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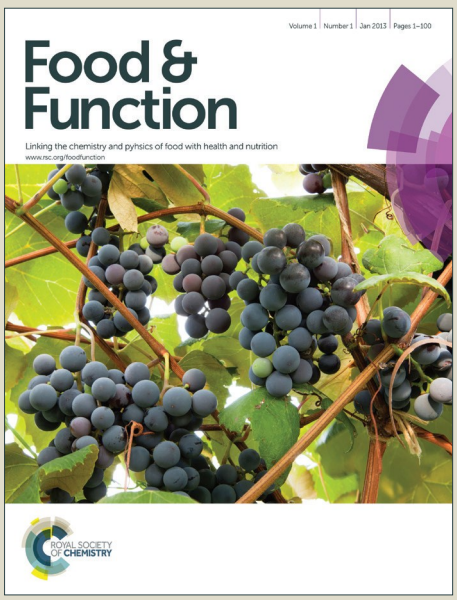


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# An effective homogenate-assisted negative pressure cavitation extraction for determination of phenolic compounds in pyrola by LC-MS/MS and the evaluation of its antioxidant activity

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**Abstract**

A novel extraction method, homogenate-assisted negative pressure cavitation extraction (HNPCE), was designed for the extraction and determination of main phenolic compounds from *Pyrola incarnata* Fisch. by LC-MS/MS. The particle sizes and extraction yields in the process of homogenate were compared with conventional pulverization. The results showed that the homogenate under 120 s could produce more suitable particle size powders for analyte extraction. The following NPCE parameters were optimized by a BBD test and under the optimal conditions, the maximum extraction yields of arbutin, epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin increased 68.7%, 72.0%, 43.3%, 62.5% and 34.5% than the normal NPCE. LC-MS/MS method was successfully applied for the quantification of five target compounds in pyrola, and the results of the precision test indicated a high accuracy of the present method for the quantification of target compounds in pyrola. Furthermore, the antioxidant activities of the pyrola extracts were also determined. The results showed that pyrola had good antioxidant activities and it was a valuable antioxidant natural source.

**Keywords:** homogenate-assisted negative pressure cavitation extraction, *Pyrola incarnata* Fisch, LC-MS/MS, BBD test, antioxidant activity

## 1. Introduction

Pyrola [*Pyrola incarnata* Fisch.] is a herbaceous plant widespread in China. Because of its function of slowing down aging and boosting immunity, it was used as a kind of tea called *Lu Shou Cha* for daily drinking in China. [1] Pyrola is widespread as an edible plant for food and healthy industry. [2] The extracts of the plant was reported to inhibit the growth of many kinds of human pathogenic bacilli *in vitro* and it can be also used in refreshing foods. [3, 4] Earlier investigations of pyrola plants led to the isolation of chimaphilin, arbutin, epicatechin, catechin, 2'-*O*-galloylhyperin, hyperin, quercetin, pyrolatin and other naphthoquinones.[5-10] According to other research, 2"-*O*-galloylhyperin has anti-inflammatory, cough, blood pressure, and lower cholesterol, the cardiovascular and cerebrovascular protective role.[11, 12] Epicatechin and hyperin have good antioxidant activity.[13, 14] Chimaphilin has the antifungal activities and antioxidant activity.[15] Thus, it is meaningful to investigate the extraction and determination of the active compounds from pyrola.

According to other reports, arbutin can be successfully extracted by ultrasound extraction, maceration and heating reflux extraction followed by HPLC-UV [16, 17] and LC-MS/MS. [18] And catechin and hyperin can be successfully extracted by ultrasound extraction, microwave extraction, heating reflux extraction determined by HPLC-UV [19-21] and LC-MS/MS.[22, 23] For 2'-*O*-galloylhyperin and chimaphilin, there was no report about the determination, only some reports about pharmacological activity can be found. As a modern separation method, LC-MS/MS presents excellent sensitivity and selectivity for the quantification of target compounds in plants. Thus, in the

present study, LC-MS/MS was used as the detection method.

Particle size is an important factor which can influence the extraction efficiency. The reduction in particle size can essentially shorten the processing time, and enhance the overall extraction yield. However, if the powder of crushed particles is too fine, it may cause difficulties in the filtration and raise the cost of processing during the subsequent industry procedures and moreover, it can also lead to dust pollution. Homogenate is an effective pulverization method. This method is wildly used in the pretreatment of animal and plant tissues.[24-27] Compared with the conventional pulverization method, homogenate can not only pulverize the samples but also mix the samples with extraction solvent effectively, which can avoid dust pollution.

Traditional extraction methods such as soxhlet extraction, heating reflux extraction or maceration reveal disadvantages, e.g. time-consuming, no environmental friendliness processes and low efficiency.[28] Because of the complexity of plant material and the low content levels of some phytochemicals, normal extraction methods are not always suitable.[29] Negative pressure cavitation extraction (NPCE) is a cheap and energy efficient extraction method. The cavitation phenomenon of NPCE is generated by negative pressure which is similar to ultrasonic cavitation. It keeps constantly lower temperature and its intensity is comparable to that of ultrasonic cavitation. Nitrogen is continuously added to the NPCE system. Under negative pressure, small nitrogen bubbles appear and ascend among the liquid-solid phase, resulting in the violent movement of solvent and the formation of a highly instable gas–liquid–solid phase.[30] When the bubbles collapse, it will cause the effect of

cavitation which can destroy the cell wall of plant samples and result in the efficient extraction of active compounds.

In the present study, a new homogenate assisted negative pressure cavitation extraction (HNPCE) device was proposed and designed, it combined the both benefits of homogenate and negative pressure cavitation extraction. HNPCE was applied for the extraction of target compounds from pyrola followed by the determination of LC-MS/MS. In the device, the samples were firstly pulverized in a homogenizer and then the mixtures (samples and solvent) were extracted by NPCE with the action of negative pressure. After that a LC-MS/MS method was applied for the determination of the target compounds in pyrola, the intra-day test, inter-day test and recovery test were conducted for the precision of the method.

## *2. Materials and methods*

### *2.1. Plant material*

Pyrola [*Pyrola incarnata* Fisch.] was collected in autumn in Heilongjiang province, China, and identified by Professor Shao-Quan Nie (Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, P. R. China). Voucher specimens were deposited in the herbarium of the same laboratory. The samples were dried in the shade, pulverized, and sieved. They were protected from light in a desiccator at room temperature until used.

### *2.2. Chemicals and reagents*

Arbutin (≥95%), epicatechin (≥98%), hyperin (≥98%), 2'-*O*-galloylhyperin (≥96%) and chimaphilin (≥95%) were purchased from Daierta (Wuhu, China).

Methanol of HPLC grade was obtained from J & K Chemical Ltd. (Beijing, China), Deionized water was purified by a Milli-Q water-purification system from Millipore (Bedford, MA, USA). Ethanol of analytical grade for extraction was bought from Tianjin Kermel Chemical Reagent Co. (Tianjin, China).

*2.3. Pulverization and extraction*

*2.3.1 Instrumentation*

HNPCE was performed on a device designed and manufactured in our laboratory. The device is shown schematically in Fig. 1. Ultrasound-assisted extraction (USE) was performed in an ultrasonic bath (Kunshan Ultrasonic Instrument, Kunshan, China).

*2.3.2 Pulverization procedures*

Conventional pulverization: 5 g of dry sample was introduced into the homogenizer and then pulverized for different time. After pulverization, the sample powders were sieved (20–90 mesh) by different sifter and then weighed.

