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RESEARCH ARTICLE

An extraction-assay system: Evaluation on flavonols in plant resistance to Pb and Cd by supercritical extraction-gas chromatography

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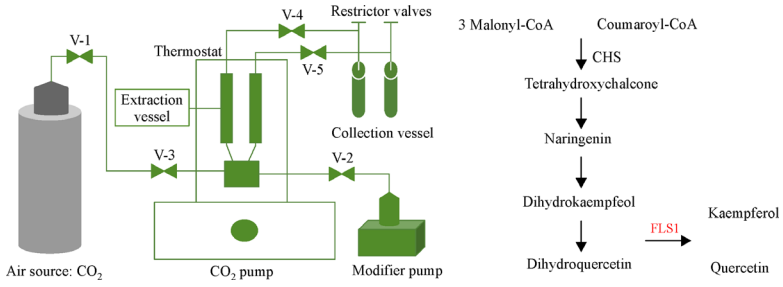
HIGHLIGHTS

- SFE-GC system is established for flavonols assessment.
- Optimal parameters of SFE-GC are evaluated and determined.
- Quercetin and kaempferol are detected in plant under heavy metal stress.
- Gene expression analysis shows consistent regularity with content of flavonols.
- ROS level is applied for elaborating the plant resistance status.

GRAPHIC ABSTRACT



ABSTRACT

In this research, supercritical carbon dioxide extraction (SFE) showed better extraction effect when compared with Solid- liquid extraction (SLE), Soxhlet extraction (SE) and Ultrasonic extraction (UE), not only in the rate but also the time. The comparison among these three extraction modifiers, including acetone, ethanol and methanol demonstrated that ethanol was preferred to SFE due to its high extraction effect and low toxicology. In addition, parameter of SFE, influence of temperature and pressure were investigated, and the best extraction effect was achieved at the optima conditions, temperature of 40°C and the pressure of 35 MPa. Thus, SFE is a highly effective method for flavonols extraction, requiring minimum energy and producing non-toxic byproduct. SFE-GC system is applied for the evaluation on flavonols that plays a key role in plant resistance to heavy metal, with its content and synthetase gene expression significantly increasing in plant when threatened by heavy metal. Besides, results indicated that flavonols can improve plant resistance to oxidative stress by quenching the redundant ROS in matrix.

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1 Introduction

The extraction of natural products from plant using green technology has been widely perceived as an alternative approach to conventional methods (Mason et al., 2016). Among the various extracts obtained from natural sources, molecules with biological activities are the main resources that drive further studies in this field (Hu et al., 2001). Supercritical fluid extraction (SFE) takes advantage of the

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properties of fluids over their critical points to selectively extract soluble components from different biological matrices (Kumar et al., 2008). In the super-critical state, solvents have properties of both liquids and gases, low viscosity and high diffusivity and density, which are convenient in extractive processes (Katherine et al., 2008). SFE has shown incredible remarks not only under the higher selectivity but also the higher extraction rate, especially when dealing with the recovery of natural compounds that exhibit antioxidant properties (Maran et al., 2015).

Consumption of flavonoids has beneficial effects in the prevention of cardiovascular, circulatory and neurological diseases due to their anti-inflammatory, anti-allergic, antithrombotic and antimicrobial activities (Benaventegarcía and Castillo, 2008). The bimolecular responsible for the chemo protective effects are found in plant resistance process under external environmental stress, especially flavonols. It is generally believed that flavonols can be used as an antioxidant and former oxidants to eliminate the stress caused by reactive oxygen damage (Skerget et al., 2005; Zhang et al., 2017c). As antioxidant compounds, flavonols are obtained from the plant and participate in many biological activities from birth to death (Jaramillo et al., 2011). Hence, finding a green-efficient method for flavonols extraction is necessary, not only to crop resistance but also to human health.

