution decreased by 89.17% in the T-6.00 PVPP group, the nor-sugar rate decreased from 37.98 to 55.93 to 17.95% (Figure 5A). The results showed that TPs played an important and key role in the hypoglycemic performance of the PYS solution.

TPs and its main catechin monomer, EGCG, are the main kinds of functional active substances enriched in tea, and studies have shown that the hypoglycemic effects of tea are closely related to TPs (Macena et al., 2022; Tan et al., 2022).

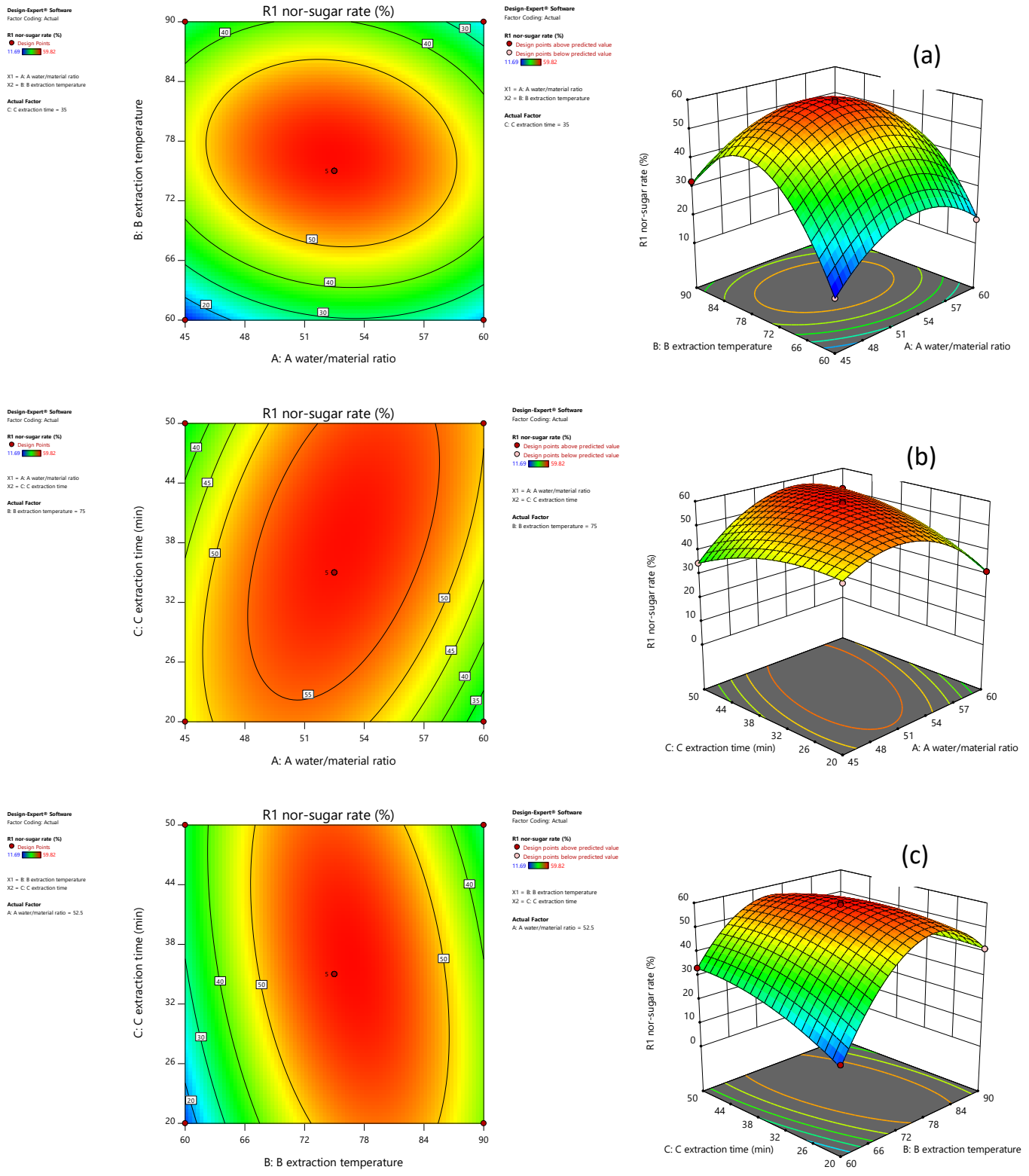

Figure 4. Response surface and contour plots showing the effects of different extraction conditions on the nor-sugar rate. Remarks: (A) the results of the interaction effect between water/material ratio and extraction temperature; (B) the results of the interaction effect between water/material ratio and extraction time; (C) the results of the interaction effect between extraction temperature and extraction time.

