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Original Research Article

Extraction, identification, and antioxidant property evaluation of limonin from pummelo seeds

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ABSTRACT

Limonin, the main bioactive phytochemical constituent of limonoids with multi-functions, is enriched in citrus fruits and often found at a high concentration in citrus seeds. The present study was attempted to introduce a new and efficient extraction method to isolate limonoids from pummelo seeds, and to evaluate the antioxidant property of the main constituent limonin in HepG2 cells. Three key single factors were identified for the extraction of limonoids from pummelo seeds using the Box-Behnken experiment design of response surface methodology (RSM), and the optimized extraction parameters were treatment with 89.68 mL of anhydrous acetone for 4.62 h at 78.94 °C, while the yield of limonoids was 11.52 mg/g. The structure of isolated main constituent of the limonoids was further identified as limonin by Fourier transform infrared (FT-IR) spectrometer and nuclear magnetic resonance (NMR) spectrum. Moreover, the molecular data in HepG2 cells revealed that limonin exerted its anti-oxidant property mainly by the activation of nuclear factor (erythroid-2)-like 2 (Nrf2)/kelch-like ECH-associated protein 1 (Keap1)- antioxidant response element (ARE) pathway in the form of transcriptional regulation of *Nrf2* mRNA and posttranscriptional regulation of Nrf2/Keap1 system. These results demonstrate that pummelo seeds are an ideal source of limonoids, and limonin is proved to exert its anti-oxidant property by the activation of Nrf2/Keap1 pathway.

1. Introduction

Limonoids are a prominent group of secondary metabolites found in the *Rutaceae* and *Meliacea* families and a group of highly oxygenated triterpenoid compounds (Manners, 2007; Roy and Saraf, 2006; Tian et al., 2001; Zhao et al., 2008). Many previous studies had shown that limonoids exhibited a number of biological and pharmacological activities, such as anti-cancer (Tian et al., 2001; So et al., 1996), anti-obesity (Ono, 2011), anti-HIV (Battinelli et al., 2003), anti-oxidant (Sun et al., 2005; Mandadi et al., 2007), antiviral (Ribeiro et al., 2008), and cholesterol lowering (Kurowska et al., 2000) properties. Extraction of limonoids by supercritical carbon dioxide (SC-CO_2) was prevalent in the limonoids extraction field (Patil et al., 2006). Although organic solvents was reduced, sophisticated devices and special expertise were required. Meanwhile, the low yield of limonoids extracted by the method restricted the development in the food industry. Recently, flash extraction (Liu et al., 2012b) and hydrotropic extraction (Dandekar et al., 2008) were reported for limonoids extraction, but these methods are not mature enough to achieve industrial requirements. Therefore, solvent extraction still remains the main method of limonoids extraction at present (Jayaprakasha et al., 2006; Liu et al., 2012; Mandadi et al., 2009; Melwita and Ju, 2010; Vikram et al., 2007). The pummelo is an important and popular citrus species in terms of cultivation and consumption

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around the world. It belongs to the *Rutaceae* family and is a native citrus species in Southern China. However, there is limited information available in literature about the extraction of limonoids from pummelo seeds. The development of new raw sources of limonoids is a great help to satisfy the increasing demand for limonoids, which is with potential health benefits.

Accumulating data have shown that nuclear factor (erythroid-2)-like 2 (Nrf2) can modulate the antioxidant or electrophile response element (ARE/EpRE) and specific nucleotide sequences to promote series of antioxidant genes (Wasserman and Fahl, 1997; Venugopal and Jaiswal, 1996; Hayes and McMahon, 2001). Nuclear factor (erythroid-2)-like 2 is a member of the Cap 'n' collar (CNC) family of bZIP proteins and has extensively been shown to be a crucial activator of antioxidant response element (ARE)-mediated gene expression, such as sulfiredoxin 1 (*SRXN1*), thioredoxin reductase 1 (*TXNRD1*), NAD(P)H dehydrogenase quinone 1 (*NQO1*), and glutathione reductase (*GSR*) (Malhotra et al., 2011; Chorley et al., 2012; Hirotsu et al., 2012). Limonin is the most prevalent member of limonoids, which has been reported to exhibit an antioxidant property and induce the expressions of glutathione S-transferase and quinone reductase (Sun et al., 2005; Jun et al., 2005). However, the effect of limonin on Nrf2/Keap1-ARE signaling pathway and the underlying mechanism remained unclear.