Arabidopsis thaliana, belonging to Cruciferae family, is applied for this research (Sung and Amasino, 2004). As a model organism, *Arabidopsis thaliana* has obtained much interests from scientific community for research on plant science, environmental science and toxicology, etc. (Rajagopalan et al., 2006; Pan et al., 2017). HPLC, as a traditional method, is applied for evaluating flavonols and oxidized flavonols with UV-, DAD- and ESI-ion trap MS detection (Jungbluth and Ternes, 2000). In this work, SFE extraction method was selected compared with Solid-liquid extraction (SLE), Soxhlet extraction (SE) and Ultrasonic extraction (UE) extraction method. Furthermore, for the SFE extraction process, optimization test of the supercritical extraction of flavonols in *Arabidopsis* was performed to find the optima temperature, pressure and modifier for maximize extraction efficiency. Plant samples in soil, treated with different concentration of Pb and Cd, were used for exploring biological status of flavonols in plant resistance process (Houben et al., 2015).

2 Materials and method

2.1 Chemical regent

Standard samples of quercetin and kaempferol were purchased from the China Standard Certification Center (CSC). Methanol, acetone and ethanol were used for preparing modifier. The supercritical grade carbon dioxide (99.99%) was supplied by Jinan Gas Factory of Shandong Province in China. Ultrapure water (18MΩ), obtained by using a Milli-Q water purification system (Millipore, USA), was used throughout. Nitrogen (99.99%, Jinan Gas Factory, China) was used as the carrier gas for GC.

2.2 Sample cultivation

Arabidopsis thaniala seeds were sterilized with 70% ethanol for 5 min, and 90% ethanol for 1 min. Afterwards, the seeds were sown on 1/2 MS medium and kept in the dark for 2 days at 4°C for vernalization(Zhang et al., 2017d). Based on the pre-experiment, water-soluble formation was used in two-heavy metal (Cd^{2+} and Pb^{2+}) experiment in a completely randomized design with three replications included: C (control, 0 $mg \cdot kg^{-1}$ of Pb and 0$mg \cdot kg^{-1}$ of Cd); Cd50 (50 $mg \cdot kg^{-1}$ of Cd), Cd100 (100 $mg \cdot kg^{-1}$ of Cd); Pb500 (500 $mg \cdot kg^{-1}$ of Pb), Pb1000 (1000 $mg \cdot kg^{-1}$ of Pb). After 21 days, old seedlings were collected for research (Ren et al., 2015; Wang et al., 2016).

2.3 Supercritical fluid extraction

Stainless-steel extraction vessels were saturated with plant samples and the remaining volume was filled with polypropylene wool which was packed firmly to ensure the uniform diffusion of supercritical CO_2 throughout the sample matrix (Emteborg et al., 2016). On laboratory-scale, two 5 mL extraction vessel were equipped with needle valves. In the operation, the optimum temperature was performed on thermostat and CO_2 was pumped to get optimum pressure. This system was kept at a certain pressure and temperature to extract flavonols in plant samples. CO_2 was introduced at a constant flow rate of 2.0 mL/min. Finally, the extract was collected in collection vessel at atmospheric pressure where CO_2 escapes as gas (Modey et al., 2015).

2.4 RT-PCR

Trizol method (Gibco BRL) was used for extraction of total RNA from 21 days seedlings under different treatments. Each RNA sample was reversely transcribed using oligo (dT) primers and a Superscript II RNase H reverse transcriptase kit (Invitrogen) following the manufacturer's protocol (Kuhn et al., 2011). The resulting cDNA was used to perform a RT-PCR with the primers of Chalcone Synthase and Flavonol Synthase. Both primer pairs were flanking intronic sequence to distinguish in the RT-PCR experiment between cDNA and contamination with genomic DNA (Diet et al., 2006; Xing et al., 2017).

2.5 Data analysis

Results were presented by Relative extraction rate and Relative concentration (Zhang et al., 2016) (Relative

