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Full Length Article

In Vitro Antioxidant and Antibacterial Activities of Quinoa Flavonoids Extracted by Ethanol-Ammonium Sulfate Aqueous Two-Phase System

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Abstract

Quinoa (*Chenopodium quinoa* Willd.) is a functional and ideal food for human nutrition and an Andean seed-producing crop. In this study, ultrasonic-assisted extraction of total flavonoids in Quinoa with ethanol (C_2H_5OH)-ammonium sulfate ($(NH_4)_2SO_4$) aqueous two-phase system was performed based on the Box-Behnken experimental design principle. The highest extraction rate of TFQ under the condition of 28% C_2H_5OH -14% $(NH_4)_2SO_4$ aqueous two-phase extraction system was used to analyze the variance of TFQ extraction rate as the response value. The multiple quadratic linear regression equation was obtained by a three-factor three-level response surface method. The extraction rate= 74.28+1.78 A+0.10 B+0.38 C+0.20 AB+0.05 AC+0.05 BC+1.000E-002 A^2-0.94 B^2-0.69 C^2. The response surface analysis showed that the best extraction conditions of aqueous two-phase were the crude TFQ mass fraction 20.6%, pH 7.18, NaCl mass fraction 2.23% and the maximum value predicted by the extraction rate model was 75.929 3% (P=0.994). The average extraction rate of TFQ was 75.3%, according to the optimal two-aqueous phase extraction conditions. The ETFQ has varying degree of scavenging effect on hydroxyl radical, oxygen free radicals, nitrite and $\cdot ABTS^+$ compared with vitamin C. Among them, the scavenging effect of the ETFQ on hydroxyl radical, oxygen free radicals and $\cdot ABTS^+$ was greater than vitamin C, except nitrite. Also, the ETFQ has the strongest inhibitory effect on *E. coli* and *Bacillus subtilis*, and the inhibitory rate can reach up to high dose 97.59 and 98.44%, MIC is 1.56 mg/mL; the second is the inhibition of S. aureus, MIC is 6.25 mg/mL. It has the weakest inhibitory effect on Salmonella. The antibacterial rate was positively correlated with the ETFQ mass concentration. The results help to discover the medicinal effects of quinoa in addition to nutrition to carry out more in-depth research and increase economic value.

Keywords: Quinoa flavonoids; Antioxidant activity; Antibacterial; Aqueous two-phase extraction

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a functional and ideal food grown in the Andean highlands. It has attracted interest in the scientific community due to its good nutritional value (Dini *et al.* 2010; Navruz-Varli and Sanlier 2016; Vilcacundo and Hernández-Ledesma 2017). European and North American consumers are increasingly aware of the exceptional nutritional qualities of quinoa seeds and sprouts, are now considered "functional foods" (Angeli *et al.* 2020). There is extensive literature on the chemical composition of quinoa seed, which cover all nutritional aspects such as chemical characterization of proteins (Dakhili *et al.* 2019; Sezgin and Sanlier 2019). As a new high-nutrient coarse grain, quinoa is also rich in flavonoids compared to corn, rice and wheat (Dini *et al.* 2010; Carciochi *et al.* 2015; Lopez *et al.* 2018). The study showed that the contents of quercetin and kaempferol in quinoa were much higher than those in buckwheat (there was no kaempferol in buckwheat) (Zhu 2018; Xiang *et al.* 2019b).

Quinoa contains natural phytoestrogens, one of the flavonoids, especially seeds with colored testa. Flavonoids are a group of phenolic compounds with 2-phenyl-1,4-benzopyrone backbone and divided into subgroups as flavones, isoflavones, flavan, roanthocyanidins, and anthocyanidins. Flavonoids such as quercetin and epicatechin exhibit antioxidant activity (Penido *et al.* 2017; Rai *et al.* 2018; Xiang *et al.* 2019a) and has a negative correlation with the risk of developing coronary heart disease (Bohn *et al.* 2012; Sanchez Hernandez *et al.* 2016) and type II diabetes mellitus (Abe *et al.* 2017). Flavonoids may significantly improve the cognitive ability of patients in acute and chronic diseases. Evidence indicates that flavonoids have the potential to reduce the risk of cervical cancer, lung cancer, leukemia, breast cancer, colorectal cancer and prostate cancer (Brend *et al.* 2012;

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Orfali *et al.* 2016; Filho *et al.* 2017; Sezgin and Sanlier 2019). Flavonoids have strong antioxidant effects, can eliminate harmful superoxide radical groups in the human body and have physiological activities such as anti-aging, enhancing immunity and so on (Perez Vizcaino and Fraga 2018, Abotaleb *et al.* 2019; Lavanya *et al.* 2019; Romano *et al.* 2020). As a result, more and more researchers are turning their attention to the active components of quinoa.