In the present study, a simple and easy solvent extraction method was used to extract limonin from pummelo seeds, and the extraction parameters were optimized by using Box-Behnken experiment design of the response surface methodology (RSM). Moreover, the structure of the extracted limonin crystals was further identified by Fourier transform infrared (FT-IR) and nuclear magnetic resonance (NMR) spectrum. Finally, a liver cell model was used to study the antioxidant property of limonin, associated with its effect on Nrf2/Keap1-ARE signaling pathway.

2. Materials and methods

2.1. Raw material, chemicals, and cell culture

The pummelo fruits were purchased from a local farmers market (Changsha, Hunan, China). Seeds were separated manually, dried, and finely powdered. The limonin standard was purchased from Sigma–Aldrich (St. Louis, MO, USA). Other chemical reagents used were of analytical grade. The HepG2 cells were purchased from ATCC (VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) plus 100 units of penicillin and streptomycin. The antibodies of anti-Nrf2, anti-NQO1, anti-HO-1, anti-GAPDH, and anti-Keap1 were procured from Santa Cruz Biotechnology Inc. (CA, USA). The secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. Isolation and quantification of limonin

The pummelo seeds were milled by a drug pulverizer after drying. Fat in the seeds powder (10.0 g) was removed using a Soxhlet apparatus (Tianbo Corp., Tianjing, China) with 40 mL of petroleum ether at 25 °C for 10 h. The crude limonoids were then extracted from the defatted seeds powder (2.5 g) with acetone at a different time, solvent dosage, temperature, and pH value in a single factor experiment. Limonin was obtained from the crude limonoids on a silica gel column (Changcheng Corp., Zhengzhou, China) with dichloromethane (CH_2Cl_2) and isopropanol. Thin layer chromatography and chemistry color response were used for qualitative and quantitative analyses of limonin.

2.3. Experiment design

Various operation parameters were investigated to extract limonoids. The extraction of limonoids from pummelo seeds was optimized by varying operating parameters according to Box–Behnken design (3^3 factorial). Box–Behnken design is an independent quadratic design in which the treatment combinations are multiples of the edge of the process space and the center. It is known that the extraction efficiency mainly depends on solvent dosage, time, pH, and temperature variations (Toshinao and Hideaki, 2003). Based on the single factor experiment, 3 variables, namely solvent dosage, time, and temperature of extraction were selected for each set of experiments while keeping the pH of extraction (pH = 7) constant throughout the experiments (data not shown). The following 3 variables were selected for the extraction process output, viz. solvent dosage (60, 80, and 120 mL), temperature (70, 80, and 85 °C) and time of extraction (3, 4, and 6 h).

2.4. Identification of limonin

The infrared spectrum of limonin in the range of 4,000 to 400 per cm was analyzed by FT-IR (510-P, Nicolet Corp., USA) using KBr wafers. Purified limonin was identified by ^{1}H- and ^{13}C- NMR spectrometer (AC-80, BRURER Corp, Switzerland) and compared with the limonin standard.

2.5. Total RNA extraction and real-time PCR

HepG2 (1×10^6) cells were pre-cultured in dishes for 24 h and then treated with various concentrations of the purified limonin (>98%, mass/volume) obtained above in 0.1% dimethyl sulfoxide (DMSO), or with 0.1% DMSO alone as a control, for 9 h. Total RNA was extracted with an Isogen RNA Kit (Nippon Gene Co., Toyama, Japan) as described in manufacturer's manual. All primers (5′ to 3′) were designed with the software PRIMER3 and were synthesized as follows: *Nrf2*, forward (AGACAAACATTCAAGCCGCT) and reverse (CCATCTCTTGTTTGCTGCAG); *HO-1*, forward (CCAGCGGGCCAGCAACAAAGTGC) and reverse (AAGCCTTCAGTGCCCACGGTAAGG); *NQO1*, forward (AGTGCAGTGGTGTGATCTCG) and reverse (GGTGGAGTCACGCCTGTAAT); *Keap1*, forward (CCTTCAGCTACACCCTGGAG) and reverse (AACATGGCCTTGAAGACAGG). Reverse transcription and real-time PCR was performed with a DyNAmo SYBR Green 2-Step qRT-PCR kit (Finnzymes Oy., Espoo, Finland) according to the manufacturer's manual. Briefly, RNA (200 ng) was reverse-transcribed to cDNA using Oligo dT and M-MuLV RNase at 37 °C for 30 min, and the reaction was then terminated at 85 °C for 5 min. The sequences of PCR primers and other reaction conditions used in the present study were described by Qin et al (2013). The result was represented by the relative expression level normalized with control cells.