Homogenate: 5 g of dry sample was added to the homogenizer with a specified amount of solvent (20:1 mL/g), and then pulverized for different time. The mixture after homogenate were dried at 40 $^{o}$C and sieved (20–90 mesh) by different sifter.

*2.3.3 Extraction procedures*

NPCE: 5 g of pulverized sample was introduced into the NPCE device from the sample portal. After adding the solvent, the device was connected to the vacuum pump. Then nitrogen was supplied from the bottom of the device and the pressure was controlled by the valve (8).

HNPCE: 5 g of dry sample was introduced into the homogenizer with a specified amount of solvent and pulverized for different time. Connected the pump, kept the valve (1), (2) and (4) open and other valve close, the mixture was pumped into the extraction pot. Closed the valve (1) and kept the valve (2), (4), (8) and (6) open, nitrogen was introduced into the extraction pot through valve (8) and the pressure was also controlled by valve (8). The extraction solvent was filtered through a filtration net into the collection pot after extraction was conducted..

*2.4. LC-MS/MS*

The LC system consisted of an Agilent 1100 series HPLC system equipped with G1312A Binary pump and G1379A Degasser (Agilent, San Jose, CA, USA). Chromatographic separation was performed on a HIQ sil C18W reversed-phase column (250 mm × 4.6 mm i.d., 5 μm, KYA TECH Corporation, Japan). The mobile phase was consisted of aqueous solution (A) and methanol (B). The following gradient elution program was used for separation: 0-3 min 20-45% (B), 3-8 min 45% (B), 8-9 min 45-67% (B), 9-15 min 67-76% (B), 15-20 min 76-80% (B). The flow rate was 1 mL/min, the injection volume was 10 μL. After 20 min of re-equilibration, the column was ready for a new injection. The column temperature was maintained at 30 $^{o}$C. Using this LC conditions, the chromatograms showed well-separated resolution, satisfactory peak shape as well as relatively short analysis time. Five compounds separation was achieved within 20 min.

An API3000 triple tandem quadrupole mass spectrometry with a Turbolon-Spray interface from Applied Biosystems (USA) was operated in negative electrospray

ionisation ($ESI^-$) source mode. All mass spectra were acquired in multiple reaction monitoring (MRM) transitions. The ESI-MS of five phenolic compounds was recorded using direct infusion of each reference compound. The analytical conditions were as follows: nebulizing gas (NEB), curtain gas (CUR) and collision gas (CAD) 12, 10 and 6 a.u.; dwell time 1.5 s; ion spray voltage -4500 V; the ion source temperature 300 $^oC$; focusing potential (FP) and entrance potential (EP) -400 and -10 V, respectively. The other parameters for LC-MS/MS analysis of seven phenolic compounds including declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were further studied. Peak areas obtained from the selected reaction monitoring (SRM) were utilized for the quantification of five compounds. Analyst Software (version 1.4) installed on a DELL computer was used for data acquisition.

*2.5 SEM*

A Hitachi S-520 field emission scanning electron microscope (Hitachi, San Jose, CA, USA) was used to observe the morphological alteration of dried samples with different extraction methods. After removing the solvent, the remaining pyrola samples were fixed on an adhesive tape and then sputtered with gold. All the samples were examined under high vacuum condition and an accelerating voltage of 15.0 kV.

*2.6. Validation study*

The linear range, limit of detection (LOD), limit of quantification (LOQ), precision and recovery were studied for the developed method. The linearity of calibration curve was tested by analysis of individual reference compound at eight

concentrations. LOD and LOQ for each analyte were evaluated at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively. Intra-day and inter-day variations were chosen to determine the precision of the developed method. For the intra-day variability test, the samples were analyzed in triplicate five times within one day, while for the inter-day variability test, the samples were examined in triplicate on three consecutive days. The RSDs for the retention time and peak area were calculated as measures of precision. Recovery was determined using the spiked samples with the pyrola matrix. A portion of 5.0 g of pyrola matrix was individually spiked with certain amount of reference compound of arbutin, epicatechin, hyperin, 2'-O-galloylhyperin and chimaphilin respectively. Three replicated samples were extracted and analyzed with the same procedures as described in Sections 2.3 and 2.4 for evaluating the accuracy.

*2.8 Antioxidant activity*

*2.8.1 DPPH radical scavenging activity assay*

The free radical-scavenging activities of samples were measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH. [31] The different concentrations of the samples in 50% ethanol (100 μL) were mixed with 50% ethanol (1.4 mL) and then added to 0.004% DPPH (1 mL, Sigma-Aldrich) in ethanol. The mixture was shaken vigorously and then immediately incubated in darkness. After 70 min, the reaction reached a plateau. The decreasing of the DPPH solution absorbance was determined in a UV-Vis spectrophotometer (UNICO, Shanghai, China) to monitor absorbance at 517 nm. Ascorbic acid (Sigma-Aldrich), a

193 stable antioxidant, was used as a positive reference. The DPPH radical-scavenging
194 activity in percentage of sample was calculated as follows: DPPH scavenging activity
195 (%) = (1-$A_{517}$ sample/$A_{517}$ DPPH solution) ×100.

196 *2.8.2 The reducing power*

197 The reducing power was measured according to the method of Wu et al. (2010) [32]
198 with some modification. An aliquot of each sample (0.5 mL), with different
199 concentrations, was mixed with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5
200 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The reaction mixture was incubated at
201 50 $^oC$ for 20 min. After incubation, 0.5 mL of 10% trichloroacetic acid (TCA) was
202 added, followed by centrifugation at 650 xg for 10 min. The supernatant (0.5 mL) was
203 mixed with 0.5 mL of distilled water and 0.1 mL of 0.1% ferric chloride ($FeCl_3$). The
204 absorbance of all sample solutions was measured at 700 nm. An increased absorbance
205 indicated increased reducing power. BHT was used as the positive control.

206 **3. Results and discussion**

207 *3.1 Comparison between conventional pulverization and homogenate*

208 The particle size was first investigated and the results were shown in Fig. 2. The
209 particle size ranges were selected as > 20 mesh, 20-30 mesh, 40-90 mesh and <90
210 mesh. Early research reported that the particle sizes ranged from 40 to 90 mesh were
211 the optimum material mesh for extraction.[33, 34] For both conventional pulverization
212 and homogenate, the amount of the samples with the particle size of 40-90 mesh and
213 <90 mesh increased as the time increase, while the amount with the particle of > 20
214 mesh and 20-30 mesh decreased. After treated by conventional pulverization for 60 S,

the sample amount with the particles sizes 40-90 mesh and <90 mesh were 67.6% and 14.2% which were higher than those treated by homogenate. And for conventional pulverization, with the pulverization time increase from 60 S to 150 S, the sample amount with the particles sizes of <90 mesh increased from 6.8% to 23.8% and this amount of the sample treated by homogenate only increased from 5.0% to 8.0%. That is because the samples used in the experiment were dry and crisp, so in the conventional pulverization process it was more conducive to pulverizing the samples to fine. However, in the extraction process, too fine particles sizes were not conducive to extracting target compounds from plant materials. The particles sizes by conventional pulverization for 120 S (>90 mesh, 17.6%) and 150 S (>90 mesh, 23.8%) were much finer than that for 90 S (>90 mesh, 11.4%) but the extraction yields of target compounds by conventional pulverization for 120 S and 150 S were lower than that for 90 S (Fig. 3). These results have been proved by some other researches.[35-37] The reason is because too fine particles might lead to conglomeration, thus decreasing the contact surface area.[38] Meanwhile, the fine particles may also cause powder dust pollution which was harmful to human body.