Aqueous two-phase extraction (ATPE) technology is an effective separation technology widely used in natural product separation, biological extraction, pharmaceuticals, food chemical industry and other fields (Gu and Glatz 2007; Lee *et al.* 2017; Assis *et al.* 2020; Huang *et al.* 2020). Compared with traditional liquid-liquid extraction, an aqueous two-phase system is a milder extraction and separation technology that is non-toxic, non-flammable, low cost and not prone to emulsion. In recent years, water-soluble low-grade alcohols and salts aqueous two-phase system has overcome the problems of high cost, low efficiency and difficulty in target recovery and treatment of traditional two-phase technology. It is easy to integrate with other technologies and has attracted much attention. Aqueous two-phase extraction has been successfully applied as gentle unit operation for the purification of biomolecules such as therapeutic proteins, enzymes, and antibiotics (Garai and Kuma 2013; Shkinev *et al.* 2013). At present, there is very little information about the optimization of the extraction process of total flavonoids from quinoa. The purpose of this experiment is to study the extraction conditions of total flavonoids from quinoa by ultrasonic-assisted two-phase extraction technology. The optimal combination of extraction conditions is analyzed by response surface methodology, and then the antimicrobial activity of extracted TFQ (ETFQ) was analyzed.

In recent years, the planting area of quinoa has increased every year, but the processing is still in the relatively primitive stage, and the economic value is not very high (Ruiz *et al.* 2014; Bellemare *et al.* 2018). Here, we provide a theoretical basis for the in-depth development of quinoa, including its medicinal value, thereby increasing its economic value.

Materials and Methods

Material and strains

Mature quinoa seeds (CD-1, black) were collected from Yanyuan County, Liangshan Prefecture, Sichuan Province, China. The seeds were cleaned and dried. The dried seeds were ground and the powder was obtained through 100-mesh sieve.

The test strains were *E. coli* ACCC11864, *Salmonella.* ACCC 01319, *S. aureus* ACCC 01332, *Bacillus subtilis* ACCC01430, provided by professor Jianglin Zhao and Sichuan Industrial Institute of Antibiotics of Chengdu University (SIIAC).

Reagents and instruments

Anhydrous ethanol, sodium nitrate, aluminum nitrate, and sodium hydroxide were supplied by Merck (Darmstadt, Germany). MH and LB medium, and a Mackinot's turbidimeter were purchased from Solarbio. All reagents were of analytical grade. A small automatic crusher (Nanjing, China), Rotary evaporator (Yarong, Shanghai), LDZM-40KCM Autoclave (SHENAN, Shanghai), HZQ-C constant temperature incubator (Aohua, Changzhou) and Multimode reader (potenov, Beijing) were also used in the experiments.

Methods

TFQ extraction and quantitative determination: Ten grams of defatted quinoa powder was dispersed in 50mL 75% ethanol, followed by ultrasonic extraction for 30 min. The extract was obtained by centrifugation at 5500 r/min (Hemalatha *et al.* 2016). The three times extract was combined and concentrated into the aqueous phase by vacuum filtration and rotary evaporation. The crude TFQ content was determined by $NaNO_2$-Al $(NO_3)_3$ colorimetry according to the results of rutin standard curve Y = 0.0897X +0.0005 (R^2 = 0.9993).

Establishment of ethanol-ammonium sulfate aqueous two-phase system: The extraction ability of ethanol and ammonium sulfate aqueous two-phase system for TFQ extracts with certain mass concentration was investigated. Ethanol and ammonium sulfate were prepared to form a stable two-phase system. Crude TFQ were added to the system. The system was fully mixed by oscillation. After centrifugation, samples were taken from the upper and lower phases and the absorbance were determined by the method mentioned above using the multimode reader. The mass concentration of TFQ in the upper and lower phases was calculated according to the standard curve. The partition coefficient (*K*) and extraction rate (*Y*) of the system were calculated according to the formulas (1) and (2).

$$K = \frac{V_t}{V_h} \quad (1)$$

$$Y_t = \frac{V_t C_t}{V_t C_t + V_h C_h} x100\% \quad (2)$$

In formulas (1) and (2), *Vt* and *Vb* are the upper and lower phase volumes (mL); *Ct* and *Cb* were the mass concentration of TFQ in upper and lower phases (mg/mL). *Yt* were the TFQ extraction rate in upper phases.

Optimize design the response surface experiments: After determining the optimal two-phase composition of C_2H_5OH-$(NH_4)_2SO_4$ aqueous two-phase system, the effects of the flavonoids crude extract mass fraction, pH and NaCl concentration on TFQ extraction rate were studied. According to the single factor test results, 17 response tests with three factors and three levels were designed by

Design-Expert 8.0 to analyze the optimum technological conditions of TFQ two-phase extraction.

After obtaining the optimal aqueous two-phase system for extract TFQ, the system was amplified to extract quinoa flavonoids, and the ETFQ were concentrated and lyophilized for subsequent experiments.