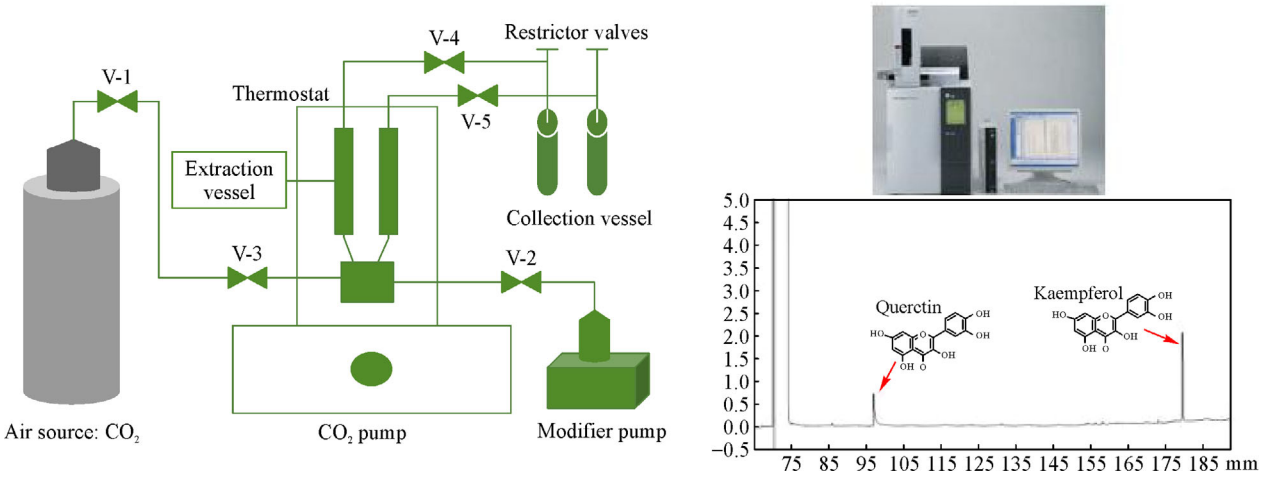

Fig. 1 SFE- GC extraction and detection system of flavonols

extraction rate = Sample extraction rate/CK Group × 100%; Relative concentration = Sample concentration/CK Group × 100%). The data were subjected to one-way analysis of variance (ANOVA) and * indicated significant differences between treatments at $p < 0.05$. All data analyses were conducted using SPSS Statistics 20.0 and represented as means±standard deviations of three replicates for each treatment. Graphs were produced by using Excel 2010 (Yang et al., 2017).

3 Results

3.1 SFE-GC extraction-detection system

Effective detection of kaempferol and quercetin using an innovative extraction- assay method was combing supercritical fluid extraction and gas chromatography spectrometry (Fig. 1). Supercritical fluid extraction instruments (Universal Analytical & Testing Instruments Ltd.) were used for extraction of flavonols. The SFE system consisted of carbon dioxide pump, thermostat, extraction vessel, co-solvent, back pressure regulator and a collection vessel (Kamali et al., 2013). The flow rate of CO_2 in the system was controlled manually by micro-metering valve. The modifier, ethanol, was added by an HPLC pump (Well-Chrom K-501, Germany) to the supercritical CO_2 stream. The extraction temperatures were monitored by a thermocouple with precision of ±1°C. After extraction, flavonols were detected by GCMS-QP2010nc-Plus (Shimadzu, Japan) gas chromatograph mass spectrometer equipped with an EI source and in full-scan operation (Dauner and Sauer, 2000). The column was a 30 m × 0.25 mmi.d. Restek Rtx-5 capillary column with a 0.25 μm film thickness (cross bond 5% diphenyl-95% dimethylpolysioxane, Restek Co.,USA).

3.2 Effect of modifier on extraction

Supercritical CO_2 is a non-polar solvent, which has a great solubility in non-polar substance but inferior in the polar substance, although its polarity can be modified by temperature and pressure variations (Kutchko et al., 2013). Flavonols with high molecular mass are hardly soluble impure CO_2, but their solubility can be improved by adding a polar modifier or by increasing pressure. Furthermore, modifier could change the critical point of mixtures and make them closer to extraction temperature (Veggi et al., 2011). As shown in Fig. 2, three effective modifiers were used for SFE extraction test. The extracts obtained by SFE with modifier varied from 72.2% to 95.8%. The addition of the polar modifier increased the polarity of the solvent, thereby enhancing the solubility of the polar substance. Moreover, the use of modifier in supercritical fluid could increase the selectivity and extraction efficiency when added with methanol, acetone and ethanol (El-Aty et al., 2009). Comparably, ethanol and