2.6. Immunoblot analysis

The HepG2 (3×10^6) cells were pre-cultured in 100 mm dishes for 24 h and treated with various concentrations of limonin for the indicated periods. After that, the cells were lysed with modified radioimmunoprecipitation assay buffer (RIPA buffer), and protein quantification was performed by a protein assay kit (Bio-Rad Laboratories, CA, USA) as describe by Qin et al, (2013). After harvest, the whole-cell lysates were collected and treated by a normal protocol, and the sample was run by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech). After blotting and antibodies incubation, the membrane was detected using an ECL western blotting system (GE

ImageQuant LAS4000mini model, Fujifilm, Tokyo, Japan) and the relative amounts of specific proteins were quantified using Lumi Vision Image software (TAITEC Co., Saitama, Japan).

2.7. Data analysis

The collected data were analyzed using RSM procedure by SAS 9.0 System for Windows. Data were expressed as means ± SD of at least 3 independent experiments. Student's *t*-tests and Tukey's test were performed to compare the means of 2 groups or selected data sets, and $P < 0.05$ was considered as significant.

3. Results and discussion

3.1. Effect of the single factor on extraction yield

The pH value played a significant role on limonoids yield. The experiments were carried out in following conditions: 100 mL acetone, 80 °C, 4 h, and the pH of 4.0, 5.5, 7.0, 8.5, 10.0, and 12.0, respectively (Fig. 1A). The extraction yield significantly increased with the pH value from 4.0 to 7.0. It reached the maximal 11.23 mg/g at pH 7.0 and significantly decreased beyond pH 7.0. It was only 0.69 mg/g at pH 12.0. The possible reason was that acidic and alkaline conditions can lead to the decomposition of limonoids and ring–opening reaction (Jitpukdeebodintra et al., 2005). Therefore, the neutral condition (pH ≈ 7) was optimum for the extraction and kept constant for all the subsequent experiments.

The solvent concentration was also an important variable. Seven acetone concentrations (acetone:H_2O; vol/vol; 80:0, 75:5, 70:10, 65:15, 60:20, 55:25) were chosen to test the effect of extraction solvent concentration on limonoids yield (Fig. 1B). The total solvent volume is 80 mL, and the experiments were conducted at 80 °C, 4 h, pH 7.0. The limonoids yield significantly decreased with increasing water content. The highest limonoids yield (10.90 mg/g) was obtained with the use of anhydrous acetone. Therefore, anhydrous acetone was chosen as the optimum extraction solvent.

3.2. Optimization of extraction parameters

After the initial study, the effects of the 3 key factors, which were solvent dosage, extraction time and temperature, were optimized using Box-Behnken experiment design of the RSM. The conventional multifactor experiment is time-consuming and ignores the combined interactions among physicochemical parameters, while the RSM can be employed as a useful approach to implement optimal process conditions by performing a minimum number of experiments. Box-Behnken experiment design is an efficient and creative three-level composite design for fitting second-order response surfaces. A total of 15 experiments were conducted to optimize the extraction conditions. Table 1 shows the experimental design and corresponding yield data. The maximal yield of 11.38 mg/g was produced at 80 °C, 4 h and 80 mL. Response surface methodology of the data shown in Table 1 demonstrates that the relationship between limonin yield and extraction parameters was quadratic with very good regression coefficient ($R^2 = 0.989$). The following equation shows the relationship: $Y = -125.1910 + 2.6958X_1 + 0.2930X_2 + 7.4845X_3 - 0.0164\,X_1{}^2 - 0.000069X_1 \times X_2 - 0.00158X_2{}^2 - 0.02353X_1 \times X_3 - 0.00084X_2 \times X_3 - 0.6025X_3{}^2$, in which Y is the extraction yield, X_1 is the extraction temperature, X_2 is the solvent dosage, and X_3 is the extraction time. This equation demonstrated that limonoid yield depended more on extraction time followed by extraction temperature, while solvent dosage was the least effect on the extraction yield.

