Screening potential mitochondria-targeting compounds from traditional Chinese medicines using a mitochondria-based centrifugal ultrafiltration/liquid chromatography/mass spectrometry method

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1. Introduction

Mitochondria play a crucial role in maintaining cellular life and are involved in various significant bio-functions and metabolic pathways, including calcium homeostasis, thermogenesis, gluconeogenesis, citric acid cycle, β-oxidation of fatty acids, urea cycle, electron transport chain and oxidative phosphorylation that end with ATP generation [1]. The integrity of mitochondrial function is fundamental to cellular homeostasis. Consequently, mitochondrial dysfunctions will contribute to many diseases with a great diversity of clinical appearances, such as neurodegenerative disorders, ischemia-reperfusion injury, diabetes, aging, inherited mitochondrial diseases and most importantly, cancer [2]. As mitochondria are key modulators of cellular homeostasis, mitochondrially-targeted compounds that remedy mitochondrial dysfunctions represent an attractive approach for the treatment of human disorders [3–5].

Traditional Chinese medicines (TCMs) have a long history of clinical testing and reliable therapeutic efficacy, which have gained attention as excellent sources of bioactive compounds for the discovery of new drugs [6]. A rapidly growing number of literatures proposed that many TCMs could regulate mitochondrial functions to perform their pharmaceutical efficacy [7]. For example, extracts of Puerariae Radix (PR) and components of Chuanxiong Radix (CR) have protective effects on mitochondria in TCMs are almost unrecognized so far, hampering the enhancement of our comprehension about therapeutic principles of TCMs, and the development of novel potential drugs from TCMs. Disclosing the presence of bioactive substances targeting to mitochondria in TCMs has thus become of utmost importance.

The traditional activity-guided isolation procedure for complex mixtures is a labor-intensive, time-consuming, and expensive process, and it is often invalid for the direct search of bioactive constituents from complex samples [10]. High-throughput...
screening [11] and high-content screening methods [12] have also been used for the efficient identification of mitochondria-targeting compounds, but they cannot be applied for direct screening of multiple ligands from complex agents simultaneously. In our previous study, we developed an efficient mitochondria-based centrifugal ultrafiltration/liquid chromatography/mass spectrometry method, which is called screening method for mitochondria-targeted bioactive constituents (SM-MBC), and our method is compatible with the searching of mitochondria-targeting compounds from complex samples [7]. It was confirmed that this method simultaneously possessed excellent recognition, separation and identification abilities, and displayed advantages as efficient use of labor, a simple procedure, and low time and sample requirements. Thus, SM-MBC, preventing unnecessary resource utilization in downstream isolation of inactive compounds from extracts used in the screening process, can be performed to the direct screening of mitochondria-targeting compounds in complex matrix such as TCMs.

In the present study, we applied SM-MBC for the direct screening of natural mitochondria-targeting compounds from TCMs (i.e. PR and CR). Bioactive molecules selectively linked to mitochondria were isolated by centrifugal ultrafiltration (CU). The isolated fractions were then collected and injected into liquid chromatography/mass spectrometry (LC/MS) for rapid isolation and identification. Our screening results were meaningful for in-depth understanding of mechanisms underlying TCMs action, but also for the development of mitochondrial modulators from TCMs. Moreover, SM-MBC was shown to be an efficient method for the rapid screening of mitochondria-targeting compounds from complex samples.

2. Materials and methods

2.1. Chemicals and materials

Puerarin (lot no. G-008–131221), daidzein (lot no. D-016–131102) and daidzin (lot no. D-013–131111) were purchased from Chengdu Herbupury Co., Ltd. (Chengdu, China), 3′-methoxypuerarin (lot no. 131217), 3′-hydroxy puerarin (lot no. 131416), senkyunolide A (lot no. 140914), ligustilide (lot no. 131204) and levistolide A (lot no. 130910) were purchased from Chengdu Pufeide Biological Technology Co., Ltd. (Chengdu, China). Formononetin (lot no. GMED-0013) was provided by Hong Kong Jockey Club Institute of Chinese Medicine (Hongkong, China). Dulbecco’s Modified Eagle’s Medium (DMEM) and Bicinchoninic Acid (BCA) protein determination kit were obtained from M&G Gene Technology (Beijing) Ltd. (Beijing, China). DMEM/F12 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). WST-8 Kit (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) was purchased from Neurobc® (Beijing, China). Nycodenz was purchased from Axis-shield PoC AS (Oslo, Norway). HPLC grade methanol and acetonitrile were provided by Fisher Scientific (Fairlawn, NJ, USA). Deionized water was purified using a Milli-Q Water Purification System (Millipore, Billerica, MA, USA). Calycosin was isolated from Astragalus Radix by our group. The structure was verified by LC/MS and nuclear magnetic resonance (NMR), and the purity was found to be > 98% by HPLC–DAD (based on the percentage of total peak area). All other reagents used were of analytical reagent grade or higher.