In Fig. 2, it indicates that with the homogenate time increases, the amount of the homogenate samples with the particles of 40-90 mesh increase. And meanwhile, the extraction yield of the target compounds also increased. That may prove 40-90 mesh is the suitable particle sizes for extraction. From Fig. 3, it is found that with the pulverization time increases, the extraction yields by the conventional pulverization method increases first and then decreases. And the optimal pulverization time of

conventional pulverization method is 90 S. For homogenate, when the homogenate time is more than 120 S, the extraction yields increased slightly. So 120 S is the optimal homogenate time. At the optimal time of conventional pulverization, the extraction yields of arbutin, epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin were 2.169 ± 0.104, 0.744 ±0.026, 1.188 ± 0.057, 4.732 ± 0.193 and 0.377 ± 0.020 mg/g which were lower than those of homogenate at its optimal time 120 S (2.677 ± 0.087, 0.844 ± 0.032, 1.371 ± 0.055, 5.039 ± 0.204 and 0.388 ± 0.017 mg/g). The reason may be because the sample amounts with 40-90 mesh by homogenate were more than those by conventional pulverization, and on other hand, the samples and extraction solvent can be mixed fully in homogenate process which was benefits to the following extraction. Thus, homogenate is more suitable than conventional pulverization for extraction.

*3.2 BBD test*

The objective of the present study was to optimize the operating conditions to achieve an efficient extraction of arbutin, epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin from pyrola for determination. A Box-Behnken design (BBD) was used to optimize the extraction conditions of target compounds (Table 1). The yields of arbutin ($Y_1$), epicatechin ($Y_2$), hyperin ($Y_3$), 2'-*O*-galloylhyperin ($Y_4$) and chimaphilin ($Y_5$) were function of these variables. Negative pressure ($X_1$), liquid/sample ratio ($X_2$) and ethanol concentration ($X_3$) are independent variables. By applying multiple regression analysis to the experimental data, the second order polynomial equations were found to represent the extraction yield adequately.

$$Y_1=-7.619+100.475X_1+0.061X_2+0.258X_3-619.000X_1^2-1.267\times10^{-3}X_2^2-2.557\times10^{-3}X_3^2-0.598X_1X_2-0.218X_1X_3-+6.4\times10^{-4}X_2X_3. \quad (1)$$

$$Y_2=-3.860+58.138X_1+0.054X_2+0.010X_3-460.500X_1^2-1.271\times10^{-3}X_2^2-8.830\times10^{-4}X_3^2+0.050X_1X_2-0.188X_1X_3+7.5\times10^{-6}X_2X_3. \quad (2)$$

$$Y_3=-6.308+98.163X_1+0.095X_2+0.157X_3-830.5X_1^2-2.246\times10^{-5}X_2^2-1.373X_3^2+0.230X_1X_2-0.273X_1X_3-1.275\times10^{-4}X_2X_3. \quad (3)$$

$$Y_4=-23.704+373.713X_1+0.318X_2+0.591X_3-3.104\times10^{4}X_1^2-6.664\times10^{-3}X_2^2-4.882\times10^{-3}X_3^2+0.888X_1X_2-1.1159X_1X_3-1.275\times10^{-3}X_2X_3. \quad (4)$$

$$Y_5=-1.389+20.463X_1+8.523X_2+0.044X_3-113.000X_1^2-1.780\times10^{-4}X_2^2-4.005\times10^{-4}X_3^2-0.090X_1X_2-0.108X_1X_+9.750\times10^{-5}X_2X_3. \quad (5)$$

The significance of each coefficient was determined using the F test and p-value. The coefficients calculated from the five regression model are listed in Table 2. The high significant levels for the five models ($p < 0.01$) were obtained by statistical analysis, results mean that they are precise and applicable models.

In the NPCE process, nitrogen is continuously added to the extraction system. Under negative pressure, small nitrogen bubbles appear and ascend among the liquid–solid phase, resulting in cavitation and turbulence. Cavitation effects can corrode the surface of solid particles. Turbulence effects can make the solid and liquid fully mixed and enhance the effect of mass transfer [39]. These effects are all generated by negative pressure. Thus, negative pressure is an important parameter influencing the efficiency of cavitation and the extraction yield.

After optimization, the optimal pressure was -0.05 MPa which was calculated

281 from the equations. And both higher and lower than -0.05 MPa, the extraction yield of
282 the target compounds decreased. This was because of an increase in negative pressure
283 resulting from reduction of the nitrogen flow rate and consequent a decrease in tiny
284 bubble formation. Hence, there were not enough nitrogen bubbles to form turbulent
285 motion for appropriate mass transfer. However, high negative pressure is not always
286 recommendable, especially if overfull gas in the liquid results in insufficient
287 cavitation effects which leads to little damage of cell wall.

288 After calculated, the extraction conditions were negative pressure -0.05 MPa,
289 liquid/solid ratio 22.74 mL/g, ethanol concentration 50.66%. Thus, in order to
290 facilitate the operation, negative pressure -0.05 MPa, liquid/sample ratio 20:1 mL/g,
291 ethanol concentration 50% were identified as optimal conditions, which were used in
292 the following tests. The optimal extraction yields were arbutin 2.718 ±0.114 mg/g,
293 epicatechin 0.859 ± 0.053 mg/g, hyperin 1.378 ± 0.043 mg/g, 2'-*O*-galloylhyperin
294 5.132 ± 0.198 mg/g and chimaphilin 0.390 ± 0.014 mg/g, respectively.

295 *3.3 Comparison of different extraction methods*

296 HNPCE and NPCE were compared for their performances of extracting target
297 compounds at the optimized conditions (Fig. 4). The extraction time was 15, 30, 45,
298 and 60 min, respectively. For HNPCE, The extraction yields of the target compounds
299 increased in the first 30 min. After 30 min, the extraction yields increased slightly. For
300 NPCE, the extraction yields of epicatechin and chimaphilin reached equilibrium at
301 around 45 min. And other compounds did not reach equilibrium until 60 min.
302 Meanwhile, the extraction yields by NPCE at 30 min were lower than these by

HNPCE at 30 min, the extraction yields of arbutin, epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin increased 68.7%, 72.0%, 43.3%, 62.5% and 34.5%, respectively. Thus, HNPCE is more effective than NPCE on the extraction of target compounds in pyrola. And this result may have two reasons. Firstly, the particle sizes of HNPCE are more suitable than these of NPCE which has been shown in section 3.1. Secondly, homogenate can mix the solvent and pyrola samples sufficiently and promote target compounds release to solvent under the effect of stirring. Moreover, HNPCE was compared with other extraction methods. From the results in Table S1, it indicated that the extraction yields of the five active compounds by HNPCE were higher than those by maceration and refluxing. Meanwhile, the extraction time and liquid/solid of maceration and refluxing were higher than that of HNPCE. Thus, the HNPCE method was more effective than maceration and refluxing on the extraction of active compounds from pyrola. In our other research, we reported a new negative pressure cavitation-microwave assisted extraction (NMAE) method. The extraction yields of hyperin, 2'-O-galloylhyperin and chimaphilin by NMAE were 1.339 ± 0.029, 4.831 ±0.117 and 0.329 ±0.011 mg/g, respectively [40] which were similar with those of HNPCE. However, in the HNPCE process, the samples can be first smashed then inhaled into extraction pot by negative pressure and followed extracted by NPCE. The grinding process and extraction process can by be auto completed without middle process. Thus, the HNPCE method was more simple and effective and this method could be a promising extraction technique for the analytical sample preparation from plants.

