Industrial Crops and Products 66 (2015) 229–238

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop

Simultaneous microwave/ultrasonic-assisted enzymatic extraction of antioxidant ingredients from *Nitraria tangutorun* Bobr. juice by-products

Dongmei Wu[a,1], Tingting Gao[b,c,1], Hongxia Yang[b,e], Yuzhi Du[b,e], Cen Li[b,e], Lixin Wei[b,e], Tongyong Zhou[d], Jingmei Lu[a,**], Hongtao Bi[b,c,*]

[a] School of Life Sciences, Northeast Normal University, Changchun 130024, China
[b] Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
[c] Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China
[d] Institute of Scientific and Technical Information of Qinghai, Xining 810000, China
[e] Key Laboratory of Tibetan Medicine Research, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China

ARTICLE INFO

Article history:
Received 2 October 2014
Received in revised form 9 December 2014
Accepted 23 December 2014
Available online 9 January 2015

Keywords:
Simultaneous microwave/ultrasonic assisted enzymatic extraction
Nitraria tangutorun Bobr. juice by-products
Antioxidant extract
UVB-oxidative phototoxicity protection
Doxorubicin-oxidative cardiotoxicity protection

ABSTRACT

By-products originating from food processing are a considerable disposal problem for the food industry. Because of the absence of specifically effective processing technology, huge quantities of by-products are often abandoned as rubbish and prone to microbial spoilage. Given this, a simultaneous microwave/ultrasonic assisted enzymatic extraction (SMU-AEE) method was established for the first time, and performed for antioxidant ingredients extraction from *Nitraria tangutorum* juice by-products (NJB) in the present study. Its experimental conditions were optimized by single factor test and response surface methodology (RSM), and gave the corresponding response values for antioxidant capacity of NJB extract (NJBE) of 219.73 ± 7.03 mg TE/g, which was 27.62%–190.23% higher than those obtained by traditional extraction methods. Chemical composition assay suggested that the increasing of antioxidant capacity of NJBE by SMU-AEE was because of the improvement of extraction efficiency of antioxidant ingredients from NJB, including phenols, flavonoids and anthocyanins. Furthermore, oxidative injury protection ability assay showed that NJBE was good at protecting cells from UVB-oxidative phototoxicity and doxorubicin-oxidative cardiotoxicity, and its protecting ability surpasses or approaches to that of grape seed extract (GSE, the positive control drug), indicating its good potential to be a natural antioxidant in food and pharmaceutical industries.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

By-products originating from food processing are a considerable disposal problem for the food industry because these waste streams emerge in huge quantities and are often prone to microbial spoilage. However, at the same time by-products constitute a rich but yet underutilized source of valuable components, which may find application as ingredients in the food, feed, cosmetics, and pharmaceutical industries (Yu et al., 2014). As a result, numerous projects are currently directed toward the utilization of by-products such as olive oil mill waste, grape pomace, potato peels, brewerís spent grains, and residues from processing of exotic fruits, oilseeds, legumes, cereal crops, meat, and seafood, to name just a few (Fu et al., 2014; Gobi and Vadivelu, 2013; Górnaś et al., 2014; Lasekan et al., 2013; Sullivana et al., 2013). Despite intense research activities during the past two decades, economically feasible processes have been established only for very few by-products, i.e., cheese whey and olive mill waste (Gobi and Vadivelu, 2014; Minhalma et al., 2007). To our best knowledge, there is no targeting effective process for antioxidant ingredients extraction from berry juice by-products. Indeed, experience has shown that the investigation of targeted applications plays a key role for the

* Corresponding author at: Qinghai key laboratory of Tibetan Medicine Pharmacology and Safety Evaluation, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810008, China. Tel.: +86 971 6143765; fax: +86 971 6143765.
** Corresponding author at: School of Life Sciences, Northeast Normal University, 5268 Renmin Street, Changchun 130024, Jilin Province, China. Tel.: +86 431 85282363; fax: +86 431 85691355.
E-mail addresses: jingmlu@163.com (J. Lu), bihongtao@hotmail.com (H. Bi).
[1] These authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.indcrop.2014.12.054
0926-6690/© 2014 Elsevier B.V. All rights reserved.

commercialization and establishment of relevant products in the market.

Nitraria tangutorum Bobr. is such a very typical example. In Qinghai–Tibet plateau, *N. tangutorum* Bobr. fruit is one of the lots of native fork medicines used to alleviate fatigue caused by oxygen deficiency for thousands of years (Dierma, 2012). Recently, some studies showed that *N. tangutorum* fruit contains abundant alkaloids and flavones including anthocyanins, allantoin, 1-methyl-1,2,3,4-tetrahydrobeta-carboline-3-carboxylic acid (MTCCA), quercetin and polysaccharide (Ni et al., 2013; Suo and Wang, 2010; Wang et al., 2007), and its extract had high FRAP values and noticeable effects on the scavenging free radicals and prolonging loading swimming time (Ni et al., 2013; Zheng et al., 2011). Qinghai–Tibet plateau has abundant resource of *N. tangutorum*. Only in Qaidam Basin (in Qinghai Province, China), there are almost 1000 km^2 of *N. tangutorum*, and the available fresh fruits are 1.5–2.5 $\times 10^5$ tons per year (Yang et al., 2013). Because of its good antioxidant and anti-fatigue activities, more and more *N. tangutorum* fruit has been processed into functional health drink, while resulting a large amount of by-products (35%~40% of fresh fruit, including peels and seeds). Because of the absence of effective processing technology, juice by-products of *N. tangutorum* fruit is being abandoned as rubbish at present.

In view of the above development, a simultaneous microwave/ultrasonic assisted enzymatic extraction (SMU-AEE) method was established for the first time, and its conditions were optimized for antioxidant ingredients extraction from *N. tangutorum* juice by-products (NJB) by using single factor test and response surface methodology (RSM) in the present study. Furthermore, the chemical composition and cell oxidative injury protection ability of *N. tangutorum* juice by-products extract (NJBE) were evaluated to assess the importance as a potential source of natural antioxidant in food and pharmaceutical industries.

2. Materials and methods

2.1. Plant material and chemicals

The fresh fruits of *N. tangutorum* Bobr. were collected from Dongshangen of Dulan Country (N36.321, E98.111; 3100 m altitude), Haixi national municipality of Mongol and Tibetan, Qinghai, China, and identified by Prof. Xuefeng Lu, Northwest Plateau Institute of Biology, Chinese Academy of Sciences. *N. tangutorum* fresh fruits were washed by purified water, and then juiced by a screw juice extractor (JYZ-E16, Joyoung, China). The *N. tangutorum* juice by-products (NJB) were collected and freeze–dried. The lyophilized NJB were grounded, passed through a 0.5-mesh sieve, and stored at −20 °C until extraction.

2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) in the crystallized diammonium form, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), peroxidase (HRP), gallic acid, quercetin, cyanine-3-glucoside and Folin–Ciocalteu reagent were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). Celluclast 1.5 L was from Novozymes (China) Investment Co., Ltd. Doxorubicin (DOX) was from Shanghai Sangon Biotech, China. Grape seed extract (GSE) was purchased from Tianjin Jianfeng Natural Product R&D Co., Ltd. (lot number: 002-1401001-02, 99.04% of polyphenols content). All other reagents were of analytical grade made in China.