***In vitro* antioxidant activity**

Scavenging of hydroxyl radical: One milliliter of 1.5 mmoL /L 1,10-phenanthroline ethanol solution was added to a 10-mL colorimetric tube. Then 2.0 mL of phosphate buffer, 1.0 mL H_2O and 1.0mL of 0.75 mmoL/L ferrous sulphate solution ($FeSO_4$) were added into the tube in turn. The solutions were fully mixed and 1.0 mL of 0.1% H_2O_2 was added. All the components were mixed and kept at 37°C for 60 min. The absorbance of the mixture was measured at 536 nm (A_0). All other conditions were held constant. The water from the tube was replaced with the different concentrations of ETFQ solution, and the absorbance was measured at 536 nm (A_1). 0.1% H_2O_2 was replaced by H_2O to get an A_2 under the same conditions. The clearance ability was calculated according to the following formula (3):

$$\bullet OH \text{ clearance ability} = \frac{A_1 - A_0}{A_2 - A_0} \times 100\% \quad (3)$$

Scavenging of oxygen free radicals: Three milliliters of Tris-HCl buffer (50 mmol/L, pH 8.2) were placed in 10-mL test tube separately and preheated in 25°C water bath for 20 min. 0.5 mL of different concentrations of ETFQ and 2.0 mL of pyrogallol solution (30 mmol/ L) was added respectively, the reaction mixture was mixed well and incubated for 8 min at 25°C. 1.0 mL HCl solution (10 mmol/ L) was added to stop the reaction, and the absorbance A_1 was measured at 320 nm with buffer solution as blank. Other conditions remained unchanged, the absorbance was measured at 320 nm with distilled water instead of pyrogallol solution (A_2) and with distilled water instead of the ETFQ solution (A_3). The clearance ability was calculated according to the following formula:

$$O^{2-\bullet} \text{ clearance ability} = \frac{A_3 - A_1 + A_2}{A_3} \text{x} 100\% \quad (4)$$

Scavenging of nitrite: To determine the nitrite scavenging ability, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL of ETFQ solution (1.0 g/L) was respectively absorbed and placed in 10 mL test tube. To the tube, 2.5 mL of 10 mg /L $NaNO_2$ were added respectively, and the reaction took place in a water bath at 37°C for 30 min. After reaction completion, 0.5 mL of 0.4% p-aminobenzenesulfonic acid solution and 0.25 mL 0.2% naphthalene hydrochloride was added and mixed well. Seven milliliters of distilled water were added, mixed well, and allowed to stand for 15 min. The A_1 was measured at 538 nm with 50% ethanol as a blank. The A_0 was determined at 538 nm without the addition of the ETFQ under the same conditions. Among them, the background value of the ETFQ was A_i. The clearance ability was calculated according to the following formula:

$$NO_2^- \text{ clearance ability} = \frac{A_0 - A_1 + A_i}{A_0} \text{x} 100\% \quad (5)$$

Scavenging of ABTS$^+$: $\cdot ABTS^+$ was generated by the reaction of a 7 m*M* ABTS aqueous solution with a 2.4 m*M* $K_2S_2O_8$ aqueous solution in molar equivalents, and the mixture was incubated in the dark at 23°C for 12–16 h. The solution was then diluted with ethanol to acquire an absorbance of 0.7 ± 0.02 at 734 nm (A_0). Next, 0.02, 0.04, 0.06, 0.08, 0.10 and 0.12 g/ L of ETFQ were absorbed and placed in a 10-mL test tube. Then 3.9 mL of the abovementioned solution was added, and the reaction took place at 23°C in the dark for 6 min. The absorbance at 734 nm (A) was measured with distilled water as a blank. The clearance ability was calculated according to the following formula:

$$ABTS^+ \text{ clearance ability} = \frac{A_0 - A}{A_0} \text{x} 100\% \quad (6)$$

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ETFQ

According to the types of test bacteria, the sterilized test tubes were divided into four groups, 12 in each group under sterile conditions. In the first test tube, two milliliters of LB liquid medium with two times mass concentration were added. Two milliliters of LB liquid medium were added to the 2–10 test tubes, respectively. Two milliliters of ETFQ were added to the first test tube. According to the double dilution method, the ETFQ mass concentration of each tube was 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.195 and 0.00 mg/mL, respectively. Then 0.1 mL suspension of each strain, which were diluted to 0.5 McFarland turbidity, were added to the 10 test tubes. The 11th tube was used as the positive control, and only 2 mL of medium LB were added. The 12th tube was used as a negative control and 1 mL of medium LB with two times mass concentration and 1 mL ETFQ were added. The abovementioned test tubes were cultured in a constant temperature incubator at 37°C for 24 h. From each of the 12 test tubes, 0.1 mL was taken and the aliquots were spread on the solid LB medium plate and cultured in a constant temperature incubator at 37°C. The concentration of ETFQ with bacterial free in 24 h was determined as the MIC, and that in 48 h was the MBC.