According to Table 1, the prediction model between the extraction yield and the 3 key factors were produced and illustrated in Fig. 2, which shows the relationship between the RSM generated

Table 1
Extraction of limonoids from pummelo seeds. [1]

Run	Temperature, °C	Acetone, mL	Time, h	Yield, mg/g
1	70	60	4	8.48
2	70	120	4	8.59
3	85	60	4	9.29
4	85	120	4	9.50
5	80	60	3	8.74
6	80	60	6	9.07
7	80	120	3	8.51
8	80	120	6	8.81
9	70	80	3	8.18
10	85	80	3	9.54
11	70	80	6	9.24
12	85	80	6	9.50
13	80	80	4	11.00
14	80	80	4	11.38
15	80	80	4	11.23

[1] Extraction solvent is anhydrous acetone; extraction pH is 7.0.

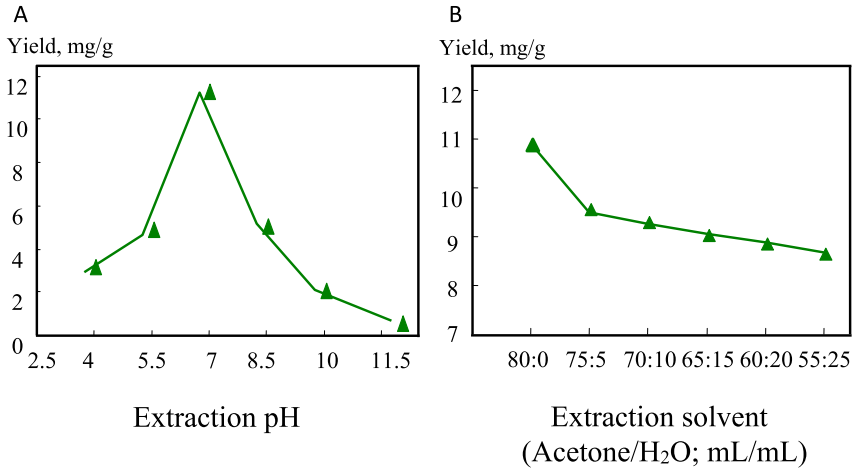

Fig. 1. Effect of extraction pH and solvent on the yield of limonoids.

extraction yield, time, and acetone dosage. The extraction yield increased with the extraction time and acetone dosage. Fig. 2B demonstrates that the extraction yield of limonin sharply improved with increasing extraction time and temperature. In Fig. 2C, it can be seen that the extraction yield increased at first and then decreased with the increase of both temperature and acetone dosage beyond the optimized extraction parameters. These behaviors can be explained by the fact that limonin can easily decompose with the increase of extraction temperature, time and solvent dosage beyond the optimized extraction parameters. By the experimental data and RSM, the predicted maximum yield of limonin was 11.58 mg/g, and the extraction parameters were optimized at 78.94 °C, 89.68 mL anhydrous acetone, and 4.62 h of extraction time. In order to verify the credibility of the optimized extraction parameters, we carried out another experiment using the optimized extraction parameters, and we obtained the extraction yield of 11.52 mg/g, which is close to 11.58 mg/g mentioned above. The results indicated that the optimized extraction parameters are credible. Comparing with the methods and results from other previous studies, the limonoid concentration range in pummelo seeds was only from 2.3 to 4.7 mg/g, by using solvent extraction method and a novel process consisting of water extraction, ammonium sulfate precipitation and resin adsorption (Wang et al., 2016; Yang et al, 2017). Therefore, the concentration of limonin obtained from this study was much higher than those from those studies.