2.2. Simultaneous microwave/ultrasonic assisted enzymatic extraction (SMU-AEE)

2.2.1. Solvent extraction

Different solvents were used for antioxidant ingredients extraction from NJB, including methanol, ethanol, and acetone. Methanol, ethanol, and acetone were used in the pure form (absolute) and also in mixtures with water to obtain different concentrations.

For the extractions, 1 g of dried NJB powder was mixed with 10 mL of solvent in a glass flask, which were duly covered to avoid solvent loss, and placed in a microwave/ultrasonic synergistic extraction apparatus (XH-300B, Beijing Xianghu Science and Technology Development Co., Ltd. China). The reaction mixture was simultaneously irradiated at 300 W of microwave power and 600 W of ultrasonic power at a temperature of 60 °C for a fixed reaction time of 20 min. Upon completion of the treatment, the resulting mixture was centrifuged at 10,000 rpm for 15 min. The supernatant was collected and vacuum dried at 30 °C, generating *N. tangutorum* juice by-products extract (NJBE). NJBE was dissolved in 10 mL of water, and then purified on an AB-8 macroporous resin column by successively elution with water and ethanol. The ethanolic eluate was collected and vacuum dried at 30 °C, and redissolved in water for antioxidant assay.

2.2.2. SMU-AEE procedure

1 g of dried NJB powder was mixed with 3~9 mL phosphate buffer (0.1 M, pH 3.0~6.0) and 1~10 mg Celluclast 1.5 L. The mixture was incubated at 45 °C for 20 min in a rotary shaker at 160 rpm. Then, absolute ethanol was added to the mixture until its ethanol concentration reaching to 70%. The reaction mixture was simultaneously irradiated at 100~600 W of microwave and 200~1200 W of ultrasonic at the temperature of 50~90 °C for 10~60 min. The resulting mixture was treated following the procedure described as the Section 2.2.1.

2.2.3. The experimental design for response surface methodology (RSM)

To analyze and optimize the influence of four independent parameters for SMU-AEE, namely: pH value of solvent (X_1, 4~5), temperature for extraction (X_2, 60~70 °C), extraction time (X_3, 30~50 min) and microwave power (X_4, 300~500 W) on Trolox equivalent antioxidant capacity (TEAC) of NJB extract, a 4^3 factorial experimental design with response surface methodology (RSM) was used. The mean values of TEAC (Y, dependent parameter) obtained from the triplicate experiments were fitted to a quadratic polynomial model which reads as follows:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad (1)$$

where, Y is the predicted response (TEAC of NJB extract in mg TE/g), β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, while X_i and X_j are the independent variables. The adequacy of the model was expressed by the coefficient of determination (R^2) and adjusted R^2, while the F-test and p-value were used to check the significance of the regression coefficient. Finally, in order to determine the adequacy of the fitted model, the experimental and predicted values were compared.

2.3. Chemical characterization of NJBE

2.3.1. Total phenols

Total phenols were determined by using the Folin–Ciocalteau reagent according to the colorimetric method described by Singleton and Rossi, 1965, adapted for use in a 96-well microplate. Briefly, 5 μL of the filtered extracts duly diluted in the same solvent for extraction were mixed with 60 μL of sodium carbonate solution (7.5% w/v) and 15 μL of Folin–Ciocalteau reagent in a 96-well microplate. Then, 200 μL of distilled water was added and mixed. After standing for 5 min at 60 °C, the samples were allowed to cool at room temperature, and the absorbance was measured at 700 nm in a spectrophotometric microplate reader (EnSpire® Multimode

Plate Readers, PerkinElmer Inc., USA), using each respective solvent for extraction as blank. A calibration curve was prepared using standard solution of gallic acid (200–3000 mg/L). The total phenols content was expressed as milligram gallic acid equivalent per dry weight of extract (mg GAE/g).

2.3.2. Flavonoids

Flavonoids were quantified by colorimetric assay (Chang et al., 2002). Briefly, 30 μL of the filtered extracts duly diluted in the same solvent for extraction were added to 90 μL methanol in a 96-well microplate. Subsequently, 6 μL aluminum chloride (10% w/v), 6 μL 1 M potassium acetate and 170 μL distilled water were sequentially added to the mixture. After 30 min of reaction, the absorbance of the mixture was measured at 415 nm, using each respective solvent for extraction as blank. A calibration curve was prepared using standard solution of quercetin (25–200 mg/L). The total content of flavonoids was expressed as milligram quercetin equivalent per dry weight of extract (mg QE/g).

2.3.3. Determination of total anthocyanin contents (TAC)

The spectrophotometric pH singe and differential methods were used to determine monomeric and total (monomeric plus condensed) anthocyanins in NJBE (Nicoué et al., 2007). Two probes of 1.5 mL extract solutions were transferred to volumetric flasks of 10 mL which were filled up to a mark with buffers of pH 1 and pH 4.5 for each probe. After allowing 15 min standstill, the absorbance at 520 nm and 700 nm was measured. The concentration of total anthocyanins was expressed as cyanine-3-glucoside equivalent (mg CGE/g) and was calculated according to:

$$C_{\text{tot}} = \frac{A_{\text{tot}} M}{\epsilon l} \quad (2)$$

$$A_{\text{tot}} = (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH}=1} \quad (3)$$

where, A_{tot}—the total solution absorbance, $A_{520\,\text{nm}}$—the solution absorbance at pH 1 and 520 nm, $A_{700\,\text{nm}}$—the solution absorbance at pH 1 and 700 nm, M—the molecular mass of cyanine-3-glucoside (449.2 g/mol), ϵ—the molar absorption coefficient of cyanine-3-glucoside, and l—the optical path (10 mm).

2.4. Total antioxidant capacity assay

The method was based on the extracts radical scavenging activity against stable $ABTS^{\bullet+}$, compared with that of Trolox, according to the procedure described as Labrinea and Georgiou (2004), with slightly modification. Each extract was dissolved in distilled water immediately before analysis. ABTS, H_2O_2, and HRP stock solutions (respectively at concentrations of 20, 20, and 1.5 mM) were prepared in 0.02 M phosphate buffer (pH 7.4), and stable for over a month at 0∼4 °C. This reaction solution (1000 μL, contained 2 mM ABTS, 15 μM H_2O_2, 0.25 μM HRP) was mixed with the extract solution (10 μL) (or blank) and vortexed, and then kept in the dark for 5 min at 25 °C. The decrease in absorption was measured at 530 nm. Trolox was used as the standard reference antioxidant at six different concentrations, and results were expressed as TEAC in mg of Trolox per gram of extract on dry weight basis (mg TE/g).

2.5. Cell oxidative injury protection assay

Rat cardiac H9c2 cells and HS68 cells were obtained from American type culture collection (ATCC), USA. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum (BS), 2 mM glutamine and 1% streptomycin/penicillin on 100 mm plastic culture dishes (BD Falcon, USA), at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed every 2–3 days. Cells were incubated up to about 24 h and grown to about 80% confluence before experiments.

Cells were treated with 0, 10, 20, or 40 μg/mL NJBE or 40 μg/mL GSE or vehicle for 24 h prior to oxidative injury. H9c2 cells were then exposed to 10 μg/mL DOX for 24 h for being injured by oxidative cardiotoxicity, and HS68 cells were then exposed to a 120 mJ/cm^2 dose of UVB light (Philips TL 20 W/12RS fluorescent sun lamp with an emission spectrum between 285∼350 nm and peak at 310∼315 nm, Amsterdam, Holland) for being injured by oxidative phototoxicity. The same volumes of corresponding solvents were added to the controls. Cell viability was determined using the MTT assay (Song et al., 2012).