325 *3.4 SEM observation*

326 Pyrola samples were examined by SEM to elucidate the morphological changes
327 of samples using different extraction methods, which is helpful in understanding the
328 extraction mechanism. Fig. 5A-D shows the samples micrographs of pulverized,
329 homogenate, NPCE, and HNPCE, respectively. Some differences were observed on
330 the parenchyma of different samples. In Fig. 5A, there was no destruction on the
331 parenchyma for the pulverized sample while little destruction of the microstructure of
332 sample occurred on the homogenate samples. That may be caused by the shear force
333 in the homogenate process. In NPCE and HNPCE, the parenchymas of samples were
334 all greatly changed or destroyed (Fig. 5C and D). And especially in the HNPCE
335 samples, there are nearly no complete parenchyma resulting in more serious
336 destruction than that of the pulverized samples. That meant the HNPCE method could
337 destroy the parenchyma of pyrola samples more seriously than NPCE in the extraction
338 process.

339 3.5 *LC-MS analysis*

340 The composition of the mobile phase was investigated first. A methanol–water
341 system and a acetonitrile–water system were both used in the selection of LC–MS/MS
342 conditions. After optimization, the methanol–water system was found to be more
343 suitable for the separation of the five target compounds. Then the mass spectrometric
344 parameters including precursor ion and product ion, declustering potential, collision
345 energy and collision cell exit potential were optimized and the results were shown in
346 Table 3 and Fig. 6A. Under the optimal LC-MS/MS conditions, the five compounds

can be separated adequately.

Quantification was performed using an external eight-point calibration curve covering the range from 20-5000 ng/mL. All calibration curves exhibited an excellent coefficient of determination ($r^2 \geq 0.99$) within the range of tested concentrations. The LODs (S/N = 3) and LOQs (S/N = 10) for the analytes were less than 0.92 and 3.74 ng/mL, respectively (Table 4). The results of the precision test were summarized in Table 5. The intra-day variations of retention time and peak area were less than 0.51% and 4.11%, and the corresponding inter-day variations were less than 0.62% and 5.64%, respectively. The recovery test had also been done. Table 5 showed that the recoveries varied between 95.84% and 104.19% and the RSD values were between 2.14% and 3.45% for five phenolic compounds. At last, pyrola samples were extracted under the optimal HNPCE conditions and then analyzed by LC–MS/MS. After determined three times, the contents and RSD of the target compounds were calculated and shown in Table 6. The RSD of the target compounds was from 3.20% to 1.96% which meant the HNPCE-LC–MS/MS can be successfully used on the determination of the compounds in pyrola.

*3.6 Antioxidant activity*

After analyzed by LC-MS, it indicated that there are many phenolic compounds in pyrola. Some reports showed that the phenolic compounds exhibit extensive antioxidant activity through their reactivity as hydrogen or electron-donating agents, and metal ion chelating properties. Thus, the antioxidant activity of the extracts by HNPCE was analyzed by DPPH test and reducing power test. The result of DPPH test

was shown in Fig. 7A. When the concentration of sample was 0.25 mg/mL, its DPPH radical scavenging activity was more than 85% which was very close to that of ascorbic acid. And when the concentration was 0.5 mg/mL, the DPPH radical scavenging activity can reach 93.01%. The result of reducing power was shown in Fig. 7B. As shown in Fig 7B, the reducing power of the pyrola extract was higher than the positive control (BHT). The $IC_{50}$s of DPPH test and reducing power test were also calculated. The $IC_{50}$ of HNPCE extract in DPPH test was 0.137mg/mL higher than that of maceration method and refluxing method (Table S1) and moreover, this value was very close to the positive control and the extract of the NMAE method in other research [40]. The $IC_{50}$ of the HNPCE extract in reducing power test was 0.075 mg/mL. This value was higher than the positive control (BHT) and those of maceration method and refluxing method (Table S1). From the results of antioxidant activity, the HNPCE extracts possess better antioxidant activity. Hence, HNPCE is a more effective method to extract active compounds from pyrola. Moreover, the present result provides evidence that the pyrola extracts had a remarkable antioxidant capacity, and it could be used as a valuable antioxidant natural source in food industry. From the results of the LC-MS analysis, it showed that the contents of arbutin, hyperin and 2'-*O*-galloylhyperin were higher than other compounds. Thus, the antioxidant activity of pyrola extract may be related to these compounds.

## 4. Conclusion

A new extraction method HNPCE was developed for extraction of five active compounds from *P. incarnata* Fisch. followed by liquid chromatography–tandem

391 mass spectrometry. Compared with the conventional pulverization method, the
392 homogenate method can produce more sample powders with suitable particle size for
393 extraction. The HNPCE parameters were optimized and the optimal conditions were
394 homogenate time 120 s, -0.05 MPa, liquid/solid ration 22.74 mL/g, ethanol
395 concentration 50.66% and time 30 min. At these conditions, the extraction yields
396 reached arbutin 2.718 ± 0.114 mg/g, epicatechin 0.859 ± 0.053 mg/g, hyperin 1.378 ±
397 0.043 mg/g, 2'-*O*-galloylhyperin 5.132 ± 0.198 mg/g and chimaphilin 0.390 ± 0.014
398 mg/g. Compared with the NPCE at 60 min, the HNPCE had higher extraction yields
399 at 30 min, and the SEM results also indicated that the HNPCE method could destroy
400 the parenchyma of pyrola samples more seriously than NPCE in the extraction
401 process. LC-MS/MS method was then successfully applied for the quantification of
402 five target compounds in pyrola, the intra-day variations of retention time and peak
403 area were less than 0.51% and 4.11%, and the corresponding inter-day variations were
404 less than 0.62% and 5.64%. Thus, H-NPCE as an environmentally friendly and
405 effective extraction method, it possesses more advantages such as no powder dust
406 pollution, easy operation, shorter time, energy saving and higher recoveries, it would
407 be an alternative extraction for the determination of active compounds from plants. At
408 last, the antioxidant activities of the pyrola extracts were also determined. The results
409 showed that pyrola had good antioxidant activities and it was a valuable antioxidant
410 natural source.

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554 **Figure Captions:**

555

556 **Fig. 1** The HNPCE device used in the present study (A) and schematic representation
557 of the HNPCE device (B).

558

559 **Fig. 2** Percentages of different particle size by homogenate and conventional
560 pulverization at different pulverization time.

561

562 **Fig. 3** The extraction yields of the five target compounds by homogenate and
563 conventional pulverization at different pulverization time. (operating parameters of
564 the extraction process: negative pressure -0.05 MPa, liquid/sample ratio 20:1 mL/g,
565 ethanol concentration 50%, extraction time 45 min.)

566

567 **Fig. 4** The comparison of HNPCE and NPCE on the extraction yields of arbutin,
568 epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin

569

570 **Fig. 5** SEM of the pyrola samples: pulverized samples (A); homogenate samples (B);
571 NPCE samples (C); and HNPCE samples (D);

572

573 **Fig. 6** Product ion mass spectra of $[M-H]^-$ ions of arbutin, epicatechin, hyperin,
574 2'-*O*-galloylhyperin and chimaphilin (A) and representative LC–MS/MS
575 chromatogramof pyrola samples. 1: arbutin, 2: epicatechin, 3: hyperin, 4:

576 2'-*O*-galloylhyperin, 5: chimaphilin (B).

577

578 **Fig. 7** The free radical-scavenging activity (A) and reducing power (B) of HNPCE

579 extracts.