Determination of bacteriostatic rate of ETFQ

The Oxford cup plate assay was used to detect the bacteriostatic rate of ETFQ (Shi *et al.* 2011). The double-layer medium was prepared. Four Oxford cups were placed

in the cooled lower layer, then the upper layer medium (MH) with tested strains, which was diluted to 1 McFarland turbidity standard with physiological saline, was added. Four holes were formed after the medium was cooled. To the four pores, 200 uL of different concentrations ETFQ solution and 0.5% potassium sorbate (positive control) were added. ETFQ were tested at low, medium and high doses, respectively. The dishes were sealed and cultured in a constant temperature incubator at 37°C for 12 h. The diameter of the bacteriostatic zone was measured by crossover method. The antibacterial activity of the tested solutions was evaluated by the diameter of the bacteriostatic zone (A) and the bacteriostatic rate (R), as shown in formula (7).

$$R = \frac{A-c}{B-c} \times 100\% \quad (7)$$

In the formula, R is the bacteriostasis rate (%); A is the diameter/mm of the bacteriostasis circle of the ETFQ; B is the diameter/mm of the bacteriostasis circle of the potassium sorbate; and c is the diameter/mm of the hole formed by Oxford cup.

Results

Effect of system composition of ATPEs on quinoa flavonoids extraction

The effect of system composition on flavonoids was studied by single factor. In a certain range, the extraction rate of flavonoids increased with the concentration of ethanol and ammonium sulfate in the aqueous two-phase system (Fig. 1). The extraction rate of TFQ gradually increased with the increase of ethanol volume fraction (Fig. 1A) and the mass fraction of $(NH_4)_2SO_4$ (Fig. 1B). When the volume fraction of ethanol reached 28% and the mass fraction of $(NH_4)_2SO_4$ was 14%, the TFQ extraction rate and the partition coefficient all reached the maximum. When the volume fraction of ethanol is more than 28% and the mass fraction of $(NH_4)_2SO_4$ is higher than 14%, both the extraction rate and the partition coefficient decreased. Thus, the composition of the two aqueous phase system for TFQ extraction is 14% $(NH_4)_2SO_4$+ 28% C_2H_5OH.

Factors affecting TFQ extraction in aqueous two-phase system

The results show that the main factors affecting the extraction efficiency of the quinoa flavonoids are the concentration of the feed solution, the pH of the system and the concentration of inorganic salts.

In the 28% C_2H_5OH-14% $(NH_4)_2SO_4$ aqueous two-phase extraction system, it showed that the extraction rate of TFQ increased with the increase of crude flavonoids volume fraction (Fig. 2A). The mass fraction of crude extract affects the extraction rate and partition coefficient of C_2H_5OH-$(NH_4)_2SO_4$ aqueous two-phase system. When the mass fraction of crude extract increased, both the extraction rate and the partition coefficient showed similar trends. When the mass fraction of crude extract was 18%, the two values reach the maximum (77.2% and 0.617, respectively). Then the extraction rate and the distribution coefficient gradually decreased.

When the pH < 7, the distribution coefficient and extraction rate increased gradually with the increasing pH (Fig. 2B). When the pH was 7, the distribution coefficient and the extraction rate reached the maximum. When the pH > 7, namely alkaline condition, the distribution coefficient and the extraction rate decreased with the increase of the pH.

Results showed that with the increase of mass fraction of NaCl in the extraction system (Fig. 2C), the partition coefficient and the extraction rate showed the same trend. When the mass fraction of NaCl was 2.5%, the two parameters reached the maximum. Therefore, the mass fraction of NaCl in the extraction system was selected as 2.5%.

Optimization of TFQ extraction by response surface method

The establishment and analysis of mathematical model: The response surface modeling test of TFQ yield was carried out by using Design-Expert 8.0.6 on three factors of crude extract mass fraction (A), NaCl (B) and pH (C). The design of the factor level and the result of the center combination are shown in Table 1.

With the TFQ extraction rate as the response value, the mathematical model equation of the three influencing factors was obtained through the Design Expert 8.0.6 by regression of the three influencing factors. The variance analysis of the mathematical model (Table 2) showed that the regression model was very significant (P =0.0043 < 0.01), and the lack of fit was not significant (P = 0.7042). It showed that the unknown factor has little effect on the TFQ yield, and the model fitting effect was good. The variance analysis of the model showed that A (crude extract) had a significant effect on the extraction rate, while B (NaCl) and C (pH) had no significance. The effect of each factor on TFQ extraction rate was in the order of A (Crude extract) > C (pH) > B (NaCl) mass fraction. By regression model fitting, the influence of three factors on response value Y (TFQ extraction rate) can be expressed by the following multiple quadratic equation: Extraction rate =74.28+1.78 A+0.10 B+0.38 C+0.20 AB+0.050AC+0.050BC+1.000E-002 A^2-0.94 B^2-0.69 C^2.

