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

Si Qin a, b, *, Chenghao Lv a, Qingshan Wang a, Zhibing Zheng a, b, Xi Sun a, Minyi Tang a, Fangming Deng a, *

a Key Laboratory for Food Science and Biotechnology of Hunan Province, College of Food Science and Technology, Hunan Agricultural University, Changsha 410128, China
b Hunan Co-Innovation Center for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha 410128, China

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.

© 2018, Chinese Association of Animal Science and Veterinary Medicine. Production and hosting by Elsevier B.V. on behalf of KeAi Communications Co., Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Limonoids are a prominent group of secondary metabolites found in the Rutaceae and Meliaceae 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-CO2) 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 hydrodrotropic 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.
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 Fah1, 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., 2012). Its antioxidant property and induce the expressions of glutathione S-transferase and quinone reductase (Sun et al., 2005; Jun et al., 2012). Nrf2/Keap1 is a crucial activator of antioxidant response element (ARE)-mediated 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 harvested, mashed, dried, and finely powdered. The limonin was then extracted from pummelo with petroleum ether at 25 °C for 10 h. The crude limonoids were then recovered 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 (Changchong Corp., Zhengzhou, China) with dichloromethane (CH2Cl2) and isopropanol. Thin layer chromatography and chemistry color response were used for qualitative and quantitative analyses of limonin.

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 (Changchong Corp., Zhengzhou, China) with dichloromethane (CH2Cl2) 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 (3^2 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 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 4000 to 400 cm−1 was analyzed by FT-IR (510-P, Nicolet Corp., USA) using KBr wafers. Purified limonin was identified by 1H- and 13C-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 (AGACAAACATTCAGCCCGT); reverse (CCATCTCTTATTTCTGCAG); HO-1, forward (CCAGGGGCCCAGCAAAATGTC) and reverse (AAGCTCCATGCACCGTGAAG); NQO1, forward (AGTCAGTGTGTCGACCGT); reverse (GGGGAGTCAGCCTGTAAT); Keap1, forward (CCTCAGTCACCTGCGTCC); and reverse (AAATGGGCTGACGACGG). Reverse transcription and real-time PCR was performed with a DyNaNo 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 × 10^5) 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 described 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 LAS4000 mini 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:H2O; 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: $Y = -125.1910 + 2.6958X_1 + 0.2930X_2 + 7.4845X_3 - 0.0164 X_1^2 - 0.000069X_1X_2 - 0.00158X_2^2 - 0.02353X_1X_3 - 0.00084X_2X_3 - 0.6025X_3^2$, 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.

| 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       |

* 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.

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, ¹H -NMR of the crystal was performed. Fig. 4 shows ¹H NMR of the crystal sample and the limonin standard. Table 2 lists the ¹H -NMR data. The main
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, 1H-NMR spectrum (Fig. 4A) and data (chemical shift δ) of the crystal are in excellent agreement with the standard ones (Fig. 4B). Meanwhile, the 1H-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 is 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-deacetylgedin (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

| 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 |
| 13       | 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).
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 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 1H-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.

Acknowledgment

This work was partially supported by Natural Science Foundation of China (31101268) and Core Research Program 1515 of Hunan Agricultural University of China to Si Qin.

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.

References

Battinelli L, Mengoni F, Lichtner M, Mazzanti G, Saia A, Mastroianni CM, Vullo V. Effect of limonin and nonolin on HIV-1 replication on infected human mononuclear cells. Planta Med 2003;69(10):910–3.
Breksa AF, Dragulj K, Wong KY. Isolation and identification of the first C-17 limonin epimer, epilimonin. J Agric Food Chem 2008;56(14):5595–8.
Chen JY, Zhu GY, Su XH, Wang R, Liu J, Liao K, et al. 7-deacetylgedunin suppresses inflammatory responses through activation of Keap1/Nrf2/HO-1 signaling. Oncotarget 2017;8(33):55051–63.
Chorley BN, Campbell MR, Wang X, Karaca M, Sambandan D, Bangura F, et al. Identification of novel Nrf2-regulated genes by ChIP-Seq: influence on retinoid X receptor alpha. Nucleic Acids Res 2012;40(15):7416–29.
Dandekar DV, Jayaprakasha GK, Patil BS. Hydroextracive extraction of bioactive limonin from sour orange (Citrus aurantium L) seeds. Food Chem 2008;109(3):515–20.
Hayes JD, McMahon M. Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. Cancer Lett 2001;174:103–13.
Hirotsu Y, Katsuoka F, Funayama R, Nagashima T, Nishida Y, Nakayama K, et al. Nrf2-MafG heterodimers contribute globally to antioxidant and metabolic networks. Nucleic Acids Res 2012;40:10228–39.
Jayaprakasha GK, Brodbelt JS, Bhat NG, Patil BS. Methods for the separation of limonoids from citrus. In: Patil BS, Turner ND, Miller EG, editors. Potential health benefits of citrusvol. 936; 2006. p. 34–51.
Jiutujikdeebodintra S, Chantachum S, Ratanaphan A, Chantapromma K. Stability of limonin from lime seeds. J Food Agric Environ 2005;3(2):99–100.
Jun Y, Limin W, Rosemary LW, Edward GM, Leonard MF, Bhimanagouda SP. Antioxidant activity of citrus limonoids, flavonoids, and coumarins. J Agric Food Chem 2005;53:2009–14.
Kuroska EM, Manthey JA, Hasegawa S. Regulatory effects of tangeretin, a flavonoid from tangerines, and limonin, a limonoid from citrus, on apo B metabolism in HepG2 cells. Farab J Biochem 2000;14(4). A298 – A298.
Liu C, Liu J, Rong YH, Liang NY, Rong L. Aqueous extraction of limonin from orange (Citrus reticulata blanco) seeds by the flash extraction method. Solvent Extr Res Dev Jpn 2012;19:137–45.
Malhotra D, Thimmulappa RK, Mercado N, Ito K, Kombairaju P, Kumar S, et al. Denitrosylation of HDAC2 by targeting Nrf2 restores glucocorticoid sensitivity in macrophages from COPD patients. J Clin Invest 2011;121:4289 – 302.
Mandadi KK, Jayaprakasha GK, Bhat NG, Patil BS. Red Mexican grapefruit: a novel source for bioactive limonoids and their antioxidant activity. Zeitschrift Fur Naturforschung C J Biosci 2007;62(3–4):179–88.