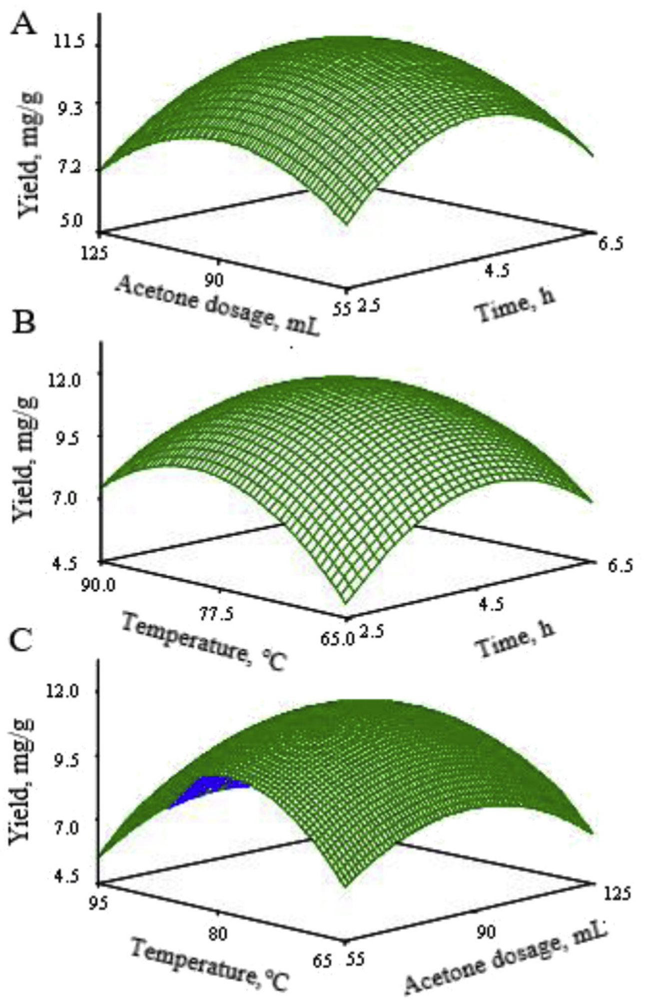

Fig. 2. Box–Behnke experiment result of optimal parameters for the extraction of limonoid.

3.3. Determination of limonin

Limonoids, including limonin, nomilin ichangin, and obacunone, are a group of highly oxygenated, tetracyclic triterpene secondary metabolites derivatives (Roy and Saraf, 2006). Limonin is the most important bioactive limonoid. The purification of limonin from limonoids is a universal and significant process. The detailed purification process is described in the materials and methods. Fig. 3 shows the infrared (IR) spectra of the limonin sample and the chemical structure of limonin. The IR spectra with potassium bromide pressed-disk technique are exhibited (Fig. 3A). The characteristic absorption peaks were: β-furan ring (2,966, 1,065 and 875 per cm), δ-lactone (1,759 per cm), cyclic ether (1,285 per cm), ketone (1,708 per cm), and methyl groups (1,365 and 1,165 per cm). The results of the IR analysis were consistent with the chemical structure of limonin.

To further determine the crystal, ^{1}H -NMR of the crystal was performed. Fig. 4 shows ^{1}H NMR of the crystal sample and the limonin standard. Table 2 lists the ^{1}H -NMR data. The main

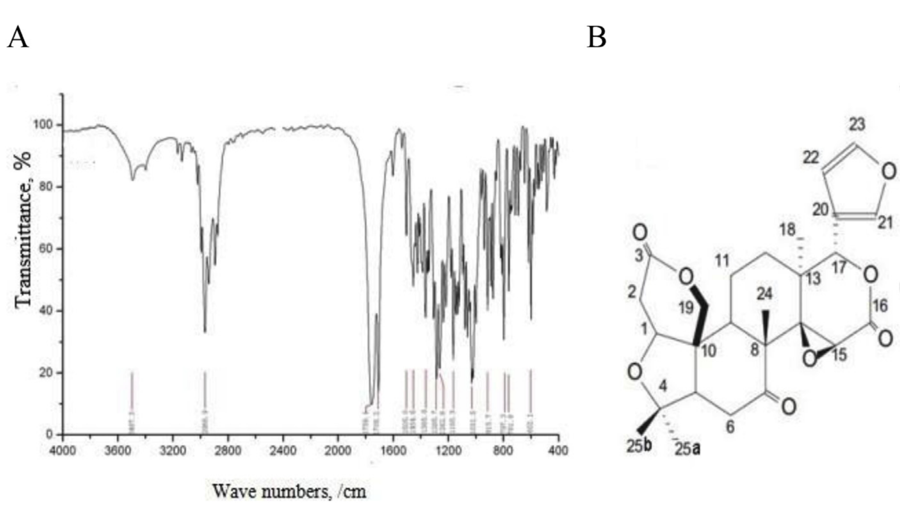

Fig. 3. Infrared (IR) spectra data of the limonin sample (A) and the chemical structure of limonin (B).