2.6. Statistical analysis

Data are reported as the mean ± SD of three measurements. Design-Expert (Version 8.0, Stat-Ease) was used for the experimental design and the regression analysis of experimental data. Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc comparisons. A P-value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Effect of SMU-AEE conditions on antioxidant capacity of NJBE

3.1.1. Solvent

To evaluate the effects of different extraction solvents (methanol, ethanol, acetone, and their aqueous solutions) on the antioxidant ability of NJBE, the other factors were fixed and set as follows: microwave power 300 W, ultrasonic power 600 W, extraction time 20 min, extraction temperature 60 °C and solvent to raw material 10:1 (w/w). As shown in Fig. 1A, the antioxidant capacities of extracts varied significantly ($P < 0.05$) among each solvents, and peaked at 70% ethanol aqueous solution which was considered in the BBD experiment.

To evaluate the effects of key factors of SMU-AEE procedure on antioxidant capacity of NJBE, single factor test was carried out, and the fixed parameters were set as the follows: phosphate buffer (pH 5.5, 0.1 M), 0.6% added content of cellulase, the solvent to NJB ratio 10:1, extraction temperature 60 °C, extraction time 30 min, microwave power 300 W and ultrasonic power 600 W. The data were subjected to analysis of variance by general liner model (GLM) in software SPSS 13.0.

3.1.2. PH of phosphate buffer

PH of solvent affects the cellulase activity as well as the extraction efficiency of phenols (Puri et al., 2012). To investigate the effect of pH value of phosphate buffer on antioxidant capacity of NTBE, the pH of phosphate buffer was set at 3.0∼6.0 in the present study. As shown in Fig. 1B, the antioxidant capacity of NJBE increased obviously at pH 3.0∼4.5, reached the peak value at pH 4.5, and then decreased at pH 4.5∼6.0.

3.1.3. Added content of cellulase

In enzyme-assisted aqueous extraction, enzyme concentration is one of the key parameters which influences the extraction efficiency of bioactive compounds, and various for different plant sources (Huynh et al., 2014). In the present experiment, added content of cellulase (Celluclast 1.5 L) was set at 0.1%∼1.0% (wt.%). As shown in Fig. 1C, the antioxidant capacity of NJBE increased obviously with the increasing added content of cellulase, reached the peak value at 0.6%, and remained almost constantly. The result indicated that 0.6% of cellulase could completely destroy the cellulose

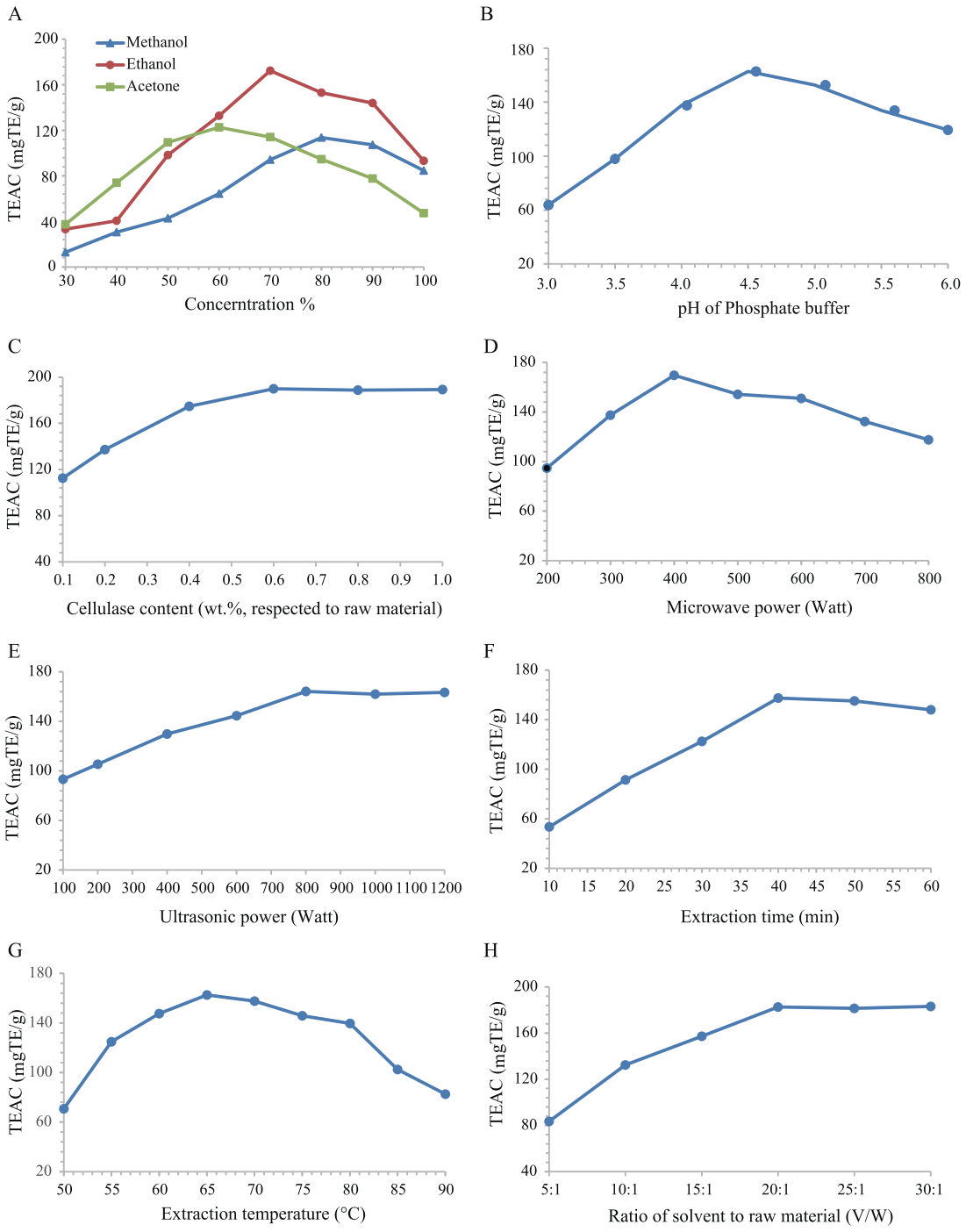

Fig. 1. Effect of SMU-AEE conditions on antioxidant capacity of NJBE.

structure of *N. tangutorum* fruit cell wall to release the intracellular antioxidant components.

3.1.4. Microwave power

The influence of microwave power on the antioxidant capacity of NJBE was examined by varying magnetron power, maintaining the heating conditions in all cases, whereas other experimental conditions were held constant for all of the reactions in this set of experiments. As shown in Fig. 1D, the TEAC of NJBE varied from 94.7 to 169.7 mg TE/g when changing the magnetron power from 200 W to 800 W, and peaked at 400 W. It was suggested that the increasing microwave power could promote the dissolution of antioxidant components of NJBE, until it reached to 400 W. Otherwise, the further increasing microwave radiation will accelerate their degradation.

3.1.5. Ultrasonic power

The optimization of ultrasonic power to the reaction mixture allowed for the achievement of the highest rates of extraction and derivatization from the lowest possible energy input, reducing the cost of sample preparation (Liu et al., 2013). Hence, the effect of ultrasonic power on antioxidant capacity of NJBE was studied. The power was varied from 100 to 1200 W as specified, and other experimental conditions were held constant as described in Section 3.1.1. The result was shown in Fig. 1E. The TEAC of NJBE

was 93.2–164.2 mg TE/g at the tested ultrasonic power, and reached plateau phase at 800 W.