PR (purchase date 2014/04) and CR (purchase date 2009/12) were purchased from the Tianheng Drug Store (Beijing, China) and the Hebei Anguo Yaoxing Pharmaceutical Co. (Anguo, Hebei, China), respectively. All the samples were authenticated by Professor Shao-Qing Cai and the voucher specimens of PR (No. 7546) and CR (No. 6332) have been deposited in the Herbarium of Pharmacognosy, School of Pharmaceutical Sciences, Peking University (Beijing, China).

2.2. Experimental animals

Experimental protocols involving conscious animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Biomedical Ethical Committee of Peking University (No. SYXK2011-0039). Healthy male Sprague-Dawley rats (300 ± 50 g) provided by the Department of Laboratory Animal Science, Peking University Health Science Center (Beijing, China) were used in this study. Rats were bred under an environmentally controlled room with ad libitum access to food and tap water. In addition, neonatal Sprague-Dawley rats (< 24 h) provided by the Vital River Laboratories (Beijing, China) were used for the primary culture of cardiomyocytes. All efforts were made to minimize the number of the animals used and their suffering.

2.3. Preparation of analytical solutions

Working solutions of PR (450 mg/mL) and CR (600 mg/mL) were prepared by dissolving the freeze-drying powder of PR and CR extract (the preparation methods are described in the Supplementary material) in dimethyl sulfoxide (DMSO). For the pharmacological test, analytical solutions of standard compounds from PR and CR extracts, including puerarin, daidzein, daidzin, formononetin, ligustilide and levistolide A were prepared in DMSO and diluted with physiological saline to the demanded concentration. All solutions were stored at 4 °C in the dark.

2.4. Preparation of mitochondrial suspension

Mitochondrial suspension was prepared as previously described [7]. Briefly, hearts isolated from Sprague-Dawley rats were quickly placed into ice-cold isolation buffer (buffer A: 210 mM mannitol, 70 mM sucrose, 10 mM Tris base, 1 mM EDTA and 0.5 mM EGTA, pH 7.4) to remove blood, mired into 1 mm 3 pieces, and homogenized by using a Dounce glass homogenizer (Kimbale/Kontes, Vineland, NJ, USA) with buffer B (buffer A plus a mixture of 1 mM PMSF and protease inhibitor cocktail). After centrifugation at 1000 × g for 10 min, the supernatant was collected and centrifuged at 10,000 × g for 10 min to obtain crude mitochondria. Nycodenz was dissolved to 34%, 30%, 25%, 23% and 20% (w/v) with buffer B. The crude mitochondria were suspended in 1.3 mL of 25% Nycodenz and layered on a discontinuous Nycodenz gradient of 0.5 mL of 34% and 0.9 mL of 30%, which was topped off with 0.9 mL of 23% and finally 0.4 mL of 20%. The gradient was centrifuged at 52,000 × g for 90 min in a Beckman SW 60 Ti rotor (Beckman Coulter, Inc., Fullerton, CA, USA). The band at the 25%/30% interface was collected, diluted with the same volume buffer A and then centrifuged at 10,000 × g for 10 min. The pellet was washed with buffer B and collected as purified mitochondria that were resuspended with buffer A to obtain a 1.0 g/L of mitochondrial suspension. Mitochondrial protein concentration was determined using BCA method. Furthermore, a part of the mitochondrial suspension was persistently heated for 2 h in boiling water to produce denatured mitochondria without their original biological functions.

2.5. Screening process with SM-MBC

Screening process using SM-MBC was based on previously reported method (Fig. 1) [7]. Briefly, 5 μL PR working solution and CR working solution were separately incubated with 200 μL of the
mitochondrial suspension at 37 °C for 60 min (90 min for the CR working solution), and then were filtered through a 10 kDa molecular weight cutoff ultrafiltration membrane (Microcon YM-10, Millipore Co., Bedford, MA, USA) by centrifugation at 14,000 × g for 25 min at 4 °C. Pellets entrapped in the membranes were washed three times with 200 μL ammonium acetate buffer (50 mM, pH 7.5) through centrifugation at 14,000 × g at 4 °C for 25 min. Afterward, bound molecules were released from mitochondria by adding 400 μL of 80% aqueous methanol followed by centrifugation at 14,000 × g for 25 min at room temperature. Purified ultrafiltrates were dried under a stream of nitrogen and reconstituted with 100 μL of 80% methanol. Samples containing screened compounds were analyzed by LC/MS (The conditions of LC/MS analysis are described in the Supplementary material). Control experiments for nonspecific binding were performed in a similar way with denatured mitochondria. LC/MS peaks obtained with and without denatured mitochondria were compared. When the percentage of discrepancy of peak area between experiment and control (ΔP) was > 20%, this increase in peak area indicated a specific binding that was deemed as the mitochondria-targeting compounds. ΔP value was calculated using the formula:

\[ ΔP = \frac{(P_c - P_e)}{P_e} \times 100 \]

where \( P_c \) and \( P_e \) are the peak areas in the experiment and control, respectively.

2.6. Determination of mitochondrial permeability transition pore opening in isolated myocardial mitochondria

The opening of the mitochondrial permeability transition pore (mPTP) was determined by \( \text{Ca}^{2+} \)-induced swelling of isolated myocardial mitochondria and was performed to interact the interaction between the hit compounds and the mitochondria. The mPTP opening results in mitochondrial swelling, which will induce a decrease of absorbance at 520 nm (\( A_{520} \)). This absorbance fluctuation provides a convenient and frequently used determination of the mPTP opening [7,13]. Briefly, isolated cardiac crude mitochondria (0.25 g/L) were resuspended by swelling buffer (120 mM KCl, 20 mM MOPS, 10 mM Tris-HCl, 5 mM KH₂PO₄, pH 7.4), and then preincubated with tested compounds for 3 min at room temperature followed by the addition of 250 μM \( \text{CaCl}_2 \) to induce mPTP opening for 15 min. Afterward, the \( A_{520} \) was immediately measured with UV–VIS Spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China). The experiment was performed five times.

2.7. Evaluation of cancerous cell proliferation

For cancerous cell proliferation assay, aliquots of HepG2 cells (\( 2 \times 10^4 \) cells/well) were seeded in 96-well plate, and cultivated in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂. Confluent beating cells were then treated with tested compounds at different concentrations, and imaged using the CloneSelect Imager system (Genetix, UK) after 0, 24, 48, 72 and 96 h treatment. The proliferation curves were drawn with the cell confluence (%) at the indicated time points to evaluate the inhibition effects of compounds on cell proliferation. The assay was performed five times.

2.8. Assessment of cellular viability in cardiomyocyte model of hypoxia/reoxygenation (H/R) injury

Cellular viability was measured using the cell counting kit (CCK)-8 assay, according to the manufacturer’s recommendations. Confluent beating cardiomyocytes from neonatal rats primarily cultured for 4 days were exposed to hypoxia for 2 h, and then were incubated with tested compounds. Subsequently treated cells were reoxygenated for 1 h. After experimental treatment, 10 μL of WST-8 solution was added to each well, and cardiomyocytes were incubated for additional 2 h at 37 °C. The absorbance of each well at 450 nm was determined using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The percentage of cellular viability was calculated by the following formula [14]:

\[ \% \text{ cell viability} = \left( \frac{\text{mean absorbance in test wells} - \text{mean absorbance in blank wells}}{\text{mean absorbance in negative control well}} \right) \times 100 \]

2.9. Data treatment

All data are presented as mean ± SD. For statistical analysis, one-way analysis of variance (ANOVA; Dunnett’s method) was performed using SPSS software (13.0 for Windows; SPSS, Chicago, IL, USA). \( P < 0.05 \) (two-tailed) was considered significant.

3. Results and discussion

3.1. Principle of SM-MBC

SM-MBC was previously developed by combining CU and LC/MS techniques to perform the direct search for mitochondria-targeting compounds in complex samples as TCMs compounds [7]. In this method, active constituents against specific targets in mitochondria could be specifically recognized and isolated. Structural characteristics of corresponding constituents were identified by LC/MS (Fig. 1). SM-MBC showed the recognition, separation and identification capabilities that were sufficiently validated using positive and negative controls, avoiding the use of unnecessary resources on downstream isolation of compounds of little or no value from extracts used in the screening process. Thus, SM-MBC can be efficiently applied for the identification of mitochondria-targeting compounds in complex preparations.
3.2. Effect of screening conditions

The developed SM-MBC was used to identify natural mitochondria-targeting compounds from PR and CR extracts. In order to acquire the best screening performance for active compounds from the extracts and investigate the major influencing factors in SM-MBC, the effects of several screening conditions were investigated, such as mitochondrial concentration, sample concentration and incubation time.