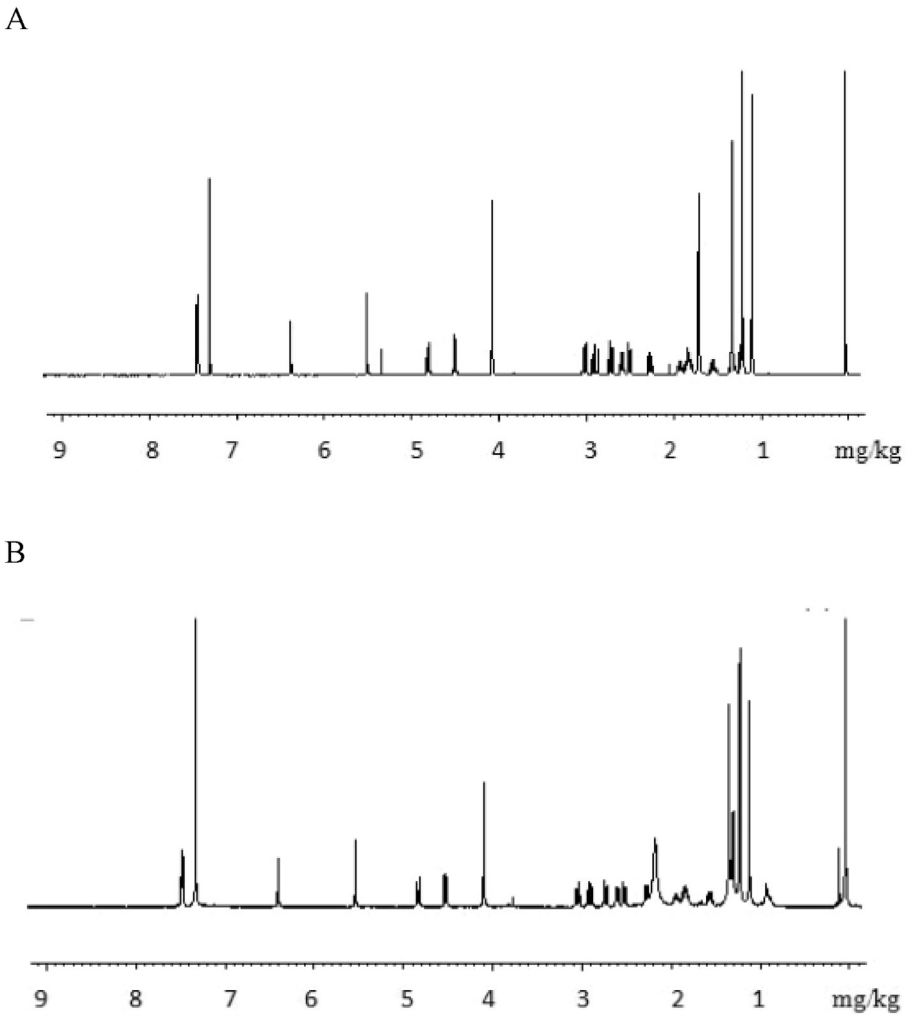

Fig. 4. Proton nuclear magnetic resonance (^{1}H NMR) spectrum data (A) of the crystal obtained versus that of its corresponding standard (B).

characteristic peaks list as follows: H-1 (δ 4.10), H-2a (δ 2.69), H-2b (δ 2.97), H-15 (δ 4.04), H-17 (δ 5.47), H-19a (δ 4.78), H-19b (δ 4.45 to 4.47), H-21 (δ 7.27), H-22 (δ 6.34), H-23 (δ 7.40 to 7.42). Compared with the limonin standard, ^{1}H -NMR spectrum (Fig. 4A) and data (chemical shift δ) of the crystal are in excellent agreement with the standard ones (Fig. 4B). Meanwhile, the ^{1}H -NMR data are consistent with the reference of (Breksa et al., 2008).

3.4. Antioxidant property assay in in vitro level

Indirect antioxidant property of phytochemical seems more attractive than its direct reactive oxygen species (ROS) scavenging ability, which was why we performed *in vitro* test by detecting the expressions of mRNA and protein of typical biomarkers related to Nrf2-ARE pathway in HepG2 cells. As shown in Fig. 5, limonin (5 to 10 μmol/L) enhanced the transcription of *Nrf2* and its downstream genes *HO-1* and *NQO1*, in a dose-dependent manner. Similarly, limonin also stimulated the expressions of *Nrf2*, *HO-1*, and *NQO1*, but had no effect on *Keap1* expression. These results are in accordance with the study of Chen et al (2017), in which limonin 7-deacetylgedunin (7-DGD) was also reported to induce the expressions of *Nrf2* and *HO-1*, at a dose of 25 μmol/L in RAW264.7 cells. Thus, the results obtained here indicated that limonin could