3.1.6. Extraction time

As reported elsewhere, a long extraction time had a favorable effect on the production of antioxidant compounds, such as phenols and vitamins (Vuong et al., 2013). The effect of extraction time (10~60 min) on the antioxidant capacity of NJBE was shown in Fig. 1F. The variance was relatively rapid when extraction time varied from 10 to 40 min, and reached a maximum at 40 min. Then, the antioxidant capacity of NJBE was decreased slowly with increasing time, indicating that some ingredients without antioxidant activity might be acceleratedly dissolved out or the antioxidant ingredients might be decomposed by the further increasing time.

3.1.7. Extraction temperature

Some studies have shown that high temperature can increase solubility of antioxidant compounds such as phenols in the solvent, meanwhile over high temperature can also destroy their structures (Majd et al., 2014). The effect of extraction temperature on the antioxidant capacity of NJBE was investigated, and the result was shown in Fig. 1G. The antioxidant capacity of NJBE exhibited a temperature-dependent manner, and the peaked TEAC value was 162.7 mg TE/g at 65 °C.

3.1.8. Ratio of solvent to raw material

Solvent to raw material ratio, as another important extraction parameter could affect the extraction yield of antioxidant compounds dramatically (Kaiser et al., 2013). If solvent to raw material ratio is too low, antioxidant components in raw material cannot be completely extracted up. On the contrary, if ratio of solvent to raw material is too high, it will lead to a higher process cost and other components without antioxidant activity dissolution. In the present experiment, the solvent to raw material ratio was set at 5:1~30:1. As shown in Fig. 1H, it was founded that the TEAC value of NJBE was 83.6~183.1 mg TE/g, and reached plateau phase at solvent to raw material ratio 20:1.

According to the single-parameter study, four key parameters remarkably affected antioxidant capacity of NJBE were selected to be optimized by RSM, which were adopted for RSM experiment as follows: pH of phosphate buffer 4.5~5.0, extraction temperature 60~70 °C, extraction time 30~50 min, and microwave power 300~500 W. Whereas other experimental conditions were held constant for all of the reactions in this set of experiments as follows: extraction solvent 70% ethanol, 0.6% added content of cellulase, ultrasonic power 800 W, ratio of solvent to raw material 20:1.

3.2. Optimization of key parameters of antioxidant components extraction from N. tangutorum juice by-products

3.2.1. Statistical analysis and the model fitting

The BBD in the experimental design consisted of four factors, three levels and three replicates and the five center point runs were carried out to measure the process stability and inherent variability. The experimental conditions and the results of 29 runs with BBD design were presented in Table 1, and the TEAC of NJBE varied from 89.8 to 218.7 mg TE/g. The results of antioxidant capacity affected by four factors were fitted with a second order polynomial equation, and the values of regression coefficients were calculated. The effects of three variables were highly significant on antioxidant capacity of NJBE (Table 2). The extraction yield value could be expressed by the following second order polynomial equations:

Table 1
Coded and uncoded Box–Behnken design with the experimental result.

Run	pH X_1	Extraction Temperature X_2 (°C)	Extraction time X_3 (min)	Microwave power X_4 (W)	TEAC Y (mg TE/g)
1	4.0	65	50	400	145.9
2	5.0	65	50	400	150.8
3	4.5	60	40	300	129.5
4	4.5	65	40	400	217.5
5	4.5	60	50	400	145.7
6	4.5	70	40	500	171.2
7	4.5	70	40	300	152.4
8	4.0	65	40	300	109.5
9	4.5	65	40	400	217.9
10	4.0	70	40	400	130.1
11	4.5	65	50	300	165.6
12	5.0	65	40	500	153.4
13	4.5	65	40	400	217.3
14	4.0	65	30	400	89.8
15	4.5	65	30	500	137.7
16	4.0	60	40	400	112.1
17	4.5	65	50	500	195.5
18	4.5	60	40	500	149.2
19	4.5	65	30	300	155.9
20	4.5	65	40	400	218.3
21	4.5	65	40	400	218.7
22	5.0	65	30	400	142.7
23	4.0	65	40	500	125.7
24	4.5	70	50	400	190.3
25	5.0	70	40	400	159.6
26	5.0	65	40	300	102.2
27	4.5	70	30	400	165.9
28	4.5	60	30	400	120.5
29	5.0	60	40	400	135.5

$$Y = 217.94 + 10.93X_1 + 14.75X_2 + 15.11X_3 + 9.80X_4 + 1.52X_1X_2 - 12.00X_1X_3 + 8.75X_1X_4 - 0.20X_2X_3 - 0.23X_2X_4 + 12.03X_3X_4 - 57.50X_1^2 - 31.92X_2^2 - 26.38X_3^2 - 33.69X_4^2 \quad (4)$$

where, Y is the TEAC value and X_1, X_2, X_3, and X_4 are the coded values for pH value, extraction temperature, extraction time and microwave power, respectively.

The ANOVA of the quadratic regression model showed that the value of the determination coefficient ($R^2 = 0.9629$). The value of the adjusted determination coefficient (Adj. $R^2 = 0.9258$) was rea-

Table 2
ANOVA for response surface quadratic model.

Source	Sum of squares	df	Mean Square	F Value	Prob > F
Model	37077.91	14	2648.42	25.97	<0.0001**
X_1	1432.27	1	1432.27	14.04	0.0022**
X_2	2610.75	1	2610.75	25.60	0.0002**
X_3	2739.14	1	2739.14	26.86	0.0001**
X_4	1152.48	1	1152.48	11.30	0.0047**
X_1X_2	9.30	1	9.30	0.091	0.7671
X_1X_3	576.00	1	576.00	5.65	0.0323*
X_1X_4	306.25	1	306.25	3.00	0.1051
X_2X_3	0.16	1	0.16	1.569×10^3	0.9690
X_2X_4	0.20	1	0.20	1.986×10^3	0.9651
X_3X_4	578.40	1	578.40	5.67	0.0320*
X_1^2	21448.43	1	21448.43	210.31	< 0.0001**
X_2^2	6607.27	1	6607.27	64.79	< 0.0001**
X_3^2	4513.40	1	4513.40	44.25	< 0.0001**
X_4^2	7362.63	1	7362.63	72.19	< 0.0001**
Residual	1427.81	14	101.99		
Lack of Fit	1426.50	10	142.65	434.91	< 0.0001**
Pure Error	1.31	4	0.33		
Cor. Total	38505.72	28			

$R^2 = 0.9629$; = 0.9258.
* $p < 0.05$.
** $p < 0.01$.