Interaction response surface of various factors and optimal validation: As shown in the Fig. 3, the response surface of extraction rate opens downward, and the three restrictive factors on extraction rate and aqueous two-phase extraction system show an obvious quadratic parabolic relationship. With the increase of each factor level, the extraction rate of response value also increased. According to the theory of extraction kinetics, with the increase of three

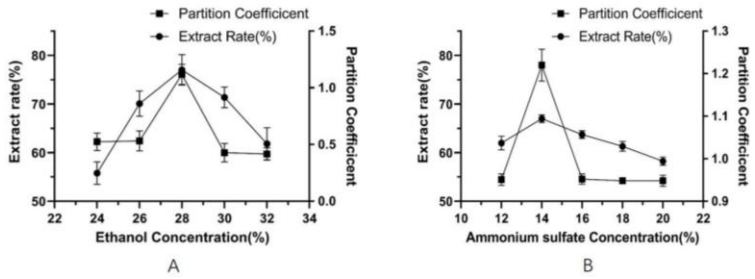

Fig. 1: Effect of system composition of ATPEs on quinoa flavonoids extraction

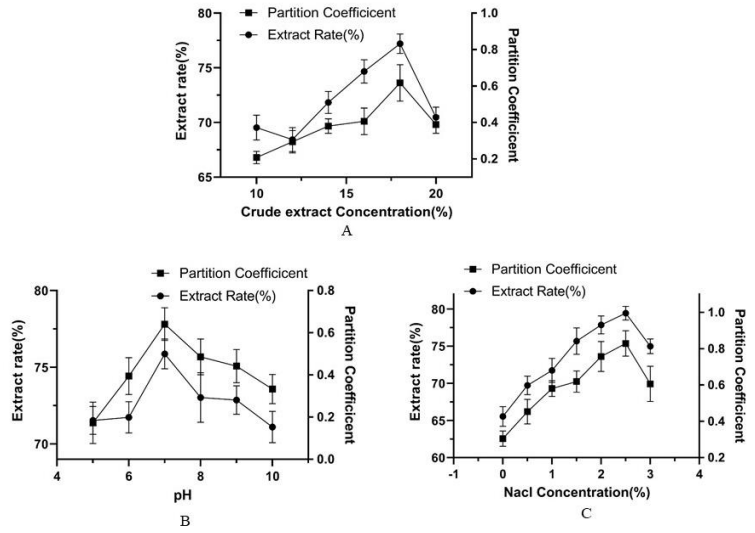

Fig. 2: Factors affecting TFQ extraction in aqueous two-phase system

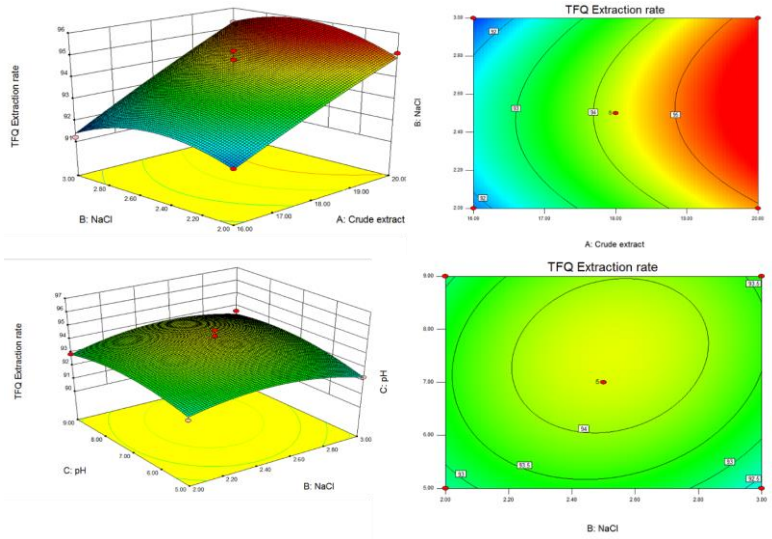

Fig. 3: The interaction effects of three factors on extraction rate of TFQ

factors, the extraction rate of response value reaches its maximum and then decreases with the increase of three factors. The regression model has a stable point and a stable point is the maximum. The extraction rate of TFQ was estimated by means of the multiple quadratic regression model, and the extremum value of the quadratic parabolic function model was analyzed. The best combination coordinates Z (1, 0.29, 0.31) of the three factors was

Table 1: Arrangement and results of response surface methodology

Test number	A: Crude extract (%)	B: NaCl (%)	C: pH	TFQ Extraction rate (%)
1	16	2.0	7	71.8
2	18	3.0	9	73.6
3	20	3.0	7	75.3
4	20	2.5	5	75.1
5	18	2.5	7	75.2
6	20	2.5	9	75.5
7	18	2.5	7	74.8
8	18	2.5	7	73.7
9	18	3.0	5	72.3
10	18	2.0	9	72.9
11	20	2.0	7	75.1
12	18	2.5	7	73.5
13	16	3.0	7	71.2
14	16	2.5	5	71.8
15	16	2.5	9	72.0
16	18	2.5	7	74.2
17	18	2.0	5	71.8