Table 2
Proton nuclear magnetic resonance (^{1}H NMR) data for the purified crystal, limonin standard, and the reference. [1]

Position	Sample	Standard	Reference
1	4.03	4.03	4.03
2a	2.69	2.67	2.67
2b	2.97	2.97	2.98
6a	2.48	2.48	2.46
6b	2.86	2.85	2.85
9	2.54	2.54	2.55
11	1.802	1.77 to 1.89	1.72 to 1.95
12	1.68	1.50 to 1.53	1.46 to 1.58
15	4.04	4.04	4.05
17	5.47	5.47	5.47
18	1.18	1.18	1.18
19a	4.78	4.77	4.76
19b	4.45 to 4.47	4.45 to 4.48	4.46
21	7.27	7.26	7.4
22	6.34	6.34	6.34
23	7.40 to 7.42	7.40 to 7.41	7.41
24	1.07	1.07	1.08
25a	1.30	1.30	1.29
25b	1.17	1.18	1.18

[1] Source: Breksa, Dragull and Wong (2008).

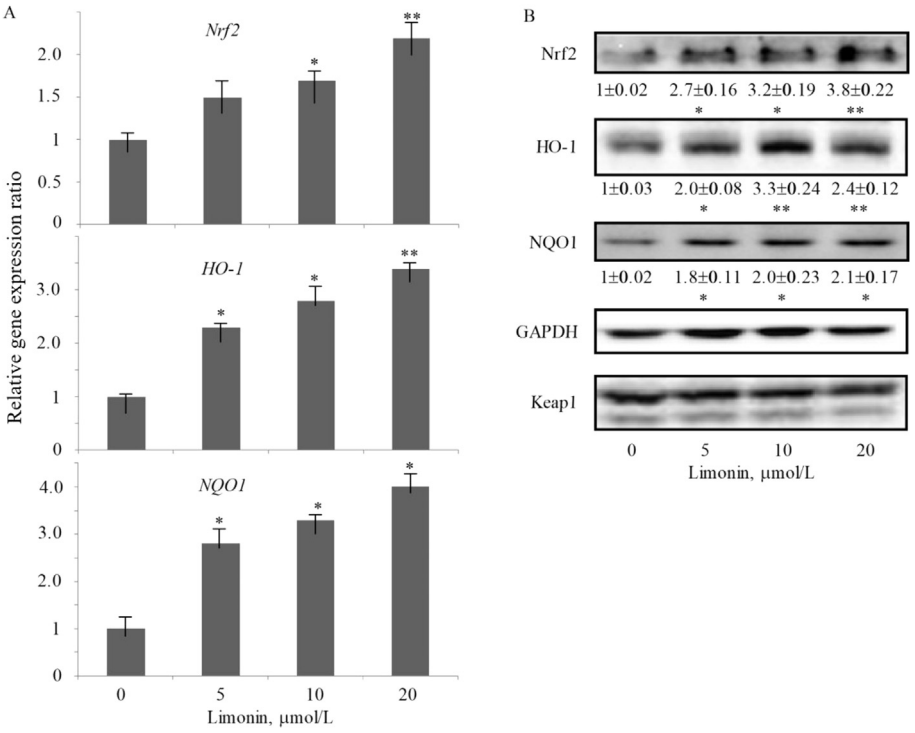

Fig. 5. Effect of limonin on the transcriptional (A, gene expressions) and posttranscriptional of Nrf2-ARE pathway (B, protein synthesis). Significance is marked by star bars. * stands for $P < 0.05$, ** stands for $P < 0.01$.

activate Nrf2-ARE pathway in both transcriptional and post-transcriptional levels.

4. Conclusions

In conclusion, the present study demonstrated that pummelo seeds are a potent source of limonoids. The extraction of limonoids was optimized by using Box-Behnken experiment design of the RSM. The highest yield of limonoids was 11.52 mg/g at 78.94 °C of extraction temperature, 89.68 mL anhydrous acetone, and 4.62 h of extraction time. The isolated limonin crystals were identified by the FT-IR and ^{1}H -NMR spectrum. These results opened a new way to improve the development of deep processing industry of pummelo seeds. Moreover, the *in vitro* data obtained in HepG2 cells by treatment of limonin revealed that pummelo seeds could be deemed as a potential candidate for dietary source with potent antioxidant property.

Conflicts of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work; there is no professional or other personal interest of any nature or kind in any product, service and company that could be construed as influencing the content of this paper.

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