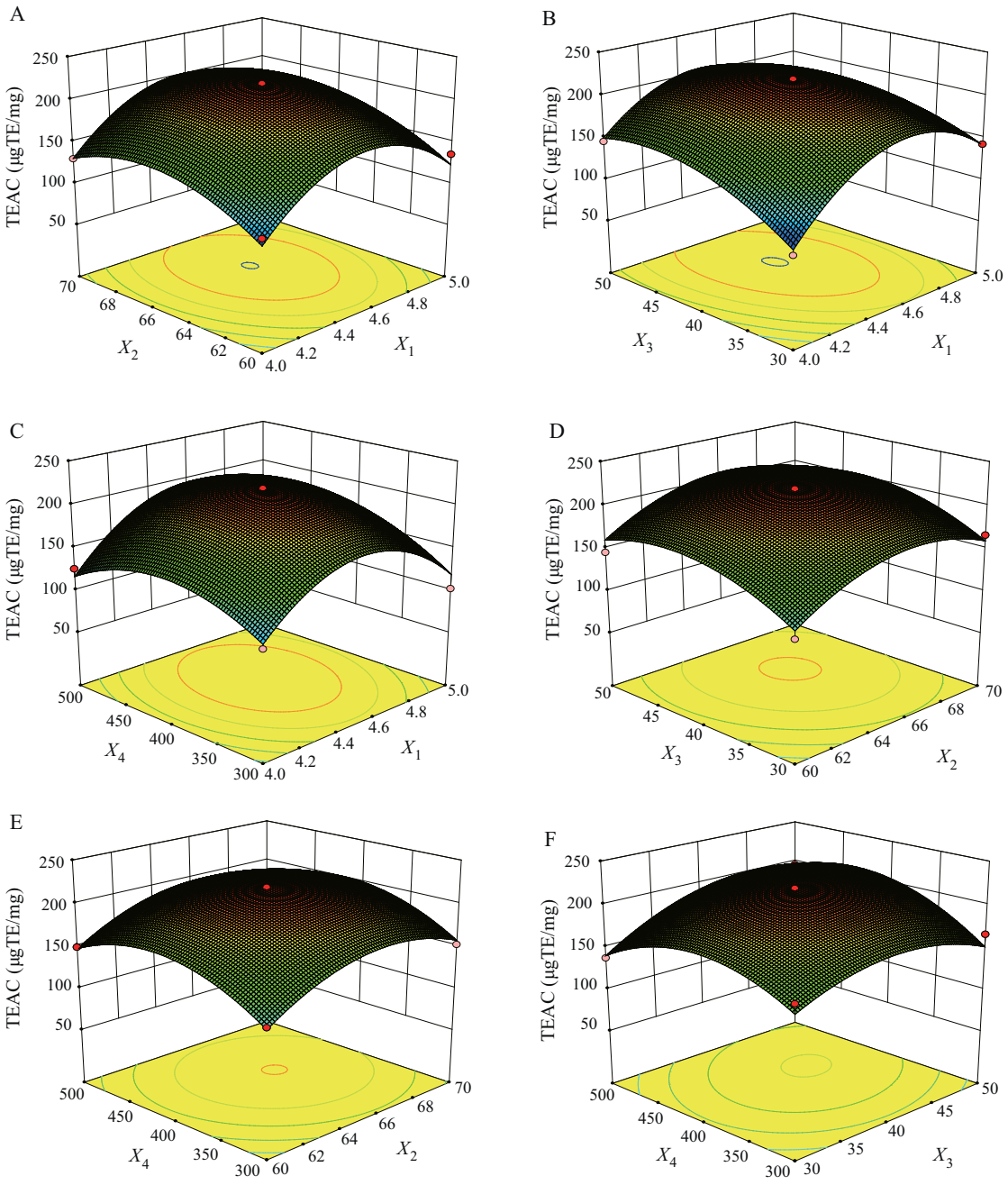

Fig. 2. Response surface plots showing the effect of pH value (X_1), extraction temperature (X_2), extraction time (X_3) and microwave power (X_4) on the antioxidant capacity of NJBE.

sonably close to 1, which indicated a high degree of correlation between the observed and predicted values.

The P values are used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The coefficient estimate for the parameter optimization suggested that the independent variables (X_1, X_2, X_3, and X_4), quadratic terms (X_1X_1, X_2X_2, X_3X_3, and X_4X_4) and cross product coefficient (X_1X_3 and X_3X_4) significantly affected the antioxidant capacity of NJBE ($P < 0.05$). The results of the study showed that extraction time was the most significant single parameter which influenced antioxidant capacity followed by extraction temperature, pH value and microwave power.

3.2.2. Analysis of response surfaces and contours

Eq. (4) allowed the prediction the effects of the four parameters on the antioxidant capacity of NJBE. Six independent response surface plots and their respective contour plots are shown in Figs. 2 and 3. Two variables within the experimental range were depicted in one 3D surface plots while the two other variables were kept constant at zero level. The shapes of the contour plots, circular or elliptical indicated whether the mutual interactions between the variables were significant or not (Muralidhar et al., 2001).

In the case of antioxidant capacity of NJBE, all of pH value (X_1), extraction temperature (X_2), extraction time (X_3) and microwave power (X_4) used had quadratic effects on the antioxidant com-

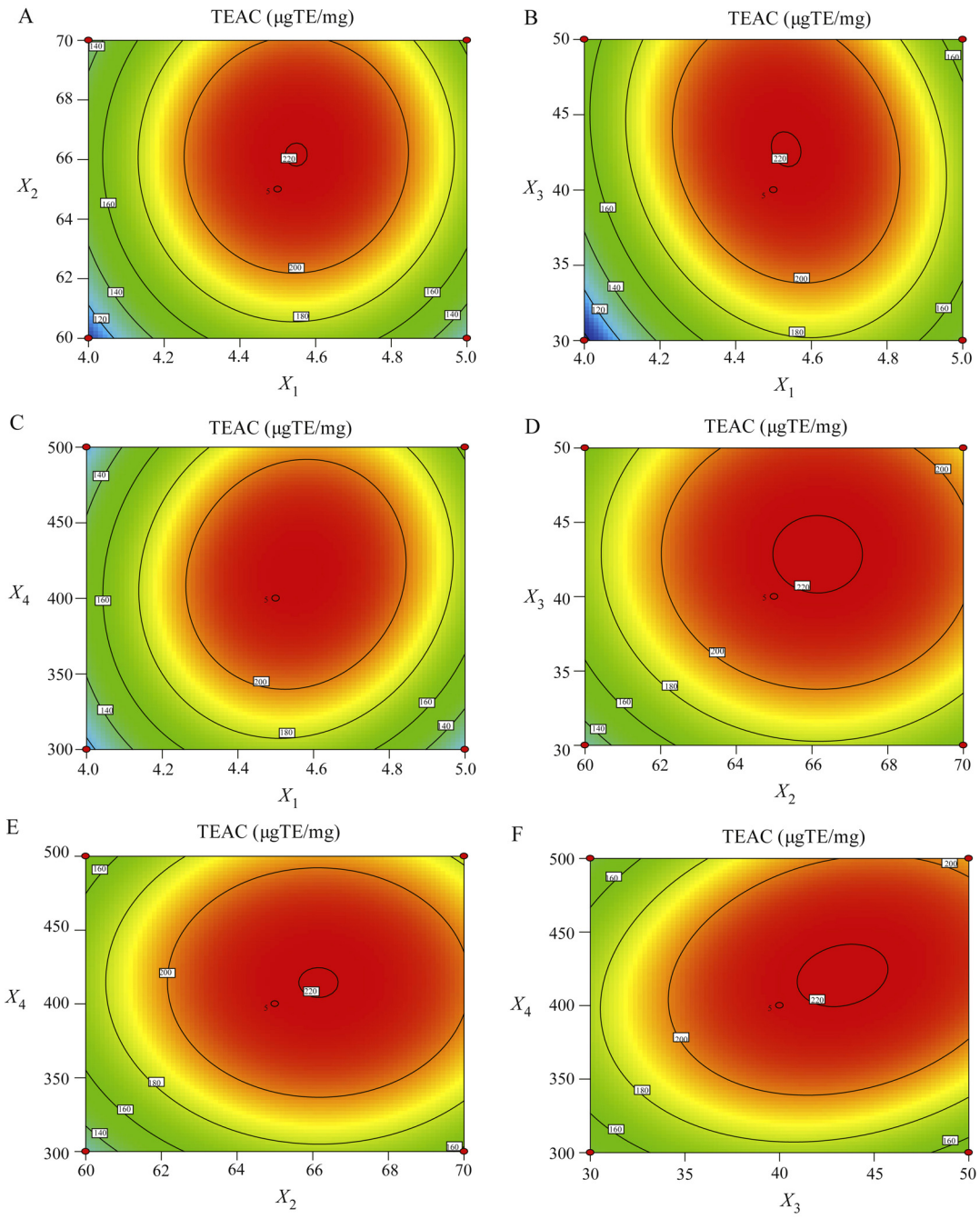

Fig. 3. Contour plots showing the effect of pH value (X_1), extraction temperature (X_2), extraction time (X_3) and microwave power (X_4) on the antioxidant capacity of NJBE.