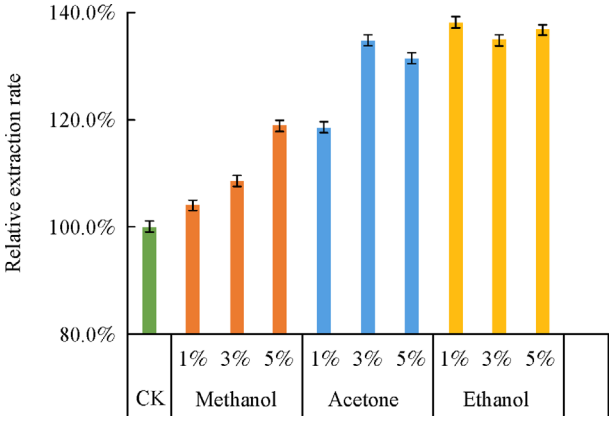

Fig. 2 Modifier comparison among methanol, acetone and ethanol

acetone showed better extraction efficiency than methanol. Regarding to the toxic effect of acetone, ethanol was preferred due to its clean and safe characteristic, which had been proven to be an alternative to extract flavonoid from fruit (Espinosa-Pardo et al., 2016). The moderate polarity of flavonoid helped their recovery with co-solvents in SFE extraction process, indicating that addition with ethanol was much more effective to obtain flavonols.

3.3 Effect of pressure and temperature on extraction

As shown in Fig. 3, extract rate of flavonols under different pressures and temperatures were represented by different colors. It can be observed that pressures and temperatures, as important factors, showed similar characteristic. The density and solvation power of the supercritical CO_2 increased with the increase of pressure in certain range, which improved the extraction efficiency (Ferrentino et al., 2014). However, when the pressure reached a threshold value, the extraction rate was retarded due to volatilization. Increasing temperature made contribution to the thermal motion that was beneficial for solutes to overcome the adsorbing energy fortress of the matrices (Lummaetee et al., 2017). The solvation power of supercritical CO_2 rose with constantly increasing temperature due to the higher solvent density. Higher temperature can increase the vapor pressure, enhancing their solubility in the fluid phase and the extraction yield. Temperature and pressure coordinate and constraint with each other in a complicated way (Spence et al., 2009). In this study, optimum combination of temperature and pressure were 40°C and 35 Mpa.

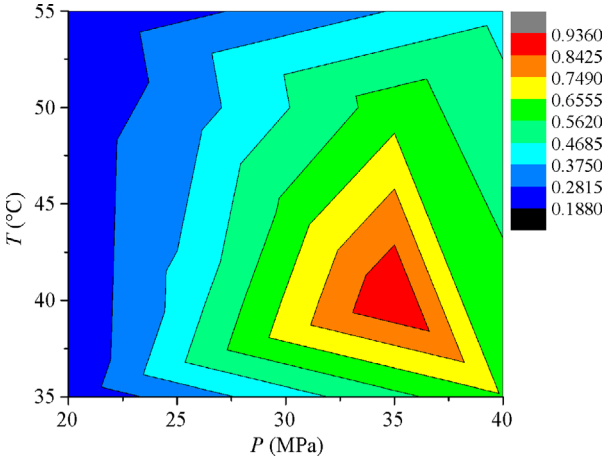

Fig. 3 Optimum parameter analysis of pressure and temperature

3.4 Ratio of static time and dynamic time

The effect of flow rate of supercritical CO_2 on the recovery was investigated. The increasing flow rate improved the extraction efficiency because higher flow rate of supercritical CO_2 could enhance the mass-transfer efficiency (Taher et al., 2014). However, the extraction efficiency decreased with the further increase of the flow rate, which may be attributed to the formation of the aerosols. Therefore, the flow rate of supercritical CO_2 was controlled at 2.0 mL/min. The effect of combination of extraction time was shown in Fig. 4 and the results indicated that the recovery rate increased with the extension of extraction time. The reason was obvious that mass-transfer efficiency and the extraction ability enhanced as the dynamic extraction time prolonged (Hossein and Karamatollah, 2009). On one hand, the long- static time influenced the extraction efficiency and selectivity of the fluid, which can thus improve the penetration of supercritical CO_2 into the interstices of matrix. On the other hand, long-dynamic time kept the matrices continually exposed to fresh supercritical fluid, finally enhancing the recovery efficiency (Kazazi et al., 2007). However, the extraction rate could not increase and keep stable when exceeding a certain time range. In this study, 30 min static time and 60 min dynamic time were selected as the optimum combination of extraction time.