Table 2: ANOVA for Response Surface Quadratic Model for TFQ

Source	Sum of Squares	df	Mean Square	F-Value	p-Value
Model	32.646706	9	3.627412	8.947104	0.0043significant
A-Crude extract	25.205	1	25.205	62.16878	< 0.0001
B-NaCl	8.00E-02	1	8.00E-02	1.97E-01	0.6703
C-pH	1.125	1	1.125	2.774841	0.1397
AB	0.16	1	0.16	0.394644	0.5498
AC	0.01	1	0.01	0.024665	0.8796
BC	0.01	1	0.01	0.024665	0.8796
A^2	0.0004211	1	0.000421	0.001039	0.9752
B^2	3.7204211	1	3.720421	9.176514	0.0191
C^2	2.0046316	1	2.004632	4.944475	0.0615
Residual	2.838	7	0.405429		
Lack of Fit	0.77	3	0.256667	0.496454	0.7042 not significant
Pure Error	2.068	4	0.517		
Cor Total	35.484706	16			

* notes the difference was significant (P < 0.05). **notes the difference was extremely significant (P < 0.01)

predicted, that is, the mass fraction of crude extract was 20.6%, pH 7.18, NaCl 2.23%. Under these conditions, the maximum value of the model was Y = 75.929 3% (*P* = 0.994). Three groups of repeated experiments were carried out in the Z coordinate. The average extraction rate of TFQ reached 75.3%. The results showed that the regression model can accurately predict TFQ extracted by the aqueous two-phase system.

Evaluation of antioxidant in vitro of the ETFQ

Scavenging of hydroxyl radical: The hydroxyl radical belongs to a kind of strong oxidant, and the most active oxygen molecule in the organism. Thus, it is highly destructive (Sander *et al.* 2014). It can react with almost any biological molecule in a living cell to cause damage. Therefore, the elimination of excessive ·OH in the body has a very important biological significance. As shown in Fig. 4A, with the increase in mass concentration, the clearance rates of sample solution and vitamin C to·OH increased. When the concentration of ETFQ in the test solution was 0.3 g/L, the clearance rate of OH was 94.63%. When the mass concentration of vitamin C was 0.6 g/L, the clearance rate of ·OH was 39.9%. Therefore, the ETFQ has a strong scavenging effect on ·OH and its scavenging ability is significantly higher than that of vitamin C.

Scavenging of oxygen free radicals: Superoxide anion can generate other oxygen free radicals through a series of reactions. It can attack cell DNA, and it has high toxicity (Janik and Tripathi 2013). Therefore, it is important to eliminate the excessive O_2^-· in biological organisms. It was shown from Fig. 4B, with the increase in mass concentration, the clearance rates of O_2^- of the ETFQ and vitamin C increase gradually. When the concentration of ETFQ was 0.1 and 0.6 g/L, the scavenging rate of O_2^- was 88.64 and 96.93%, respectively, while the clearance rate for O_2^- was only 22.31% when the vitamin C concentration was 0.1 g/L. The results show that the test solution has strong scavenging capacity for O_2^-, and its scavenging ability is higher than vitamin C at low concentration.

Scavenging of nitrite: Excessive nitrite can affect the functioning of red blood cells, making it impossible for the blood to carry oxygen. In severe cases, the brain is deprived of oxygen and may even die (Fisher *et al.* 2017). Fig. 4C