ponents extraction. When extraction parameter was kept at one level, the antioxidant capacity of NJBE increased with increasing extraction parameter within certain range and then decreased with extended it.

As shown in Table 3, optimal conditions for antioxidant capacity of NJBE were pH value of 4.54, extraction temperature of 66.15 °C, extraction time of 43.16 min, and microwave power of 421.12 W. These conditions gave the corresponding predicted response values for antioxidant capacity of NJBE of 223.51 mg TE/g. To compare the predicted result with experimental values, experimental rechecking was performed for the response using the optimum extraction conditions. Mean values of 219.73 ± 7.03 mg TE/g for antioxidant capacity obtained from real experiments validated the RSM model. The good correlation between these results confirmed that the

Table 3
Predicted and experimental values of antioxidant capacity of NJBE at optimum and modified conditions.

	pH X_1	Extraction Temperature X_2 (°C)	Extraction time X_3 (min)	Microwave power X_4 (W)	TEAC Y (mg TE/g)
Optimum conditions (predicted)	4.54	66.15	43.16	421.14	223.51
Modified conditions (actual)	4.5	66	43	420	219.73 ± 7.03

Table 4
The antioxidant capacities and chemical compositions of NJBEs obtained by different extraction methods.

Extraction method	Antioxidant capacity (mg TE/g)	Total phenols content (mg GAE/g)	Total flavonoids content (mg QE/g)	Total anthocyanin content (mg CGE/g)
SMU-AEE	219.73 ± 7.03	157.54 ± 5.42	101.28 ± 6.84	82.26 ± 4.79
Soxhlet extraction	75.71 ± 5.72***	59.35 ± 3.70***	43.37 ± 4.42***	42.75 ± 2.52***
Soxhlet enzymatic extraction	148.26 ± 6.81***	88.19 ± 5.58***	72.45 ± 3.15**	66.43 ± 3.68**
Ultrasound-assisted extraction	102.44 ± 7.69***	83.22 ± 6.31***	63.51 ± 4.77***	52.17 ± 5.35***
Ultrasound-assisted enzymatic extraction	172.18 ± 5.22**	125.97 ± 7.45**	93.04 ± 7.13*	76.38 ± 3.94*
Microwave-assisted extraction	94.53 ± 10.07***	65.24 ± 9.26***	55.92 ± 5.27***	50.85 ± 4.42***
Microwave-assisted enzymatic extraction	156.94 ± 11.46**	97.46 ± 10.53***	79.63 ± 6.43**	74.91 ± 5.86*

SMU-AEE: simultaneous microwave/ultrasonic assisted enzymatic extraction. The conditions of SMU-AEE were solvent of 70% ethanol, cellulase added content of 0.6% (wt.%, respected to raw material), pH 4.5 of phosphate buffer, ratio of solvent to raw material 20:1, extraction temperature of 66 °C, extraction time of 43 min, ultrasonic power of 800 W and microwave power of 420 W. Other extraction methods were held constant for all of the reactions in this set of experiments as above. Data are mean ± SD (n = 6).
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ compared to the SMU-AEE group.

response model was adequate in reflecting the expected optimization and the model can be used to optimize the process of antioxidant compounds extraction from *N. tangutorum* juice by-products.

3.3. Comparison of the optimized extraction procedure to traditional extraction methods

Antioxidant capacity of NJBE, which could be achieved by the optimized SMU-AEE procedure, were compared with recoveries obtained by classical Soxhlet extraction, ultrasound-assisted extraction and microwave-assisted extraction with or without cellulase. Meanwhile, the chemical compositions were analyzed to reveal the reason for the difference of antioxidant capacity of NJBE among those extraction methods. As shown in Table 4, cellulase, ultrasound or microwave assisted techniques could significantly improve the antioxidant capacity of NJBE compared with classical Soxhlet extraction method. Compared with traditional extraction methods, SMU-AEE method could improve the antioxidant capacity of NJBE by 27.62%–190.23%, indicating that the three assisted techniques had good synergistic effect on antioxidant ingredients extraction from berry juicy by-products. Chemical composition assay (Table 4) showed that the contents of phenols, flavonoids and anthocyanins of NJBE by SMU-AEE were, respectively, 157.54 ± 5.42 mg GAE/g, 101.28 ± 6.84 mg QE/g and 82.26 ± 4.79 mg CGE/g, which were severally 25.06%–141.48%, 8.86%–133.52 and 9.81%–92.42% higher than those by traditional extraction methods. The above results suggested that the increasing of antioxidant capacity of NJBE by SMU-AEE process was due to the improvement of the extraction efficiency of antioxidant ingredients from *N. tangutorun* juice by-products.

3.4. Effect of NJBE on UVB-induced oxidative phototoxicity in human skin fibroblasts

UVB, in particular, is the most hazardous environmental carcinogen known with regard to human health through generation of reactive oxygen species (ROS) (Peres et al., 2011). Recently, our research revealed that photocytotoxicity of UVB was caused by cell lipid peroxidation (Saliou et al., 1999; Song et al., 2012). To exam the effect of NJBE on UVB-induced oxidative phototoxicity in human skin fibroblasts, HS68 cells viability was measured after being treated with NJBE for 24 h and then exposed to a 120 mJ/cm^2 dose of UVB light. As shown in Fig. 4, cell viabilities of NJBE-treated groups (70.83~90.44%) were significantly higher than that of model group (65.74%). Especially, NJBE was significantly better than GSE at the dosage of 40 μg/mL for protecting HS68 cells from UVB-induced oxidative phototoxicity.

3.5. Effect of NJBE on doxorubicin-induced oxidative cardiotoxicity in rat cardiomyocytes

Oxidative stress is now considered a major contributor as a trigger for cardiomyocytes death by apoptosis or cell necrosis (Simunek et al., 2009). In the present study, H9c2 cells were treated with NJBE or GSE or vehicle for 24 h, and then exposed to 10 μg/mL DOX for 24 h. Whereafter, H9c2 cells viabilities were analyzed to evaluate the effect of NJBE on DOX-induced oxidative cardiotoxicity in rat cardiomyocytes. As shown in Fig. 5, cell viabilities of NJBE-treated groups (68.90~85.43%) were significantly higher than that of model group (53.53%). Especially, there was no significant difference between NJBE and GSE at the dosage of 40 μg/mL, indicating NJBE had good ability on protecting cardiomyocytes from DOX-induced oxidative cardiotoxicity.