3.5 Comparison of different extraction methods

The extraction efficiency of flavonols with four extraction methods, including Solid-liquid extraction (SLE), Soxhlet extraction (SE), Ultrasonic extraction (UE) and supercritical carbon dioxide extraction (SFE) showed significant differences. For each extraction process considered, extraction time and rate were shown in Fig. 5. SLE and

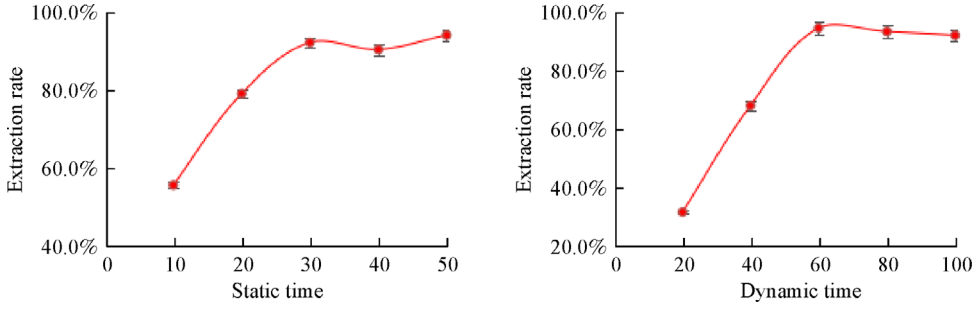

Fig. 4 Effect of static time and dynamic time

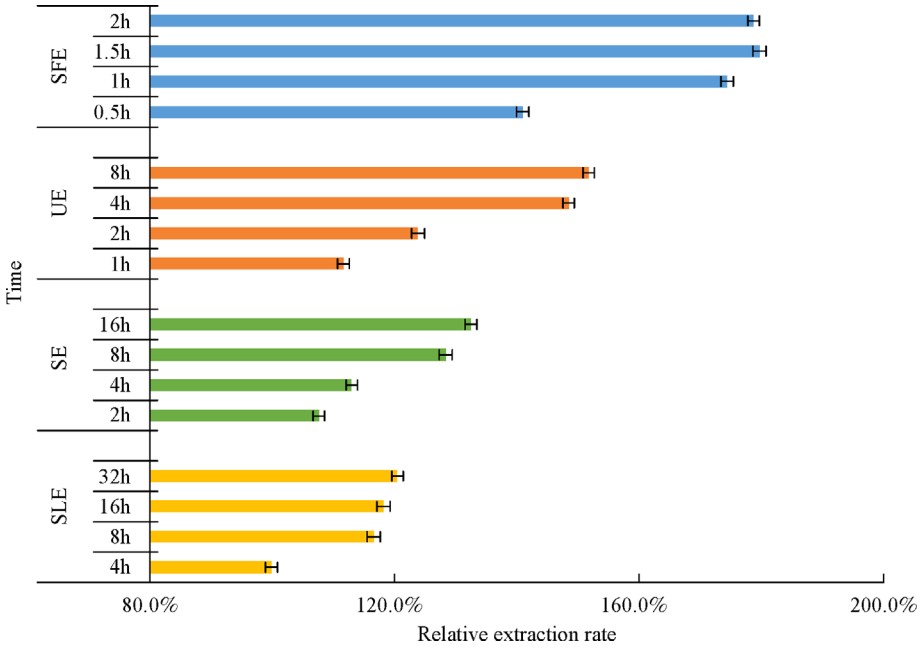

Fig. 5 Extraction efficiency of four different methods

SE, as traditional extraction methods using maceration (Bogdanov et al., 2012), consumed longer time (32 h and 16 h respectively) and lower extraction rate (64.3% and 70.9% respectively). These two traditional extractions methods were considered as references for the evaluation on new methods as the extraction by UE for 8 h had a yield of 81.2%. Especially, the extraction using ultrasonic led to an increasing recovery rate of more than 10%, furthermore, saving more than half of the extraction time compared to 32h or 16h of traditional methods. SFE is an effective and green method, showing strongest extraction ability not only in extraction content, but also in extraction time, which allowed high recovery of extractable substances (96.1%) and short time (1.5 h). Although, SLE, SE and UE are cheap and simple methods, but the lower extraction efficiency are unable to apply for the rapid and accurate extraction, finally affecting the real-time detection (Shen and Shao, 2005). These results allow us to affirm that SFE is much more qualified as an essential part of extraction-detection system for qualitative and quantitative analysis of flavonols than other three methods.

4 Discussion