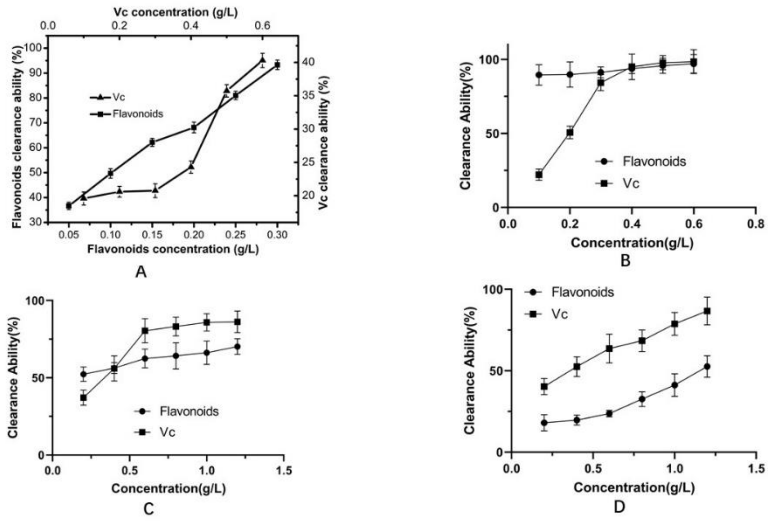

Fig. 4: The antioxidant activity of the ETFQ *in vitro*

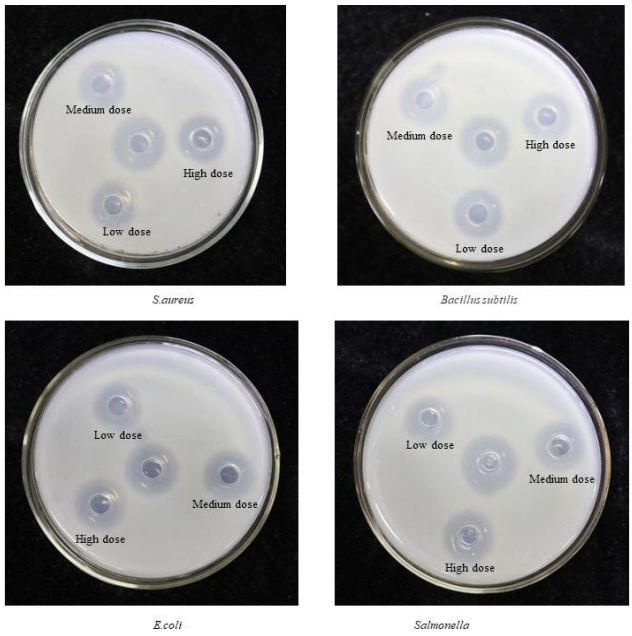

Fig. 5: The bacteriostasis diameter and bacteriostasis rate of ETFQ

shows that with increasing concentration and volume, the clearance of $NaNO_2$ by the flavonoids solution and vitamin C increased gradually. When the concentration of ETFQ was 1 g/L and the volume was 1.2 mL, the scavenging rate of $NaNO_2$ was 66.34%, while the clearance rate of $NaNO_2$ was up to 84.92% when the concentration of vitamin C was 0.1 g/L and the volume was 1.2 mL. The results show that the ETFQ had a certain scavenging effect on $NaNO_2$, but its scavenging ability was weaker than vitamin C.

Scavenging of ·ABTS$^+$: ABTS can be oxidized to green $ABTS^+$ with proper oxidant, and the production of ABTS is restrained when the antioxidant is present (Bora *et al.* 2019). The total antioxidant capacity of the sample can be determined by measuring the absorbance of ABTS. With the increase in concentration, the scavenging rate of ·ABTS+ of the ETFQ and vitamin C increased gradually. When the concentration of ETFQ was 1.2 g/L, the scavenging rate of ·$ABTS^+$ was up to 87.24%, while the clearance rate of vitamin C was only 52.58% (Fig. 4D). The results show that the ETFQ has strong scavenging effects on $ABTS^+$ and its scavenging ability is significantly higher than that of vitamin C.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

As shown in Table 3, the MIC of ETFQ for *Bacillus subtilis* ACCC01430 and *E. coli* is 1.56 mg/mL. The MIC of ETFQ for *S. aureus* ACCC01332 is 6.25 mg/mL and for *Salmonella* ACCC01319 is 12.50 mg/mL. The results show that ETFQ has the strongest inhibitory activity against *Bacillus subtilis* ACCC01430 and *E. coli*; *Salmonella* ACCC01319 exhibited a strong tolerance to the ETFQ.

In vitro bacteriostasis diameter and bacteriostasis rate of ETFQ

As shown in Table 4, ETFQ showed good bacteriostatic activity to all four tested strains, and the tolerance of four tested strains to ETFQ was significantly different among individuals. The diameter of the bacteriostasis circle (Fig. 5) of *Bacillus subtilis* and *E. coli* was 16.64 ± 0.12 mm and 14.28 ± 0.13 mm, respectively, under high dosage. The results were similar to that of the control potassium sorbate. The bacteriostasis rate were 98.44 and 97.59%, respectively. This indicated that the bacteriostasis circle had a strong inhibition on the mycelial growth of *Bacillus subtilis* ACCC01430 and *E. coli* ACCC11864, followed by *S.*

Table 3: MIC and MBC of ETFQ

Strains	MIC (mg/mL)										MBC (mg/mL)
	0	0.2	0.39	0.78	1.56	3.12	6.25	12.5	25	50	
E.coli ACCC11864	-	-	-	-	+	+	+	+	+	+	3.12
S.aureus ACCC01332	-	-	-	-	-	-	+	+	+	+	12.5
Salmonella ACCC01319	-	-	-	-	-	-	-	+	+	+	25
Bacillus subtilis ACCC01430	-	-	-	-	+	+	+	+	+	+	3.12

"+" indicates bacteriostasis; "-" means no bacteriostasis

Table 4: Diameter of inhibition zone and bacteriostasis rate of testing strains exposed to ETFQ