Studies have shown that tea has an intervention effect on the digestion and absorption of nutrients in the gut and the oxidation and utilization of nutrients in the body. The well-known intervention pathway is to inhibit intestinal digestive enzyme activities, including amylase and lipase activities (Li et al., 2018; Zhang et al., 2018); specifically regulate intestinal

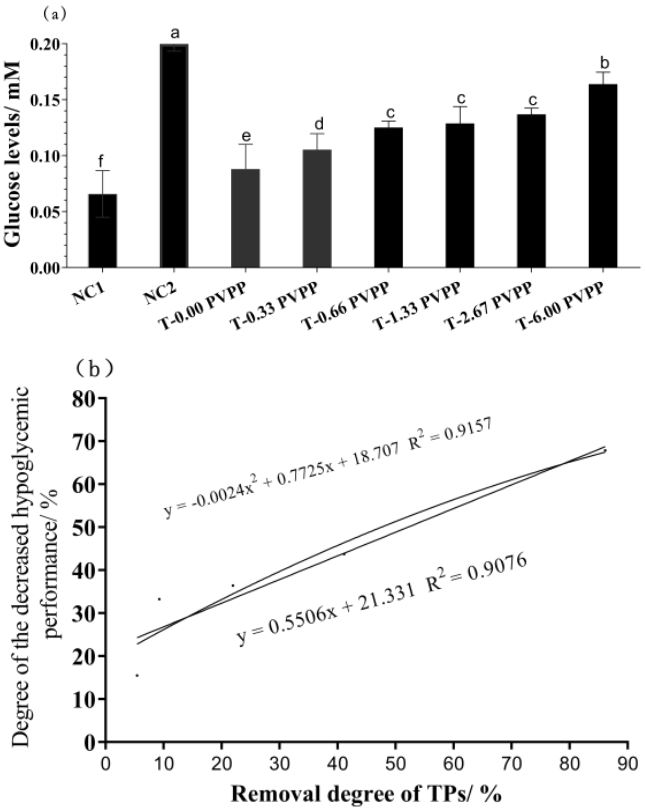

Figure 5. Effects of Pingyang Yellow Soup (PYS) on the hypoglycemic effects in zebrafish with non-insulin-dependent diabetes mellitus (NIDDM). (A) The effect of Pingyang Yellow Soup (PYS) within polyphenols directed by PVPP on the hypoglycemic effect of zebrafish with non-insulin-dependent diabetes mellitus (NIDDM); (B) the correlation between the removal degree of tea polyphenols (TPs, %) and the degree of decreased hypoglycemic performance (%) of PYS.

microecological balance (Liu et al., 2022a; Zhao and Zhang, 2020); and affect and change the internal and external transport efficiency of nutrients, including downregulating the expression of dependent glucose cotransporter 1 (Hossain et al., 2002), protecting islet cells and promoting insulin secretion (Wang et al., 2010), alleviating insulin signal blockade, and promoting sugar oxidation (Bose et al., 2008) and lipid metabolism in vivo (Huang et al., 2013).

In addition, after intake of the active ingredients of tea, they are initially degraded in the intestine, especially under the fermentation of intestinal microorganisms, which can promote the degradation of the large molecular components that are difficult to cross the intestinal wall into small molecular active components. These small molecular components also can play a key role in antioxidant and free radical scavenging in the body (Vaquero et al., 2004), which is beneficial to the physical health. For the TPs themselves, the hypoglycemic effects of catechin are obviously related to the structure of the B ring in catechin gallic acyl or galloyl moieties (Lu & Hwang, 2008).

Although TPs obviously had hypoglycemic effects, the degree of hypoglycemic effects of TPs in tea is still unknown, particularly in yellow tea. Our results clearly revealed and made clear the contribution degree of hypoglycemic effects of TPs in yellow tea. As we know, tea contains lots of functional active substances, and TP is one among them. Meanwhile, TPs also belong to mixture, containing many catechin monomers. Ho ever, the effects and roles of TPs and its monomer on hypoglycemic effects in tea (not for single pure materials), particularly in yellow tea, still remain unknown. Therefore, in the next section, we deeply discussed the effects of polyphenols and alkaloids on hypoglycemic effects in PYS solutions.

3.3.2 Analysis of the effect of phenolic components on the hypoglycemic performance of PYS

In view of the important role of phenolic substances in the hypoglycemic effects of PYS, 33 peaks with the same peak emergence time were obtained after elution and separation analysis of phenolic substances, which were labeled as peak 1-peak 33 according to the peak emergence time. The results are shown in Figure 6. Then, the correlation analysis was carried out to preliminarily explore the possible and potential mechanisms from the aspects of exponent, linearity, logarithm, polynomial (quadratic), and power exponent, and the final relationship was determined based on the highest value of R^2. For details, see Table 4.