So far, flavonoid biosynthesis process has been well characterized, and several key genes functioning on the synthesis of enzymes that participate in different steps have been identified in *Arabidopsis thaliana* (Fig. 6). SFE-GC was used for extracting and detecting the content of kaempferol and quercetin in plant under the stress of Pb and Cd. Furthermore, the expression of key genes in flavonols synthetic, Chalcone synthase (CHS) and Flavonol synthase (FLS) were evaluated (Zhang et al., 2018a). As shown in Fig. 6, the concentration of kaempferol and quercetin increased significantly under Pb and Cd treatment. Additionally, results of RT-PCR showed that gene expression level of CHS and FLS were upregulated when plants were under the stress of Pb or Cd. It has been reported that Pb and Cd have considerably toxic effects on plant growth cycle (Zhang et al., 2018b), including germination stage and seedling stage, perhaps by combining with proteins and inhibiting the activity of some key enzymes, then influencing a series of physiologic and biochemical processes in cells and causing metabolic disorder in plants (Zhang et al., 2017a; 2017b). Previous study has shown that exogenous flavonols can alleviate the adverse effect which was caused by exogenous Pb and Cd stress, and enhance the growth and biomass of stressed seedlings, thus demonstrating the amelioration action of flavonols in reducing phytotoxicity [50]. Moreover, endogenous evaluation on the content of flavonols and gene expression level analysis fully proved and improved this research. However, some interesting results occurred that the content of kaempferol and quercetin showed different characteristics in Pb and Cd group. On one hand, the concentration of quercetin was higher than kaempferol when under Pb stress; On the other hand, kaempferol accounted for more in Cd group. The results indicated that different flavonols may modulate different immune pathways due to the specific characteristics of heavy metal.

As shown in Fig. 7, DAB and NBT staining methods were used for analyzing the level of ROS in plants. NBT staining showed an increased amount of O^{2-} as scattered