Strains	Diameter of inhibition zone (mm)					Inhibition rate (%)		
	Blank (mm)	Potassium sorbate (0.5%)	Low dose	Medium dose	High dose	Low dose	Medium dose	High dose
E.coli ACCC11864	7. 80±0. 00	14.44±0. 31	13.08±0.31	13.45±0.04	14.28±0.13	79.51	85.09	97.59
S.aureus ACCC01332	7.80±0.00	18.91±0.35	15.71±0.43	16.64±0.33	17.37±0.72	71.20	79.57	86.14
Bacillus subtilis ACCC01430	7.80±0.00	16.78±0.04	15.90±0.11	16.21±0.02	16.64±0.12	91.11	94.6	98.44
Salmonella ACCC01319	7.80±0.00	17.11±0.11	13.21±0.42	13.45±0.37	13.82±0.58	54.26	56.67	60.38

Low dose,3mg/mL; Medium dose, 3mg/mL; High dose, 3mg/mL for *E. coli* and *Bacillus subtilis*; Low dose,15mg/mL; Medium dose, 20mg/mL; High dose, 25mg/mL for *S. aureus* and *Salmonella*

aureus ACCC01332 and *Salmonella* ACCC01319. In a word, the diameter of the bacteriostasis circle of the tested bacteria increased with increasing ETFQ concentration, and the bacteriostasis rate is positively correlated with the concentration of ETFQ.

Discussion

Different solvents were found to have different extraction efficiencies for flavonoids, which exhibited high levels of antioxidant activity. In the aqueous two-phase extraction system composed of ethanol and ammonium sulfate, the environment formed by ethanol and water is favorable for the dissolution of flavonoids. The presence of ammonium sulfate can adjust the charge distribution of the extraction system (Li *et al.* 2010), which is beneficial to the extraction of flavonoids, and the trace amounts of protein in the crude extract can be removed by salting-out of ammonium sulfate. Meanwhile, aqueous two-phase systems are easily scalable and conditions can be controlled, making them suitable for industrial-scale production.

Flavonoids are bioactive compounds that are found in the form of pigments in plant parts, such as fruits and flowers (Carciochi *et al.* 2015). These are synthesized by plants as a response to environmental stress and microbial infections, and are known to have antioxidant, anti-inflammatory and also antimicrobial properties, especially their free radical-scavenging ability (Kalogeropoulos *et al.* 2009). Antioxidant activity is largely attributable to the amount of phenolic compounds, including flavonoids, especially in the dark color quinoa seeds (Hirose *et al.* 2010).

In this study, the ETFQ showed different degree of clearance ability to four kinds of free radicals, as shown in Fig. 4. There is a good correlation between the ETFQ contents and the radical-scavenging abilities, except for nitrite. The predominant flavonoids in quinoa samples were quercetin and kaempferol while in some varieties myricetin and isorhamnetin were also found. The dark color seeds may contain mostly quercetin and isorhamnetin with smaller amounts of myricetin, kaempferol and rhamnetin (Repo-Carrasco-Valencia *et al.* 2010). Thus, the ETFQ showed a broad spectrum of antimicrobial activity against gram-positive and gram-negative bacteria might due to the high content of quercetin and isorhamnetin. Similarly, (Zeng *et al.* 2011) working with water-soluble extract from pine needles, which is rich in flavonoids, have activity against microorganisms. The antimicrobial activity was present in the ETFQ might explain its broad-spectrum activity against microorganisms (Fig. 5). Similar observations were reported (Shan *et al.* 2007) regarding the correlation between total flavonoids and the antibacterial activity of various plant extracts.

Although ETFQ showed good bacteriostatic activity against four strains of testing bacteria, especially *Bacillus subtilis* and *E. coli*, the specific components of flavonoids that play an antibacterial role cannot be determined in this paper. Flavonoids generally refer to a series of compounds in which two benzene rings (A- and B-rings) with phenolic hydroxyl groups are connected to each other through a central three-carbon atom. The basic core is 2-phenylchromogen. Subsequent in-depth research should be conducted on ETFQ and the specific antibacterial flavonoid components should be identified. Additionally, the mechanisms of how ETFQ affects Gram-negative and -positive bacteria require further study.

Conclusion

Quinoa flavonoids are a kind of food flavone, which has the unique resource advantage of being developed into natural functional foods. Aqueous two-phase system significantly improved the antioxidant and antibacterial properties of quinoa flavonoids. This experiment can also carry out further in-depth research: structural identification and physiological activity experiments on quinoa flavonoids to

obtain products with known structure and function, so that to develop the medicinal value of quinoa as much as possible and to increase its economic value.

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Author Contributions

Xiaoyong Wu and Yanxia Sun conceived and designed the experiments; Enze Liu, Yan Wan and Qi Wu performed the experiments; Dabing Xiang and Changying Liu analyzed the data; Xiaoyong Wu and Enze Liu wrote the paper; Yanxia Sun reviewed the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

All data, models, and code generated or used during the study appear in the submitted article.

Ethics Approval

All studies involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Hebei Normal University of Science and Technology, China. Procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Council of the People's Republic of China, 2011). Animals were humanely sacrificed as necessary to ameliorate suffering.

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