In recent decades, antioxidative phytochemicals, especially phenolic and flavonoid compounds found in fruits, vegetables and medicinal plants, have received increasing attention due to their potential role in the prevention of human diseases (Cai et al., 2004). However, methods to optimize the extraction efficiency of antioxidant components from the fruits of *N. tangutorun* have not been investigated.

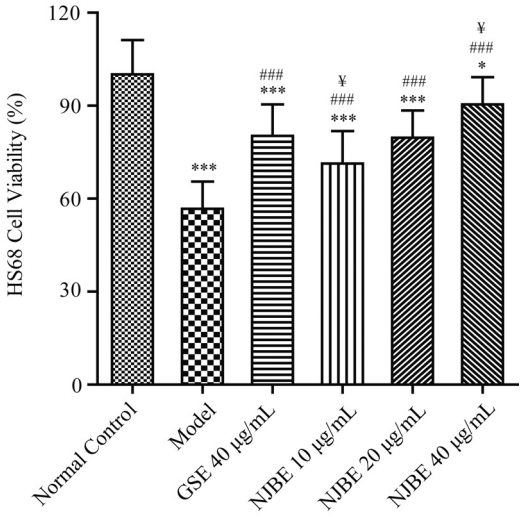

Fig. 4. Effect of NJBE on HS68 cell viability under UVB-induced oxidative phototoxicity.
HS68 cells were treated with NJBE or GSE or vehicle for 24 h, and then exposed to a 120 mJ/cm^2 dose of UVB light. The same volumes of corresponding solvents were added to the controls. Data are mean ± SD (n = 6). GSE: grape seed extract, as positive control drug. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to the normal control group; $^{\#\#\#}P < 0.001$ compared to model group; $^{¥}P < 0.05$ compared to GSE group.

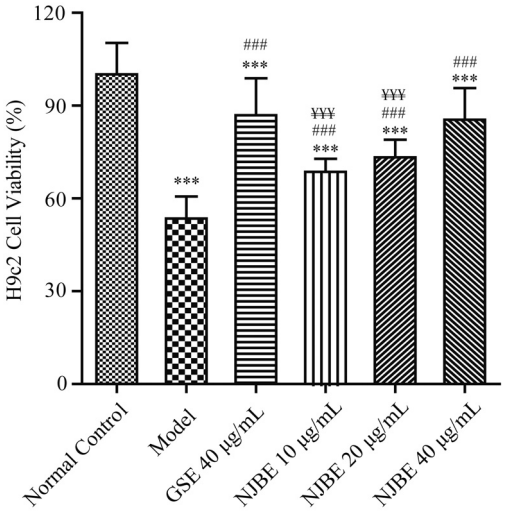

Fig. 5. Effect of NJBE on H9c2 cell viability under doxorubicin-induced oxidative cardiotoxicity.
H9c2 cells were treated with NJBE or GSE or vehicle for 24 h, and then exposed to 10 μg/mL doxorubicin for 24 h. The same volumes of corresponding solvents were added to the controls. Data are mean ± SD ($n = 6$). GSE: grape seed extract, as positive control drug. $^{***}P < 0.001$ compared to the normal control group; $^{\#\#\#}P < 0.001$ compared to model group; $^{¥¥¥}P < 0.001$ compared to GSE group.

Considering the composition of natural sources of phenolic and flavonoid compounds, as well as their structure and physicochemical properties, an universal extraction protocol is not conceivable and a specific extraction procedure must be designed and optimized for each antioxidant source (Kaiser et al., 2013; Liu et al., 2013; Majd et al., 2014; Vuong et al., 2013). Actually, some supplementary methods, such as freeze-thaw, microwave, ultrasonic, hydrolytic enzymes, micro-dynamic ultra-high pressure jet, high voltage electrical discharges and pulsed ohmic heating, have been used to extract antioxidant compounds from plant materials (Boussetta et al., 2013; Darra et al., 2013; Liu et al., 2013; Zheng et al., 2013). After all, the increase in the extract recovery by assistant methods are generally attributed to three aspects: (1) destroying the cell structure, such as enzymatic hydrolysis by cellulase, freeze-thawing, pulsed ohmic heating, ultrasonic and micro-dynamic ultra-high pressure jet; (2) improving solvent penetration into the plant tissue and capillary effects, such as ultrasonic and micro-dynamic ultra-high pressure jet; (3) volumetric heating effect, such as microwave which occurs due to the dipole rotation of the solvent in the microwave field. Moreover, some assistant methods have been applied in combination, such as microwave-ultrasonic synergistic extraction, microwave-assisted enzymatic extraction and ultrasonic-assisted enzymatic extraction. While, to the best of our knowledge, there is no report on simultaneous microwave/ultrasonic-assisted enzymatic extraction (SMU-AEE) method.

In present research, a SMU-AEE method was established for the first time, and applied in antioxidant components extraction from juice by-products of *N. tangutorun* fruits. Among various factors contributing to the recovery of antioxidant compounds from NJB, eight key parameters, including extraction solvent, pH of phosphate buffer, added content of cellulase, extraction time, extraction temperature, the solvent-to-solid ratio, microwave power and ultrasonic power, were optimized by using single factor test and RSM. Compared with traditional extraction methods, the antioxidant capacity of extract by SMU-AEE was 27.62%–190.23% higher, due to the improvement of extraction efficiency of antioxidant compounds, including phenols, flavonoids, and anthocyanins.

Free radicals have been implicated in more than one hundred disease conditions in humans (Halliwell et al., 1992). Antioxidants/free radical scavengers function as inhibitors at both initiation and promotion/propagation/transformation stages of tumor promotion/carcinogenesis, and protect cells against oxidative damage (Bagchi et al., 2002). In order to assess the cell oxidative injury protection ability of NJBE, two oxidative stress cell models were employed: UVB-induced oxidative phototoxicity in human skin fibroblasts and doxorubicin-induced oxidative cardiotoxicity in rat cardiomyocytes. The results showed that NJBE had good ability on protecting cells from oxidative phototoxicity and cardiotoxicity, and its protecting ability surpassed or approached to that of GSE (the positive control drug).

4. Conclusion

In the present study, SMU-AEE method, a combined application of three assisted techniques including microwave, ultrasonic and enzyme extraction, was established for antioxidant ingredients extraction from berry juice by-products for the first time. After optimizing by single factor test and RSM, the experimental conditions for antioxidant ingredients extraction from NJB were established to be 70% ethanol extraction solvent, 0.6% added content of cellulase, pH 4.5 of phosphate buffer, ultrasonic power 800 W, microwave power 420 W, ratio of solvent to NJB 20:1, extraction temperature 66 °C and extraction time 43 min, and gave the corresponding value for antioxidant capacity of NJBE of 219.73 ± 7.03 mg TE/g, which was 27.62%–190.23% higher than traditional extraction methods. Chemical composition assay showed that the contents of phenols, flavonoids, and anthocyanins of NJBE by SMU-AEE were, respectively, 25.06%–141.48%, 8.86%–133.52, and 9.81%–92.42% higher than those by traditional extraction methods, suggesting that the increasing of antioxidant capacity of NJBE by SMU-AEE was the result of the improvement of the extraction efficiency of antioxidant ingredients from NJB. Moreover, NJBE exhibited excellent cell protection effect from UVB-oxidative phototoxicity and doxorubicin-oxidative cardiotoxicity, indicating its good potential to be a natural antioxidant in food and pharmaceutical industries.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (No. 31300291), “The Dawn of West China” Talent Training Program and Applying Basic Research Program of Qinghai province (No. 2014-ZJ-751).