As we all know, TP is the general name of polyphenols in tea, which has a wide range of physiological activities and can be further isolated and purified into different catechin monomers such as EGCG, epicatechin gallate (EC), epicatechin gallate (ECG), and epigallocatechin catechin (EGC). Table 4 shows that the nor-sugar rate showed strong correlations ($0.6 < R^2 \leq 1.0$) with concentration levels of C, ECG, EGC, EC, EGCG, and CAF in PYS solutions.

At the same time, the analysis and determination of the influence weight coefficient of 33 detected peaks were done by using the "principal component analysis" in "dimension reduction" in SPSS Statistics 24.0. The results are shown in Table 4 and Figure 7. In terms of the single influence weight coefficient, it ranges from 0.09% to 4.76%. The cumulative influence weight coefficient above 3.0% accounted for 63.63% (Figure 7). The total influence weight coefficients of EGC (peak 14), EGCG (peak 21), ECG (peak 30), CAF (peak 17), EC (peak 18), and C (peak 15) accounted for 23.12% (Figure 7B).

The results showed that they all exhibited a downward opening parabolic shape (Table 4), and the influence weight with a higher coefficient is mainly located in the later peak. In a combined analysis of the mobile phase properties, the weight of influence of substances with strong fat solubility is relatively stronger than that of substances with strong water solubility in terms of comprehensive influence.

Polyphenol is a kind of functional mixture, containing many catechin monomers. The effects of synergistic or antagonistic effects within polyphenols and CAF on hypoglycemic performance are shown in Table 5 and Figure 8. As can be seen from Table 5 and Figure 8, within the range of observed actual dose concentration, the action curves of TPs and its main catechin monomer in PYS solution mainly showed a downward opening parabolic shape (quadratic). The results showed that, under the cross-influence of multiple factors, in

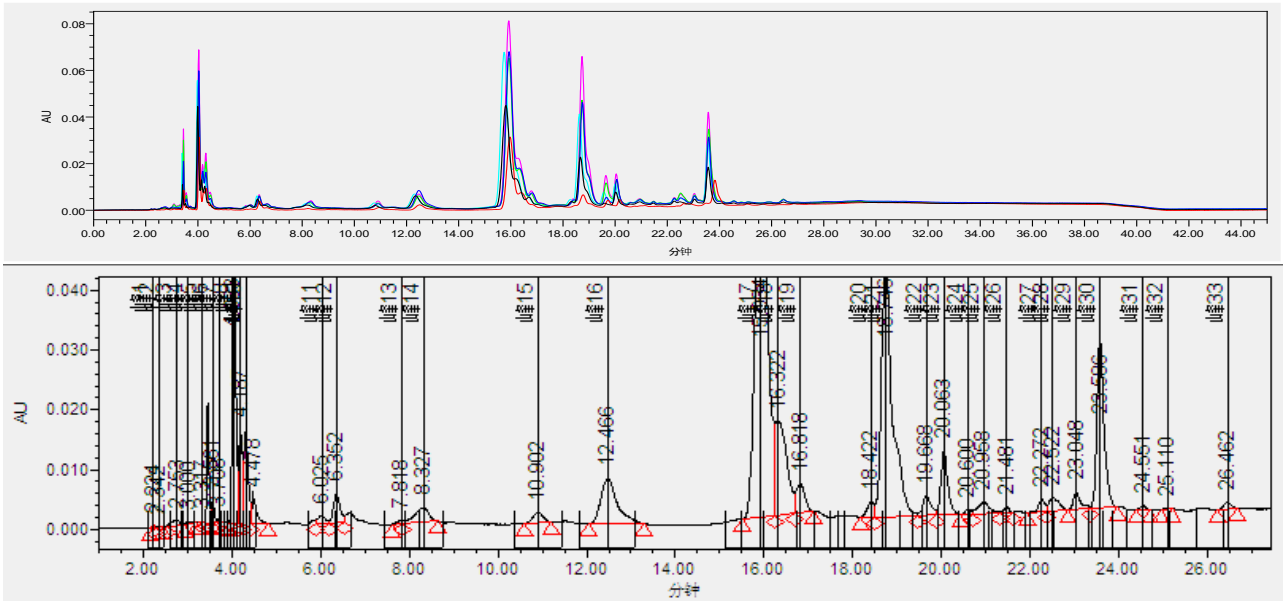

Figure 6. Analysis of phenolics in differentiated samples with hypoglycemic properties based on liquid chromatography.

Table 4. Analysis of the role of phenolic components in hypoglycemic performance in Pingyang Yellow Soup (PYS) under different extraction conditions.