**Table 1** Box-Behnken design along with experimental values of arbutin, epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin.

| No. | Negative pressure ($X_1$, MPa) | Liquid/solid ratio ($X_2$, mL/g) | Ethanol concentration($X_3$, M) | $Y_1$[a] (mg/g) | $Y_2$[b] (mg/g) | $Y_3$[c] (mg/g) | $Y_4$[d] (mg/g) | $Y_5$[e] (mg/g) |
|---|---|---|---|---|---|---|---|---|
| 1 | -1(-0.04) | -1(10) | 0(0.05) | 2.085 | 0.579 | 0.870 | 3.484 | 0.312 |
| 2 | 1(-0.06) | -1(10) | 0(0.05) | 2.512 | 0.675 | 0.995 | 4.057 | 0.376 |
| 3 | -1(-0.04) | 1(30) | 0(0.05) | 2.368 | 0.652 | 1.043 | 3.919 | 0.355 |
| 4 | 1(-0.06) | 1(30) | 0(0.05) | 2.556 | 0.788 | 1.26 | 4.843 | 0.383 |
| 5 | -1(-0.04) | 0(20) | -1(0.25) | 2.036 | 0.639 | 1.023 | 3.845 | 0.305 |
| 6 | 1(-0.06) | 0(20) | -1(0.25) | 2.402 | 0.719 | 1.150 | 4.323 | 0.360 |
| 7 | -1(-0.04) | 0(20) | 1(0.75) | 2.144 | 0.733 | 1.173 | 4.408 | 0.330 |
| 8 | 1(-0.06) | 0(20) | 1(0.75) | 2.423 | 0.738 | 1.181 | 4.440 | 0.347 |
| 9 | 0 (-0.05) | -1(10) | -1(0.25) | 2.036 | 0.532 | 0.851 | 3.197 | 0.306 |
| 10 | 0(-0.05) | 1(30) | -1(0.25) | 2.235 | 0.680 | 1.088 | 4.089 | 0.335 |
| 11 | 0(-0.05) | -1(10) | 1(0.75) | 2.010 | 0.571 | 0.913 | 3.962 | 0.301 |
| 12 | 0(-0.05) | 1(30) | -1(0.25) | 2.465 | 0.722 | 1.099 | 4.344 | 0.369 |
| 13 | 0(-0.05) | 0(20) | 0(0.50) | 2.594 | 0.850 | 1.359 | 5.109 | 0.388 |
| 14 | 0(-0.05) | 0(20) | 0(0.50) | 2.583 | 0.839 | 1.342 | 5.045 | 0.387 |
| 15 | 0(-0.05) | 0(20) | 0(0.50) | 2.601 | 0.830 | 1.328 | 4.992 | 0.389 |
| 16 | 0(-0.05) | 0(20) | 0(0.50) | 2.636 | 0.823 | 1.317 | 4.949 | 0.395 |
| 17 | 0(-0.05) | 0(20) | 0(0.50) | 2.530 | 0.876 | 1.402 | 5.268 | 0.379 |

**Table 2** Significance of regression coefficient for arbutin, epicatechin, hyperin, 2'-*O*-galloylhyperin and chimaphilin.

| Variables | Eq (1) | | Eq (2) | | Eq (3) | | Eq (4) | | Eq (5) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F-value | *p*-value | F-value | *p*-value | F-value | *p*-value | F-value | *p*-value | F-value | *p*-value |
| Model | 31.41 | < 0.0001 | 21.23 | 0.0003 | 36.05 | < 0.0001 | 18.18 | 0.0005 | 16.09 | 0.0007 |
| $X_1$ | 74.54 | < 0.0001 | 12.8 | 0.009 | 19.76 | 0.003 | 15.24 | 0.0059 | 27.72 | 0.0012 |
| $X_2$ | 45.18 | 0.0003 | 31.38 | 0.0008 | 61.77 | 0.0001 | 23.55 | 0.0019 | 23.7 | 0.0018 |
| $X_3$ | 5.21 | 0.0565 | 5.46 | 0.0521 | 4.96 | 0.0612 | 10.93 | 0.013 | 1.42 | 0.2721 |
| $X_1X_2$ | 5.36 | 0.0537 | 0.12 | 0.7433 | 1.41 | 0.2737 | 0.93 | 0.3665 | 2.84 | 0.1357 |
| $X_1X_3$ | 0.71 | 0.4271 | 1.63 | 0.242 | 1.98 | 0.2022 | 1.5 | 0.2596 | 4.06 | 0.0839 |
| $X_2X_3$ | 6.15 | 0.0422 | 2.61E-03 | 0.9607 | 0.43 | 0.5313 | 1.97 | 0.2035 | 3.34 | 0.1105 |
| $X_1X_1$ | 6.06 | 0.0434 | 10.37 | 0.0147 | 19.36 | 0.0032 | 12.28 | 0.0099 | 4.72 | 0.0664 |
| $X_2X_2$ | 25.37 | 0.0015 | 78.92 | < 0.0001 | 141.52 | < 0.0001 | 56.58 | 0.0001 | 11.7 | 0.0111 |
| $X_3X_3$ | 103.36 | < 0.0001 | 38.12 | 0.0005 | 52.91 | 0.0002 | 30.36 | 0.0009 | 59.25 | 0.0001 |
| Lack of fit | 4.24 | 0.0982 | 3.47 | 0.1303 | 1.83 | 0.2812 | 1.92 | 0.2677 | 1.44 | 0.3553 |
| $R^2$ | 0.9753 | | 0.9648 | | 0.9789 | | 0.959 | | 0.9539 | |
| Adjusted $R^2$ | 0.9435 | | 0.9196 | | 0.9517 | | 0.9062 | | 0.8946 | |

**Table 3** Mass spectrometric parameters for seven phenolic compounds.

| Analyte | DP (V) | CE (V) | CXP (V) | MRM (amu) |
|---|---|---|---|---|
| Arbutin | -56 | -23 | -3 | 271.2→161.1 |
| Epicatechin | -70 | -25 | -3 | 289.0→245.0 |
| Hyperin | -67 | -31 | -9 | 463.1→300.0 |
| 2'-*O*-galloylhyperin | -55 | -36 | -8 | 615.2→301.3 |
| Chimaphilin | -39 | -33 | -10 | 185.8→158.9 |

[a] Declustering potential

[b] Collision energy

[c] Collision cell exit potential

**Table 4** Calibration equation, LODs and LOQs for five target compounds.

| Compound | Linearity range (ng/mL) | Calibration equation[a] | LOD (ng/mL) | LOQ (ng/mL) | $R^2$ |
|---|---|---|---|---|---|
| Arbutin | 200-2000 | y = 380.4x + 42.3 | 1.86 | 6.74 | 0.9947 |
| Epicatechin | 50-500 | y = 457.2x - 56.5 | 1.21 | 4.45 | 0.9929 |
| Hyperin | 500-5000 | y = 651.8x + 132.4 | 0.92 | 3.74 | 0.9941 |
| 2'-*O*-galloylhyperin | 500-5000 | y = 738.9x + 50.3 | 1.02 | 4.31 | 0.9963 |
| Chimaphilin | 20-200 | y = 422.5x -36.8 | 1.35 | 5.48 | 0.9952 |

[a] y: peak area of analyte; x: concentration of analyte (ng/mL).