References

Bagchi, D., Bagchi, M., Stohs, S., Ray, S.D., Sen, C.K., Preuss, H.G., 2002. Cellular protection with proanthocyanidins derived from grape seeds. Ann. N. Y. Acad. Sci. 957, 260–270.
Boussetta, N., Lesaint, O., Vorobiev, E., 2013. A study of mechanisms involved during the extraction of polyphenols from grape seeds by pulsed electrical discharges. Innov. Food Sci. Emerg. 19, 124–132.
Cai, Y., Luo, Q., Sun, M., Harold, C., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157–2184.
Chang, C.C., Yang, M.H., Wen, H.M., Chern, J.C., 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 10, 178–182.
Darra, N.E., Grimi, N., Vorobiev, E., Louka, N., Maroun, R., 2013. Extraction of polyphenols from red grape pomace assisted by pulsed ohmic heating. Food Bioprocess Technol. 6, 1281–1289.
Dierma, D., 2012. Crystal Pearl of Materia Medica. Shanghai Scientific and Technical Publishers, Shanghai.
Fu, R., Zhang, Y., Guo, Y., Liu, F., Chen, F., 2014. Determination of phenolic contents and antioxidant activities of extracts of *Jatropha curcas* L. seed shell a by-product, a new source of natural antioxidant. Ind. Crops Prod. 58, 265–270.
Gobi, K., Vadivelu, V.M., 2013. By-products of palm oil mill effluent treatment plant - A step towards sustainability. Renew. Sust. Energ. Rev. 28, 788–803.

Górnaś, P., Rudzińska, M., Segliņa, D., 2014. Lipophilic composition of eleven apple seed oils: a promising source of unconventional oil from industry by-products. Ind. Crops Prod. 60, 86–91.
Halliwell, B., Gutteridge, J.M.C., Cross, C.E., 1992. Free radicals, antioxidants and human disease: where are we now? Clin. Chem. Lab. Med. 199, 598–620.
Kaiser, S., Verza, S.G., Moraes, R.C., Pittol, V., Maribel, E., Penaloza, C., Ortega, G.G., 2013. Extraction optimization of polyphenols: oxindole alkaloids and quinovic acid glycosides from cat's claw bark by Box-Behnken design. Ind. Crops Prod. 48, 153–161.
Labrinea, E.P., Georgiou, C.A., 2004. Stopped-flow method for assessment of pH and timing effect on the ABTS total antioxidant capacity assay. Anal. Chim. Acta 526, 63–68.
Lasekan, A., Abu Bakar, F., Hashim, D., 2013. Potential of chicken by-products as sources of useful biological resources. Waste Manage. 33, 552–565.
Liu, R.L., Song, S.H., Wu, M., He, T., Zhang, Z.Q., 2013. Rapid analysis of fatty acid profiles in raw nuts and seeds by microwave-ultrasonic synergistic in situ extraction-derivatisation and gas chromatography–mass spectrometry. Food Chem. 141, 4269–4277.
Majd, M.H., Rajaei, A., Bashi, D.S., Mortazavi, S.A., Bolourian, S., 2014. Optimization of ultrasonic-assisted extraction of phenolic compounds from bovine pennyroyal (*Phlomidoschema parviflorum*) leaves using response surface methodology. Ind. Crops Prod. 57, 195–202.
Minhalma, M., Magueijo, V., Queiroz, D.P., de Pinho, M.N., 2007. Optimization of Serpa cheese whey nanofiltration for effluent minimization and by-products recovery. J. Environ. Manage. 82, 200–206.
Muralidhar, R.V., Chirumamila, R.R., Marchant, R., Nigam, P., 2001. A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. Biochem. Eng. J. 9, 17–23.
Ni, W., Gao, T., Wang, H., Du, Y., Li, J., Li, C., Wei, L., Bi, H., 2013. Anti-fatigue activity of polysaccharides from the fruits of four Tibetan plateau indigenous medicinal plants. J. Ethnopharmacol. 150, 529–535.
Nicoué, E.É., Savard, S., Belkacemi, K., 2007. Anthocyanins in wild blueberries of Quebec: extraction and identification. J. Agric. Food. Chem. 55, 5626–5635.
Peres, P.S., Terra, V.A., Guarnier, F.A., Cecchini, R., Cecchini, A.L., 2011. Photoaging and chronological aging profile: understanding oxidation of the skin. J. Photochem. Photobiol. B 103, 93–97.
Puri, M., Sharma, D., Barrow, C., 2012. Enzyme-assisted extraction of bioactives from plants. Trends Biotechnol. 30, 37–44.
Saliou, C., Kitazawa, M., McLaughlin, L., Yang, J.P., Lodge, J.K., Tetsuka, T., Iwasaki, K., Cillard, J., Okamoto, T., Packer, L., 1999. Antioxidants modulate acute solar ultraviolet radiation-induced NF-kappa-B activation in a human keratinocyte cell line. Free Radical Bio. Med. 26, 174–183.
Simunek, T., Sterba, M., Popelova, O., Adamcova, M., Hrdina, R., Gersl, V., 2009. Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron. Pharmacol. Rep. 61, 154–171.
Singleton, V.L., Rossi, J.A., 1965. Colorimetry of total phenolics with phosphomolybdic- phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144–158.
Song, J., Gao, T., Ye, M., Bi, H., Liu, G., 2012. The photocytotoxicity of different lights on mammalian cells in interior lighting system. J. Photochem. Photobiol. B 117, 13–18.
Sullivana, P., Arendt, E., Gallagher, E., 2013. The increasing use of barley and barley by-products in the production of healthier baked goods. Trends Food Sci. Technol. 29, 124–134.
Suo, Y.R., Wang, L.Y., 2010. Extraction of *Nitraria tangutorum* seed lipid using different extraction methods and analysis of its fatty acids by HPLC fluorescence detection and on-line MS identification. Eur. J. Lipid Sci. Technol. 112, 390–399.
Vuong, Q.V., Hiruna, S., Roach, P.D., Bowyer, M.C., Phillips, P.A., Scarlett, C.J., 2013. Effect of extraction conditions on total phenolic compounds and antioxidant activities of *Carica papaya* leaf aqueous extracts. J. Herbal Med. 3, 104–111.
Wang, H.L., Wang, X.Y., You, J.M., Suo, Y.R., Zhao, X.N., Li, Y.L., Ding, C., 2007. Comparative analysis of allantoin quercetin and 1-methyl-1, 2,3,4-tetrahydro-beta-carboline-3-carboxylic acid in Nitraria tangutorum Bobr. Seed by HPLC-APCI-MS and CE. J. Liq. Chrom. Rel. Technol. 30, 363–376.
Yang, X., Zhang, H., Tang, X., Liu, Z., Yang, S., Ni, J., 2013. Nitraria resources in China and their utilization. World For. Res. 26, 64–68.
Yu, P.Q., Gamage, I.H., Zhang, X.W., 2014. New approaches and recent advances on characterization of chemical functional groups and structures, physiochemical property, and nutritional values in feedstocks and by-products: advanced spectroanalytical and modeling investigations. Appl. Spectrosc. Rev. 49, 585–602.
Zheng, N., Chen, F., Wang, Z., Lin, J., 2013. Modeling and optimization of artificial neural network and response surface methodology in ultra-high-pressure extraction of *Artemisia argyi Levl. et Vant* and its antifungal activity. Food Anal. Methods, 421–431.