Items	Equations of correlation	Influence weight coefficients after normalization/%	Influence weight rankings
Nor-sugar rate vs. Peak 1	$y = 23.573x^2 - 147.3x + 248.03$ ($R^2 = 0.7010$)	0.87	30
Nor-sugar rate vs. Peak 2	$y = 36.911x^2 - 268.55x + 505.94$ ($R^2 = 0.6848$)	1.11	29
Nor-sugar rate vs. Peak 3	$y = 0.5368x^2 - 25.994x + 315.23$ ($R^2 = 0.8537$)	0.09	33
Nor-sugar rate vs. Peak 4	$y = 0.0702x^2 - 5.285x + 120.77$ ($R^2 = 0.4015$)	1.45	27
Nor-sugar rate vs. Peak 5	$y = -0.5211x + 53.339$ ($R^2 = 0.8725$)	3.16	20
Nor-sugar rate vs. Peak 6	$y = -0.0006x^2 + 0.1503x + 27.72$ ($R^2 = 0.0108$)	1.93	25
Nor-sugar rate vs. Peak 7	$y = 0.4885x^2 - 14.434x + 132.91$ (R^2=0.1548)	1.60	26
Nor-sugar rate vs. Peak 8	$y = -94467x^2 + 24226x - 1508.6$ ($R^2 = 0.6016$)	4.54	4
Nor-sugar rate vs. Peak 9	$y = -0.0044x^2 + 2.4217x - 290.46$ ($R^2 = 0.2794$)	0.36	31
Nor-sugar rate vs. Peak 10	$y = -0.0186x^2 + 3.7857x - 142.57$ ($R^2 = 0.7614$)	3.32	19
Nor-sugar rate vs. Peak 11	$y = -0.3919x^2 + 34.257x - 701.61$ ($R^2 = 0.3317$)	2.59	23
Nor-sugar rate vs. Peak 12	$y = 0.0121x^{1.6409}$ ($R^2 = 0.4806$)	3.50	17
Nor-sugar rate vs. Peak 13	$y = -4.6254x^2 + 83.055x - 328.72$ ($R^2 = 0.4057$)	0.35	32
Nor-sugar rate vs. Peak 14 (EGC)	$y = -8282.3x^2 + 6295.8x - 1150.3$ ($R^2 = 0.7411$)	4.35	6
Nor-sugar rate vs. Peak 15 (C)	$y = -182566x^2 + 28481x - 1059.1$ ($R^2 = 0.8234$)	3.11	21
Nor-sugar rate vs. Peak 16	$y = 0.2368x - 62.339$ ($R^2 = 0.6215$)	2.25	24
Nor-sugar rate vs. Peak 17 (CAF)	$y = -5337.4x^2 + 6188.8x - 1725.6$ ($R^2 = 0.8312$)	3.80	13
Nor-sugar rate vs. Peak 18 (EC)	$y = -4272x^2 + 4396.4x - 1082.4$ ($R^2 = 0.6627$)	3.76	14
Nor-sugar rate vs. Peak 19	$y = 2E\text{-}07x^{3.6307}$ ($R^2 = 0.5989$)	4.27	8
Nor-sugar rate vs. Peak 20	$y = 0.2752x^2 - 33.379x + 1024.9$ ($R^2 = 0.4692$)	1.22	28
Nor-sugar rate vs. Peak 21 (EGCG)	$y = -587.87x^2 + 714.16x - 168.86$ ($R^2 = 0.7102$)	4.20	9
Nor-sugar rate vs. Peak 22	$y = -0.0011x^2 + 0.4757x + 1.5581$ ($R^2 = 0.5523$)	2.83	22
Nor-sugar rate vs. Peak 23	$y = 0.0144x^{1.3499}$ ($R^2 = 0.3395$)	4.45	5
Nor-sugar rate vs. Peak 24	$y = -1.7821x^2 + 62.848x - 508.4$ ($R^2 = 0.5202$)	4.66	3
Nor-sugar rate vs. Peak 25	$y = -0.0497x^2 + 9.1324x - 371.04$ ($R^2 = 0.4960$)	4.29	7
Nor-sugar rate vs. Peak 26	$y = 20.287e^{0.0179x}$ ($R^2 = 0.0160$)	4.12	10
Nor-sugar rate vs. Peak 27	$y = -0.351x^2 + 34.222x - 781.29$ ($R^2 = 0.6561$)	4.76	1
Nor-sugar rate vs. Peak 28	$y = -0.0058x^2 + 1.3882x - 36.61$ ($R^2 = 0.6256$)	3.38	18
Nor-sugar rate vs. Peak 29	$y = -0.0863x^2 + 15.889x - 682.99$ ($R^2 = 0.8394$)	3.92	11
Nor-sugar rate vs. Peak 30 (ECG)	$y = -4498x^2 + 2158x - 210.37$ ($R^2 = 0.8346$)	3.90	12
Nor-sugar rate vs. Peak 31	$y = 1.7838x^{1.0301}$ ($R^2 = 0.4493$)	3.67	15
Nor-sugar rate vs. Peak 32	$y = -0.6948x^2 + 10.815x - 1.195$ ($R^2 = 0.4408$)	4.66	2
Nor-sugar rate vs. Peak 33	$y = -0.077x^2 + 6.0832x - 73.741$ ($R^2 = 0.7345$)	3.53	16