**Table 5** Precision and recovery of five target compounds

| Analyte | Intra-day variations | | Inter-day variations | | Original (mg) | Spiked (mg) | Found (mg) | Recovery (%) | RSD (%) |
|---|---|---|---|---|---|---|---|---|---|
| | RSD for RT (%) | RSD for PA (%) | RSD for RT (%) | RSD for PA (%) | | | | | |
| Arbutin | 0.35 | 3.22 | 0.47 | 4.57 | 2.575 | 1.013 | 3.506 | 97.72% | 2.14% |
| | | | | | | 2.274 | 4.875 | 100.53% | 3.21% |
| Epicatechin | 0.42 | 4.11 | 0.53 | 3.92 | 0.842 | 0.684 | 1.563 | 102.45% | 2.25% |
| | | | | | | 1.421 | 2.358 | 104.19% | 3.45% |
| Hyperin | 0.51 | 3.59 | 0.62 | 5.64 | 1.345 | 0.531 | 1.85 | 98.63% | 3.03% |
| | | | | | | 1.047 | 2.373 | 99.21% | 2.27% |
| 2'-*O*-galloylhyperin | 0.47 | 3.74 | 0.44 | 5.21 | 5.177 | 1.227 | 6.138 | 95.84% | 2.75% |
| | | | | | | 3.453 | 8.828 | 102.29% | 2.36% |
| Chimaphilin | 0.31 | 2.75 | 0.38 | 4.04 | 0.386 | 0.305 | 0.666 | 96.35% | 3.11% |
| | | | | | | 0.678 | 1.034 | 97.22% | 2.98% |

**Table 6** Contents of five target compounds in pyrola with the HNPCE-LC-MS/MS method (*n*=3).

| Analyte | Content (mg/g) | RSD (%) |
|---|---|---|
| Arbutin | 2.695 | 2.25 |
| Epicatechin | 0.834 | 3.14 |
| Hyperin | 1.383 | 2.45 |
| 2'-*O*-galloylhyperin | 5.088 | 3.20 |
| Chimaphilin | 0.395 | 1.96 |