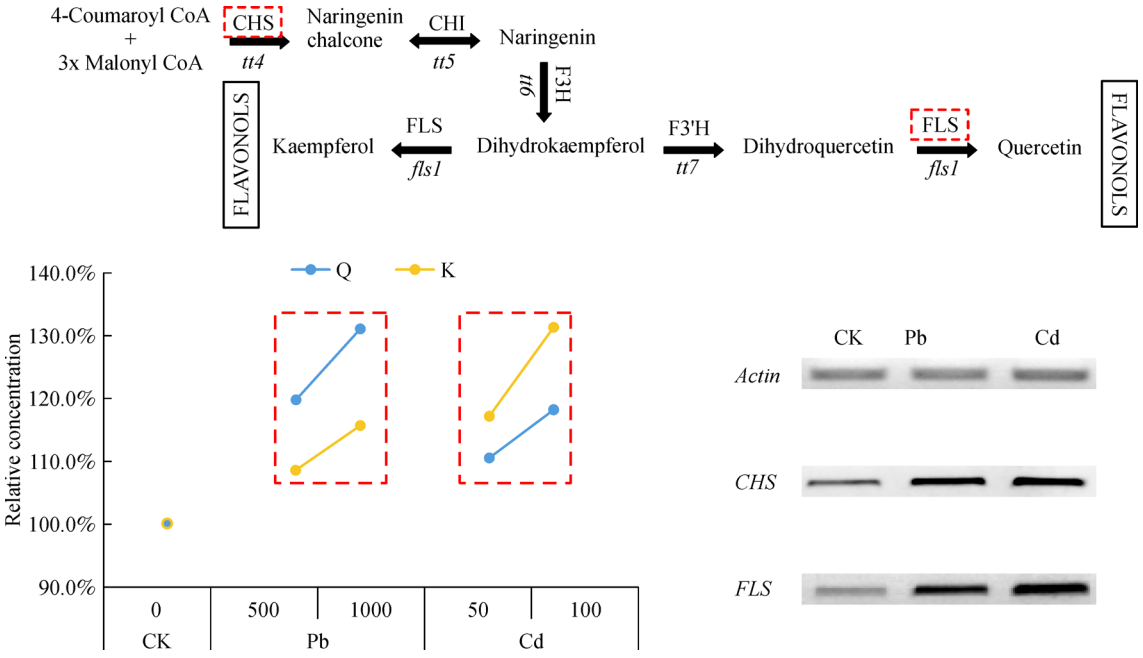

Fig. 6 Relative content and expression of quercetin and kaempferol in plant samples

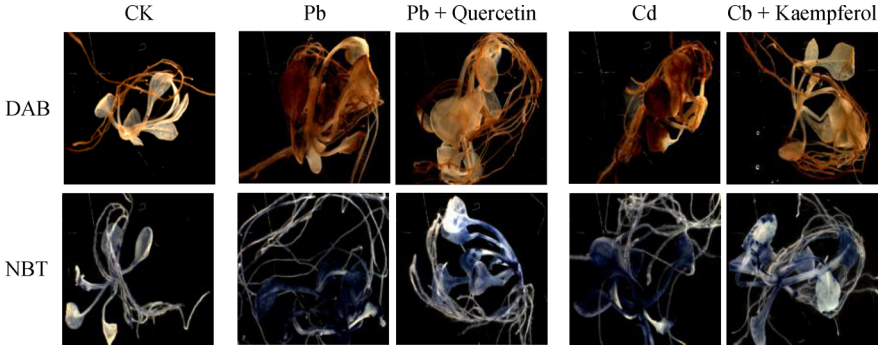

Fig. 7 DAB and NBT detection of quercetin and kaempferol groups with Pb or Cd

dark blue spots in the Pb or Cd-stressed *Arabidopsis* leaf compared with the control group. Similarly, DAB staining confirmed a remarkable increase in brown polymerization products, which indicated the over-accumulation of H_2O_2 (Yin et al., 2015). More importantly, the accumulation of ROS in Pb-stressed seedlings significantly diminished with the exogenous addition of quercetin which indicated that flavonols can enhance the tolerance of *Arabidopsis* plants against oxidative stress induced by Pb or Cd. Flavonols, including kaempferol and quercetin, are produced from dihydroflavonols via the activity of the flavonol synthase, thus representing a side branch of the flavonoid biosynthesis pathway (Jaakola and Hohtola, 2010). In the nature, abiotic stress including heavy metal stress can induce cells to produce superabundant ROS that will cause secondary oxidative stress to plants. However, kaempferol and quercetin as two main kinds of flavonols, appear to serve as an essential function in the regulation of reactive oxygen species (ROS), whose level changes have been regarded as characteristic symptoms of environmental stress (Suzuki et al., 2012; Ren, 2015).

5 Conclusions

An effective and green system was established for simultaneous extraction and determination of flavonols. SFE was selected in terms of extraction rate and time assuming compared with SLE, SE and UE methods. Ethanol was chosen as modifier which improved the extraction of flavonols and showed non-toxic effect. Optimum parameter of SFE was researched and the best combination of temperature and pressure were 40°C and 35Mpa; however, the relationship between the temperature and pressure is complex which needs further study. At last we extracted and detected the concentration of flavonols in

plant resistant to Pb and Cd, which showed consistent regularity with the expression level of enzyme-synthesis genes. In short, this extraction-assay method could be widely applied not only for the evaluation of flavonols in environmental research, but also for production at the industrial level. It is possible to greatly reduce the extraction time while simultaneously increasing the efficiency of this process.

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