"x" value: calculated by the measured dose concentration (known component) or peak area instead; "y" value: the nor-sugar rate (%); R^2: the strength of the co-relationship with "x" and "y"; $0.0 < R^2 \leq 0.2$: very weak correlation or no correlation; $0.2 < R^2 \leq 0.4$: weak correlation; $0.4 < R^2 \leq 0.6$: moderate correlation; $0.6 < R^2 \leq 0.8$: strong correlation; and $0.8 < R^2 \leq 1.0$: markedly strong correlation. The above final equation of correlation is listed in the table according to the highest level of R^2 value, which does not mean that there is only one equation of correlation.

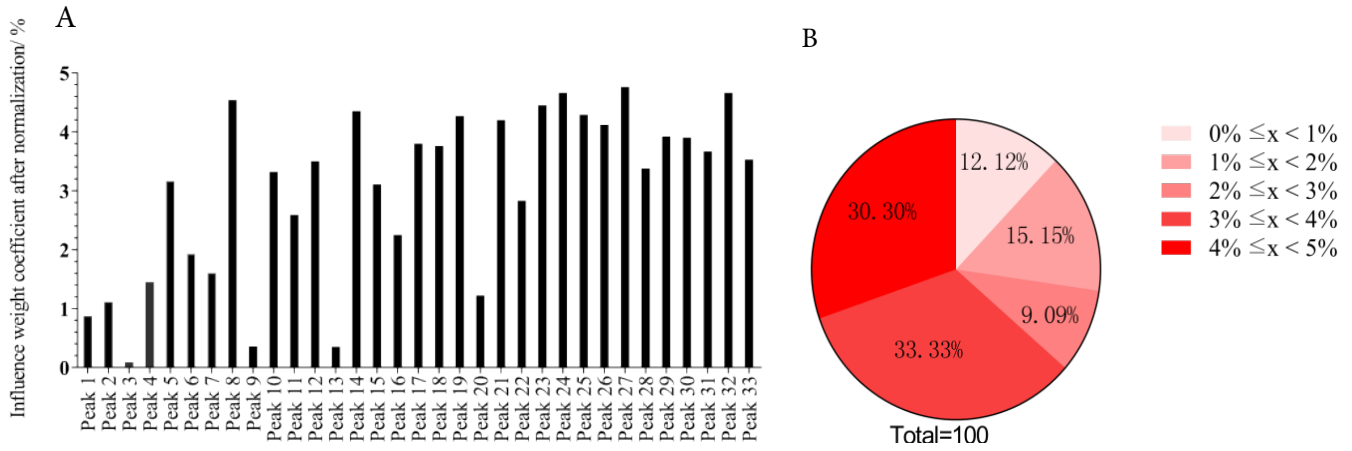

Figure 7. Analysis of the weight coefficient of each influence. (A) The influence weight coefficient of each peak after normalization; (B) the segmented statistics of influence weights.

Table 5. Mining analysis of the hypoglycemic effect of phenolic compounds in Pingyang Yellow Soup (PYS) under different extraction conditions