Mandadi K, Ramirez M, Jayaprakasha GK, Faraji B, Lihono M, Deyhim F, et al. Citrus bioactive compounds improve bone quality and plasma antioxidant activity in orchidectomized rats. Phytomedicine 2009;16(6–7):513–20.

Manners GD. Citrus limonoids: analysis, bioactivity, and biomedical prospects. J Agric Food Chem 2007;55(21):8285–94.

Melwita E, Juf YH. Separation of azadiracthin and other limonoids from crude neem oil via solvent precipitation. Separ Purif Technol 2010;74(2):219–24.

Ono E, Inoue J, Hashidume T, Shimizu M, Sato R. Anti-obesity and anti-hyperglycemic effects of the dietary citrus limonoid nomilin in mice fed a high-fat diet. Biochem Biophys Res Commun 2011;410(3):677–81.

Patil BS, Vu J, Dandekar DV, Toledo RT, Singh RK, Pike LM. Citrus bioactive limonoids and flavonoids extraction by supercritical fluids. In: Patil BS, Turner ND, Miller EG, editors. Potential health benefits of citrus vol. 936; 2006. p. 18–33.

Qin S, Chen J, Tanigawa S, Hou DX. Microarray and pathway analysis highlight Nrf2/ARE-mediated expression profiling by polyphenolic myricetin. Mol Nutr Food Res 2013;57:435–46.

Ribeiro IA, Rocha J, Sepodes B, Mota-Filipe H, Ribeiro MH. Effect of naringin enzymatic hydrolysis towards naringenin on the anti-inflammatory activity of both compounds. J Mol Catal B Enzym 2008;52:3–13.

Roy A, Saraf S. Limonoids. Overview of significant bioactive triterpenes distributed in plants kingdom. Biol Pharm Bull 2006;29(2):191–201.

So FV, Guthrie N, Chambers AF, Moussa M, Carroll KK. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr Cancer Int J 1996;26(2):167–81.

Sun CD, Chen KS, Chen Y, Chen QJ. Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation. Food Chem 2005;93(4):599–605.

Tian QG, Miller EG, Ahmad H, Tang LL, Patil BS. Differential inhibition of human cancer cell proliferation by citrus limonoids. Nutr Cancer Int J 2001;40(2):180–4.

Toshinao I, Hideaki O, Yoichi, limonoids in seeds of iyo tangor (citrus iyo hort. Ex tanaka). Food Sci Technol Res 2003;2:62–164.

Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A 1996;93:14960–5.

Vikram A, Jayaprakasha GK, Patil BS. Simultaneous determination of citrus limonoid aglycones and glucosides by high performance liquid chromatography. Anal Chim Acta 2007;590(2):180–6.

Wang F, Yu X, Liu X, Shen W, Zhu S, Zhao X. Temporal and spatial variations on accumulation of nomilin and limonin in the pummelos. Plant Physiol Biochem 2016;91:23–9.

Wasserman WW, Fahl WE. Functional antioxidant responsive elements. Proc Natl Acad Sci U S A 1997;94:5361–6.

Yang YF, Zhang LZ, Du XF, Zhang SF, Li LJ, Jiang ZD, et al. Recovery and purification of limonin from pummelo [Citrus grandis] peel using water extraction, ammonium sulfate precipitation and resin adsorption. J Chromatogr B Analyt Technol Biomed Life Sci. 2017;152:1060:150–7.

Zhao PH, Sun LM, Liu XJ, Cao MA, Yuan CS. Limonoids from the root of Dictamnus radicis cortex. Chem Pharmaceut Bull 2008;56(1):102–4.