**Fig. 1** The HNPCE device used in the present study (A) and schematic representation of the HNPCE device (B).

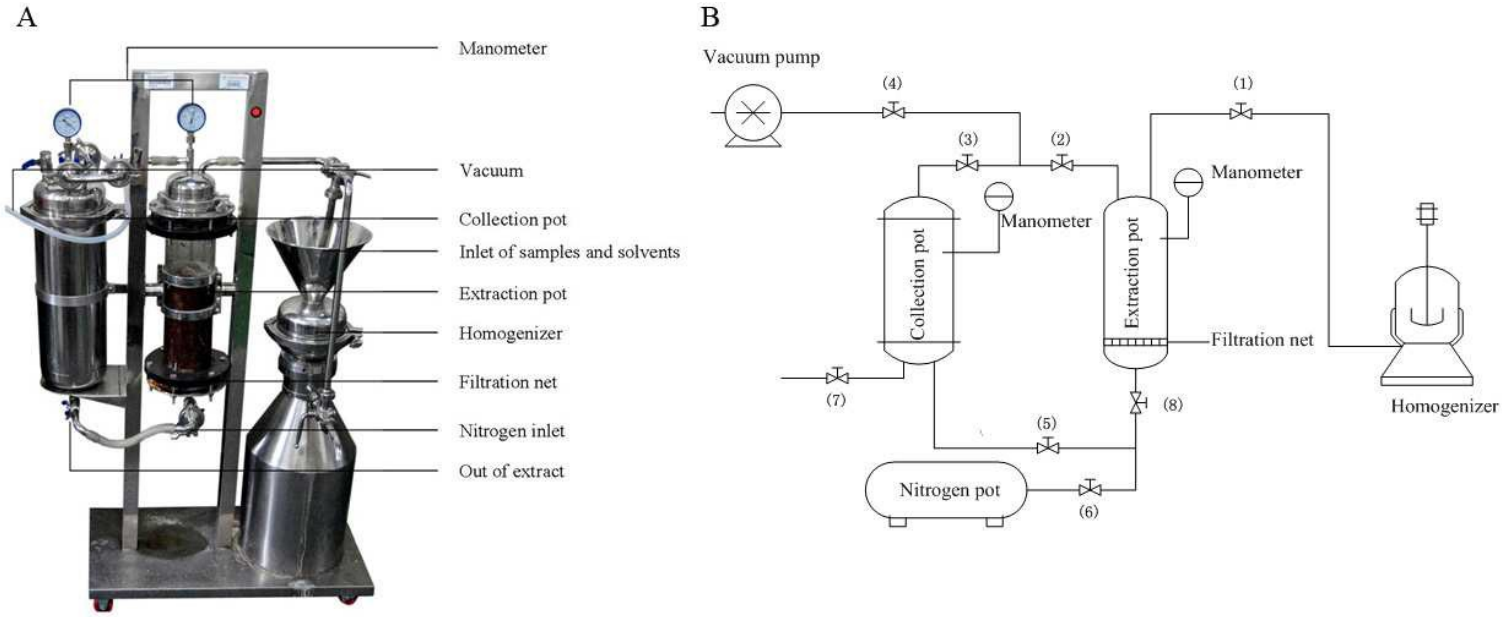


**Fig. 2**

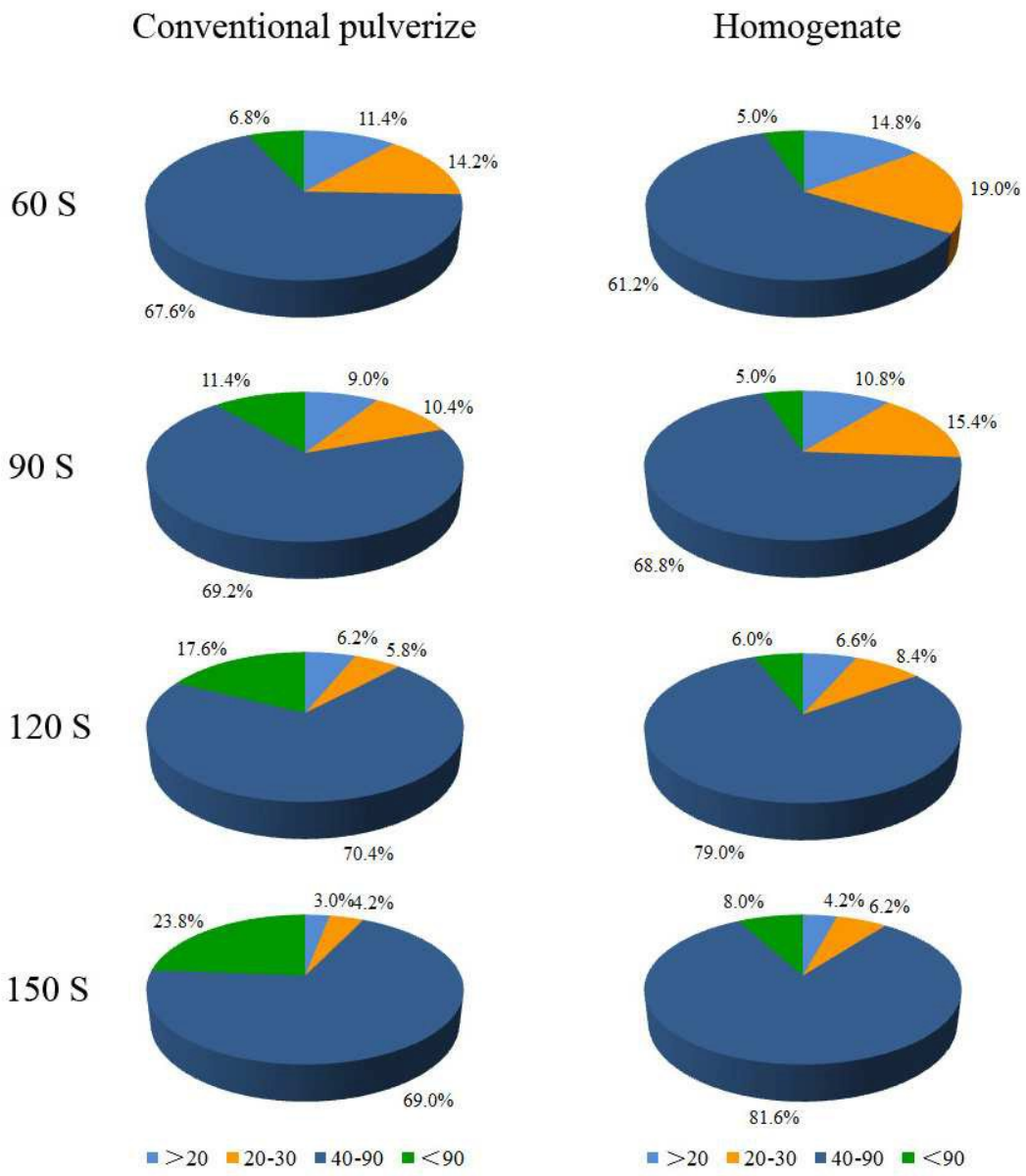


**Fig. 3**

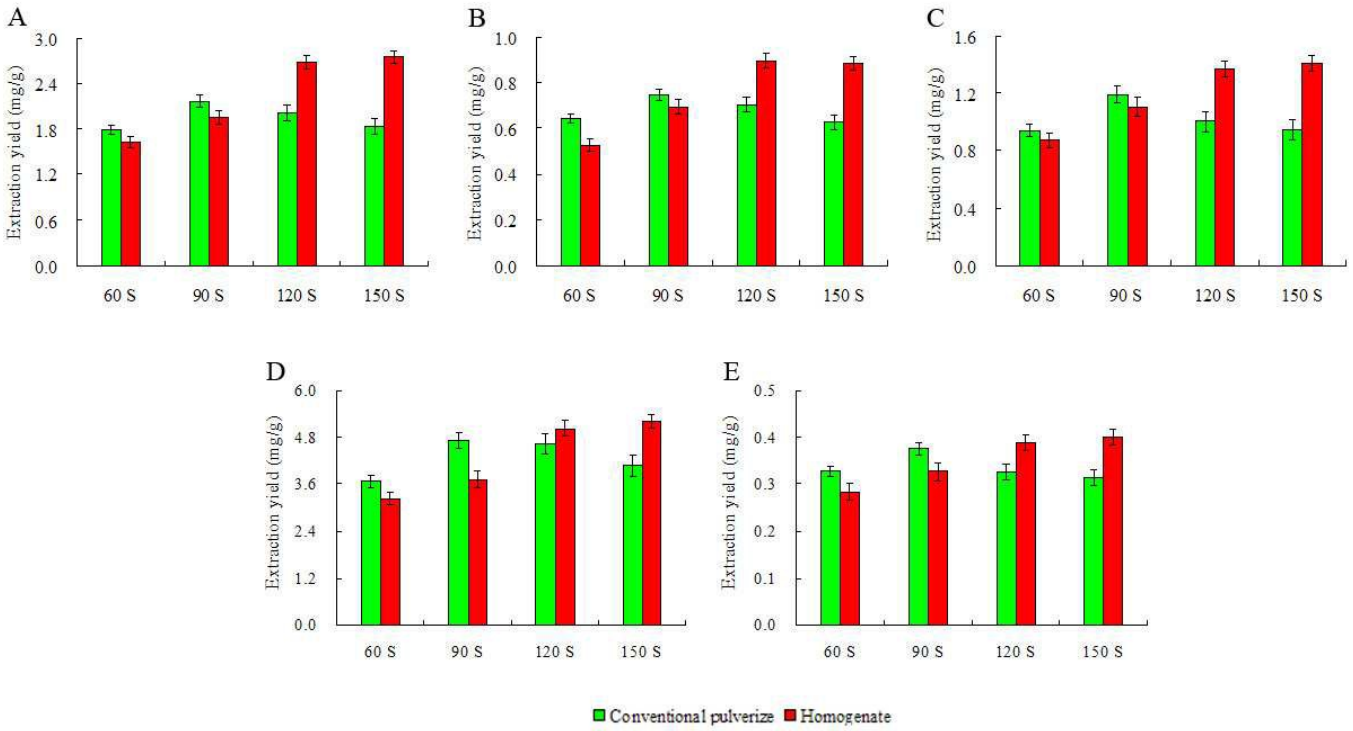