Items	Equations of correlation
Nor-sugar rate vs. TPs	$y = -57.38x^2 + 321.76x - 404.94$ ($R^2 = 0.7619$, $2.04 \le x \le 3.48$)
Nor-sugar rate vs. EGC	$y = -8282.3x^2 + 6295.8x - 1150.3$ ($R^2 = 0.7411$, $0.31 \le x \le 0.43$)
Nor-sugar rate vs. C	$y = -182566x^2 + 28481x - 1059.1$ ($R^2 = 0.8234$, $0.06 \le x \le 0.09$)
Nor-sugar rate vs. CAF	$y = -5337.4x^2 + 6188.8x - 1725.6$ ($R^2 = 0.0.8312$, $0.48 \le x \le 0.68$)
Nor-sugar rate vs. EC	$y = -4272x^2 + 4396.4x - 1082.4$ ($R^2 = 0.6627$, $0.42 \le x \le 0.59$)
Nor-sugar rate vs. EGCG	$y = -587.87x^2 + 714.16x - 168.86$ ($R^2 = 0.7102$, $0.36 \le x \le 0.80$)
Nor-sugar rate vs. ECG	$y = -4498x^2 + 2158x - 210.37$ ($R^2 = 0.8346$, $0.15 \le x \le 0.32$)
Nor-sugar rate vs. Total catechin contents	$y = -171.51x^2 + 611.58x - 494.93$ ($R^2 = 0.8349$, $1.31 \le x \le 2.18$)
Nor-sugar rate vs. Total ester catechin contents	$y = -359.68x^2 + 602.33x - 202.13$ ($R^2 = 0.8151$, $0.51 \le x \le 1.09$)
Nor-sugar rate vs. Total non-ester catechins contents	$y = -1225.9x^2 + 2204x - 946.58$ ($R^2 = 0.6493$, $0.80 \le x \le 1.11$)
Nor-sugar rate vs. TPs/CAF	$y = -33.892x^2+303.42x-641.39$ ($R^2=0.1211$, $4.24\le x\le 5.15$)
Nor-sugar rate vs. TPs+CAF	$y = -44.807x^2 + 303.6x - 467.87$ ($R^2 = 0.7750$, $2.52 \le x \le 4.15$)
Nor-sugar rate vs. C+CAF+EC	$y = -1018.5x^2 + 2383.5x - 1338.4$ ($R^2 = 0.7727$, $0.96 \le x \le 1.36$)
Nor-sugar rate vs. C+EC	$y = -3435x^2 + 4054.6x - 1146.6$ ($R^2 = 0.7396$, $0.49 \le x \le 0.68$)
Nor-sugar rate vs. C/CAF	$y = 21849x^2 - 7407.9x + 624.03$ ($R^2 = 0.8157$, $0.12 \le x \le 0.14$) $y = -1913.7x + 280.13$ ($R^2 = 0.8133$, $0.12 \le x \le 0.14$)
Nor-sugar rate vs. CAF/C	$y = 30.088x - 200.84$ ($R^2 = 0.8152$, $7.38 \le x \le 8.65$) $y = 0.6532x^2 + 19.629x - 159.15$ ($R^2 = 0.8153$, $7.38 \le x \le 8.65$)
Nor-sugar rate vs. EC/CAF	$y = -43967x^2 + 75008x - 31942$ ($R^2 = 0.7748$, $0.83 \le x \le 0.88$)
Nor-sugar rate vs. (C+EC) /CAF	$y = -34429x^2 + 67736x - 33268$ ($R^2 = 0.7619$, $0.97 \le x \le 1.02$)
Nor-sugar rate vs. EGCG/CAF	$y = -449.23x^2 + 916.82x - 421.87$ ($R^2 = 0.5707$, $0.75 \le x \le 1.23$)
Nor-sugar rate vs. CAF/EGCG	$y = -397.09x^2 + 821.81x - 378.69$ ($R^2 = 0.6343$, $0.81 \le x \le 1.33$)
Nor-sugar rate vs. ECG/CAF	$y = -6186.4x^2 + 4898.3x - 914.98$ ($R^2 = 0.8271$, $0.32 \le x \le 0.47$)
Nor-sugar rate vs. CAF/ECG	$y = -130.52x^2 + 682.3x - 838.85$ ($R^2 = 0.9034$, $2.11 \le x \le 3.18$)
Nor-sugar rate vs. EGC/CAF	$y = -11609x^2 + 13774x - 4031.9$ ($R^2 = 0.8003$, $0.55 \le x \le 0.66$)
Nor-sugar rate vs. CAF/EGC	$y = -1683.5x^2 + 5701.7x - 4771.3$ ($R^2 = 0.8220$, $1.52 \le x \le 1.80$)

"x" value: measured dose concentration (μg/mL) or value; "y" value: the nor-sugar rate (%); R^2: the strength of the co-relationship with "x" and "y"; $0.0 < R^2 \le 0.2$: very weak correlation or no correlation; $0.2 < R^2 \le 0.4$: weak correlation; $0.4 < R^2 \le 0.6$: moderate correlation; $0.6 < R^2 \le 0.8$: strong correlation; $0.8 < R^2 \le 1.0$: markedly strong correlation. The above final equation of correlation is listed in the table according to the highest level of R^2 value, which does not mean that there is only one equation of correlation.

order to achieve the optimal hypoglycemic effect, the dose of each component does not require the more the better or the less the better. The dose concentration of each component should be controlled within the appropriate range, and the balance of component proportion and concentration is the key to exert the highest hypoglycemic performance.

In terms of simple composition of the main functional substances, it indicates that the combination of various substances may play an important role in the hypoglycemic effect of PYS (Table 5). There was a strong correlation between TPs (R^2 = 0.7619) and hypoglycemic performance. Most importantly, the correlation coefficient of the total amount of ester

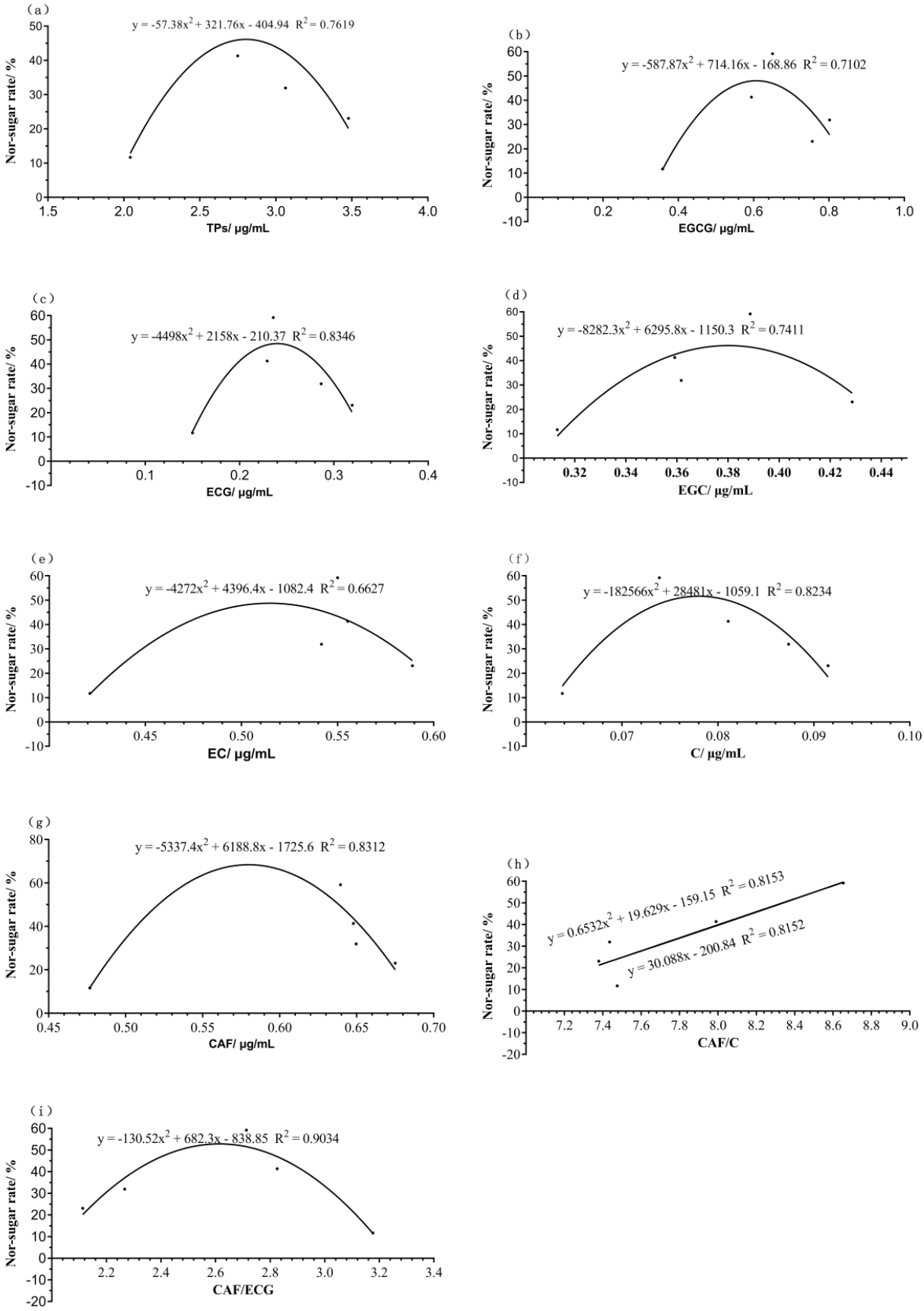

Figure 8. Comparative analysis of the hypoglycemic effect of polyphenolic fractions in Pingyang Yellow Soup (PYS) under different extraction conditions. (A–I) The action curves of single or combined components TPs, EGCG, ECG, EGC, EC, CAF, CAF/C, and CAF/ECG in the Pingyang Yellow Soup (PYS) solution system under different extraction conditions.

catechin (R^2 = 0.8151) was higher than that of the total amount of non-ester catechin (R^2 = 0.6493), which indicated that the lipid-soluble components have a stronger hypoglycemic effect. It is noteworthy that after a simple combination of C, ECG, and EGC with CAF, the R^2 values are all strongly correlated above 0.8. Especially, the correlation between C/CAF, CAF/C, and nor-sugar rate showed a significant linear relationship, and the R^2 level of CAF/ECG was obviously increased, which was worthy of further study.