**Fig. 4**

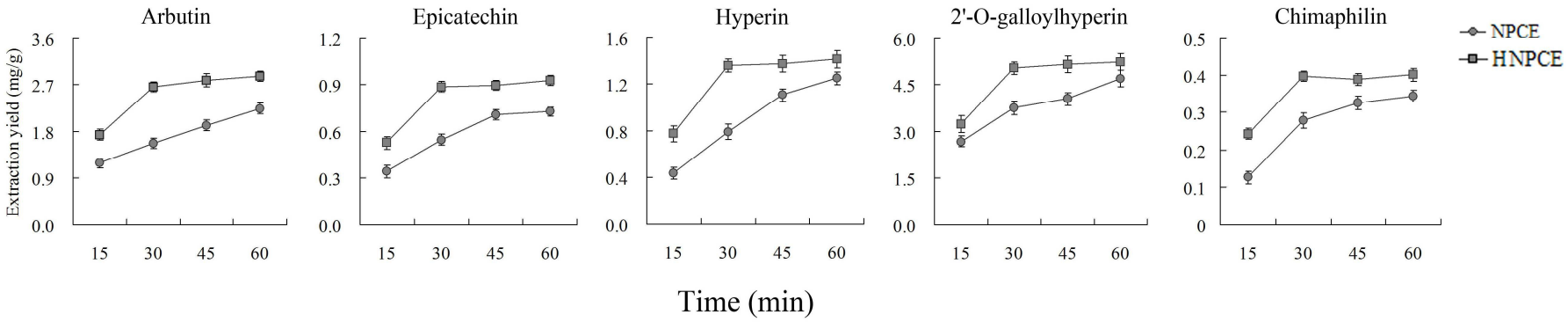


**Fig. 5**

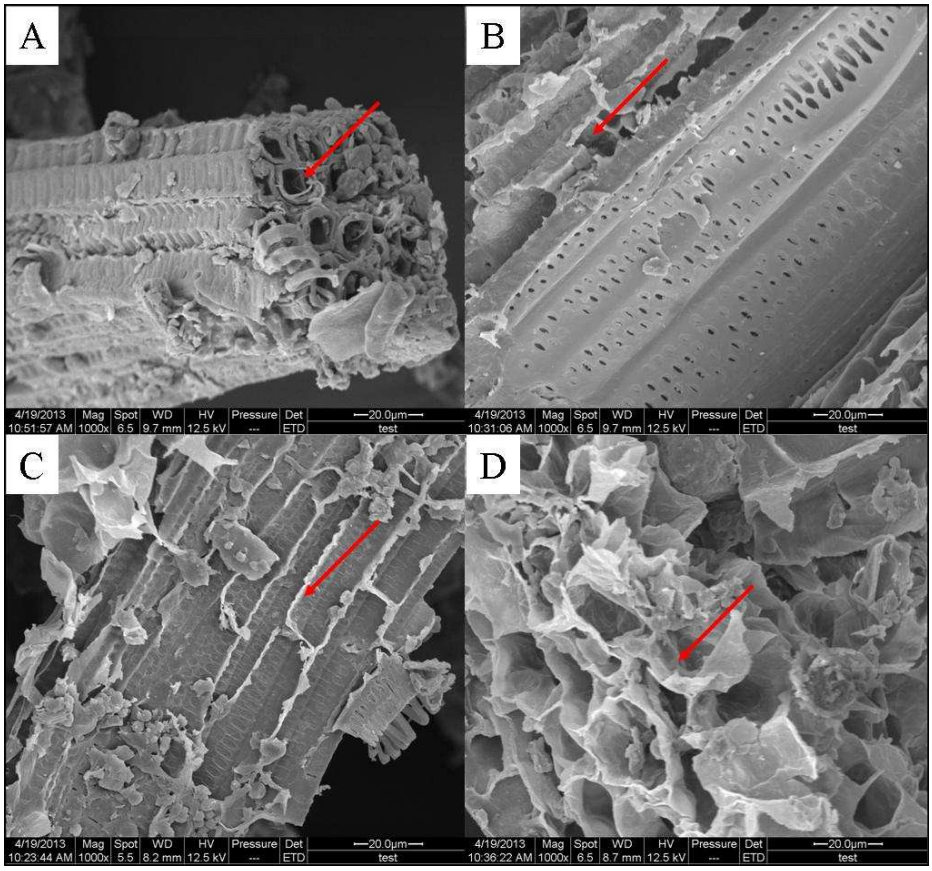


**Fig. 6A**

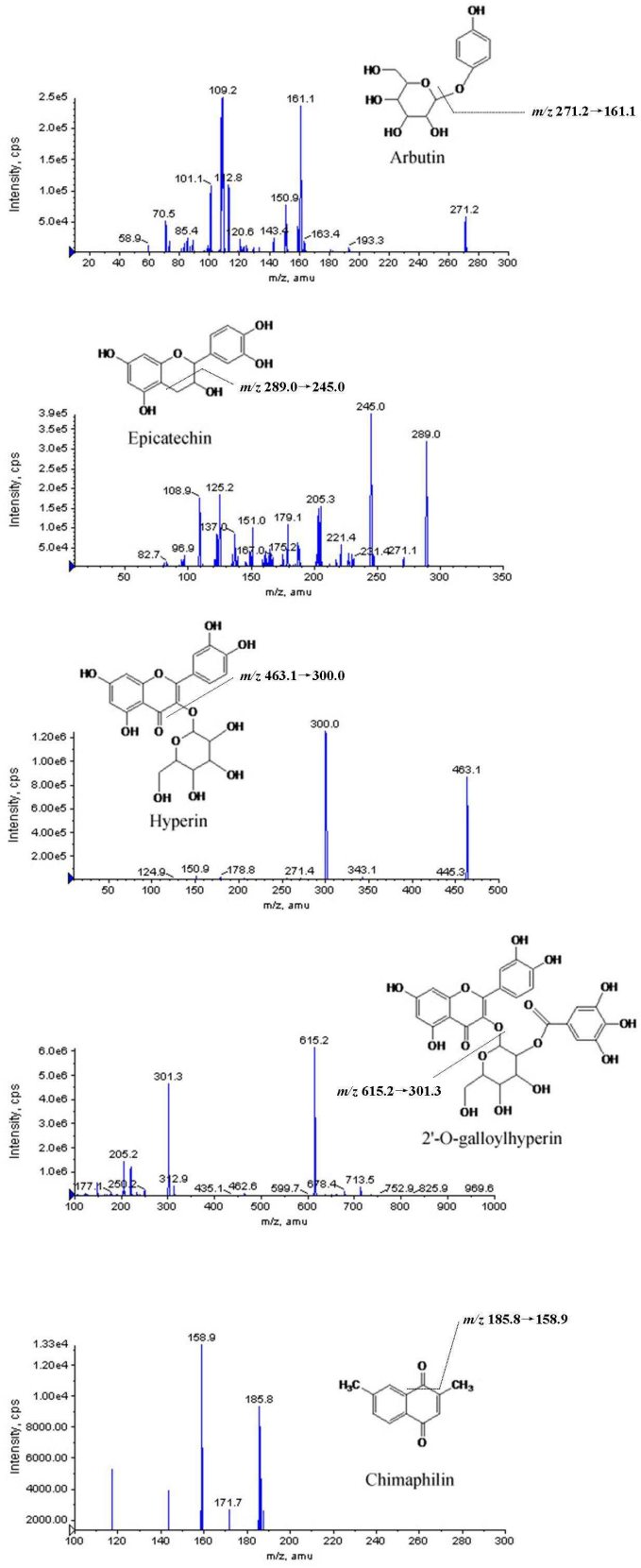


**Fig. 6B**

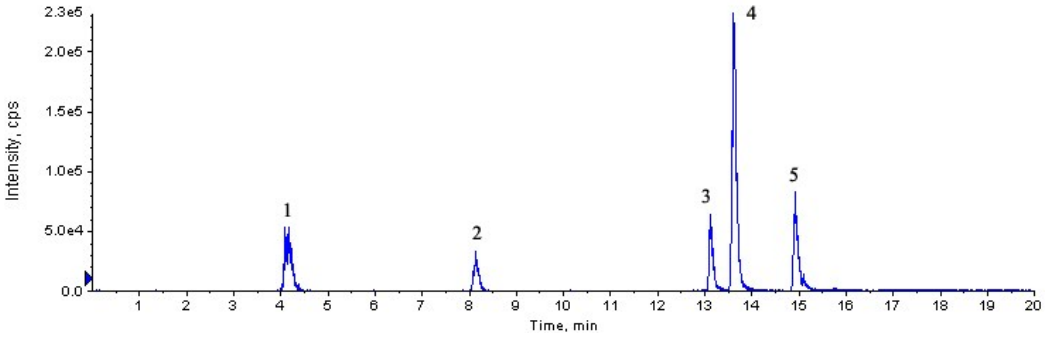
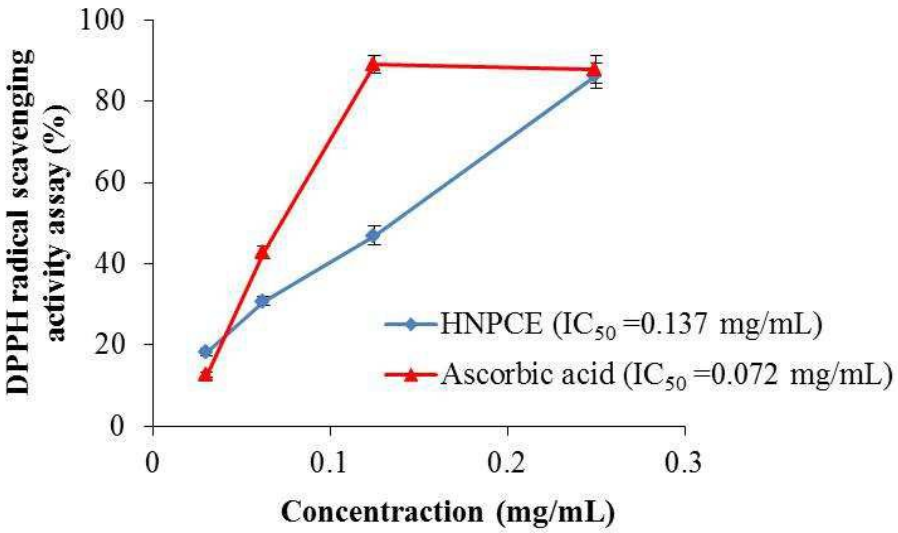


**Fig. 7**

**A**




**B**