Considering that phenolic and alkaloid components may play an important role in the hypoglycemic effect of PYS, Pearson correlation analysis and multiple linear regression analysis were conducted using the SPSS 24.0 software to reveal the correlation of the nor-sugar rate, EGC, C, CAF, EC, EGCG, and ECG. The specific results are shown in Table 6 and Figure 9. After the multiple linear regression analysis, the multiple regression equation of the nor-sugar rate and EGC, CAF, EGCG, and ECG was obtained after the removal of the influence factors of C and EC. The equation is y = -158.08 + 452.56x_{EGC} + 209.04x_{CAF} + 230.52x_{EGCG} -1027.01x_{ECG} (R^2 = 1.0000), indicating that there was a markedly strong correlation between the nor-sugar rate and the four variables of EGC, CAF, EGCG, and ECG in PYS solution.

4 CONCLUSION

Based on the functional evaluation of zebrafish, PYS has very strong and definite hypoglycemic biological activity, and the effective dose concentration of PYS is 25–50 μg/mL. Mea while, the results of the single-factor experiment showed that different water/material ratios, extraction temperatures, and extraction times had a significant influence on the hypoglycemic performance of PYS. Combined with the Box–Behnken response surface experiment design, the hypoglycemic performance was significantly affected by different extraction conditions, and the strength of influence was ranked as extraction temperature > extraction time > water/material ratio. Through the interaction analysis, the optimal extraction conditions for PYS were a water/material ratio of 52.5, an extraction temperature of 76°C, and an extraction time of 36 min.

The zebrafish model was adopted to analyze the important role of polyphenols in the hypoglycemic effect of PYS through the targeted removal of polyphenols. It was found that there was a strong linear correlation between the removal rate of the TPs in PYS solution and the degree of decreased hypoglycemic performance. When the total amount of TPs decreased by 89.17%, the nor-sugar rate of PYS decreased from 55.93% to 17.95%, with a decreased degree of 37.98 percentage points, indicating that the hypoglycemic performance of PYS was closely related to TPs.

Combined with the basic study of functional substances in PYS, it was found that the nor-sugar rate of PYS had a variety of regression relationships with its contained functional components, particularly catechin and CAF. At the same time, the best hypoglycemic performance of PYS does not require the highest content levels of each functional component but depends on what it is, and there exists a relative optimal value that provides basic information for exploring the internal mechanism of the hypoglycemic effects of PYS. Since PYS contains a large number of active substances, in addition to TPs and its main catechins, there are many unknown ingredients that also have potential hypoglycemic value. More follow-up attention and research are needed.

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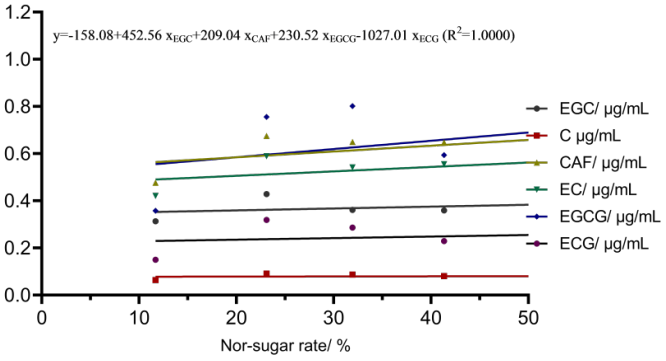

Figure 9. Multivariate linear relationship between nor-sugar rate and major catechins and caffeine (CAF) in Pingyang Yellow Soup (PYS) under different extraction conditions.

Table 6. Pearson correlation between nor-sugar rate, main catechins, and CAF of Pingyang Yellow Soup (PYS) under different extraction conditions.

	Nor-sugar rates	EGC	C	CAF	EC	EGCG	ECG
Nor-sugar rates	1	0.3380	0.0930	0.5570	0.5290	0.3640	0.1860
EGC	0.3380	1	0.7530	0.8250	0.8850	0.7510	0.8610
C	0.0930	0.7530	1	0.8730	0.8600	0.9080	0.9630
CAF	0.5570	0.8250	0.8730	1	0.9890	0.9060	0.8880
EC	0.5290	0.8850	0.8600	0.9890	1	0.8620	0.8840
EGCG	0.3640	0.7510	0.9080	0.9060	0.8620	1	0.9530
ECG	0.1860	0.8610	0.9630	0.8880	0.8840	0.9530	1

$0.0 < R^2 \leq 0.2$: very weak correlation or no correlation; $0.2 < R^2 \leq 0.4$: weak correlation; $0.4 < R^2 \leq 0.6$: moderate correlation; $0.6 < R^2 \leq 0.8$: strong correlation; $0.8 < R^2 \leq 1.0$: markedly strong correlation. The above final equation of correlation is listed in the table according to the highest level of R^2 value, which does not mean that there is only one equation of correlation.

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