First, different mitochondrial concentrations (0.25, 0.50 and 1.0 g/L) were used for screening the active compounds present in PR and CR extracts. The number of active compounds selected from the two extracts remarkably increased with the increase of mitochondrial concentration (Figs. S1 and S2), which was consistent with the previously-reported screening of other extracts [7]. Therefore, mitochondrial concentration influences SM-MBC sensitivity. Higher mitochondrial concentrations were related to higher sensitivity, which could select more active compounds from TCMs extracts. However, higher mitochondrial concentrations ( > 1.0 g/L) will block the ultrafiltration membrane, and 1.0 g/L was the optimum mitochondrial concentration for SM-MBC, enabling the screening of the maximum number of active compounds from TCMs extracts.

Next, three concentrations of PR (2.625, 5.250 and 10.50 g/L) and CR (3.875, 7.750 and 15.50 g/L) samples were separately used for the screening of active compounds present in PR and CR extracts. The number of active compounds selected from the two extracts remarkably increased with the increase of mitochondrial concentration (Figs. S1 and S2), which was consistent with the previously-reported screening of other extracts [7]. Therefore, mitochondrial concentration influences SM-MBC sensitivity. Higher mitochondrial concentrations were related to higher sensitivity, which could select more active compounds from TCMs extracts. However, higher mitochondrial concentrations ( > 1.0 g/L) will block the ultrafiltration membrane, and 1.0 g/L was the optimum mitochondrial concentration for SM-MBC, enabling the screening of the maximum number of active compounds from TCMs extracts.

Finally, the effect of different incubation times (30, 60 and 90 min) was investigated. The number of active compounds found in PR and CR extracts increased with the incubation time (Figs. S5 and S6), but identical results were obtained at 60 and 90 min for the PR extract. Thus, 60 min incubation time was sufficient for the screening of the PR extract and 90 min for the screening of the CR extract. Furthermore, we previously found that 60 or 90 min was the optimal incubation time for the screening of active compounds from other two TCMs extracts [7]. Thus, the optimal incubation time for the screening of active compounds from TCMs extracts by SM-MBC might be in the range of 60–90 min, which should be optimized to obtain the best screening performance.

3.3. Screening for the potential mitochondria-targeting compounds in TCMs extracts

Some TCMs such as PR [15,16] and CR [17,18] have been suggested to possess a range of pharmaceutical effects, including anti-cancer, cardio-, hepatic- and neuro-protection. It has been reported that several substances in PR, such as puerarin [13], daidzin
| NO. | $t_r$ (min) | $\Delta t$ (%, n = 3) | UV $\lambda_{max}$ (nm) | [M + H]$^+$ $m/z$ | ESI-MS$^+$ (+) $m/z$ (abundance) | [M - H]$^-$ $m/z$ | ESI-MS$^-$ (-) $m/z$ (abundance) | Predicted formula | Meas. ($m/z$) | Pred. ($m/z$) | Diff. (ppm) | DBE | Assigned identification |
|-----|-------------|-------------------|----------------|-----------------|------------------|-----------------|------------------|-----------------|--------------------|-----------------|-----------------|----------|---------------------|
| P1  | 17.811      | 57.6 ± 16.8       | 250,300        | 503.1198        | ---               | ---              | ---              | ---              | C$_{24}$H$_{22}$O$_{12}$ | 503.1198        | 503.1184        | 2.78     | 14                  | Malonyldaidzin       |
| P2 a| 18.441      | 50.8 ± 21.2       | 248, 289       | 433.1138        | 431.0989         | 431.0984         | 1.16             | 12              | C$_{21}$H$_{20}$O$_{10}$ | 431.0989        | 431.0984        | 1.16     | 12                  | 3'- Hydroxyperarin    |
| P3 a| 22.742      | 68.4 ± 17.8       | 248, 305       | 417.1182        | 415.1044         | 415.1104         | 0.48             | 12              | C$_{21}$H$_{16}$O$_{9}$ | 417.1182        | 417.1180        | 0.48     | 12                  | Puerarin               |
| P4 a| 24.507      | 54.1 ± 20.4       | 249, 290       | 447.1298        | 445.1146         | 445.1140         | 1.35             | 12              | C$_{22}$H$_{22}$O$_{10}$ | 445.1146        | 445.1140        | 1.35     | 12                  | 3'-Methoxyperarin     |
| P5  | 24.956      | 49.6 ± 16.7       | 248, 304       | 549.1607        | 547.1456         | 547.1457         | 0.18             | 13              | C$_{26}$H$_{28}$O$_{13}$ | 547.1456        | 547.1457        | 0.18     | 13                  | Puerarin-xyloside or Mirificin |
| P6 a| 28.378      | 56.3 ± 21.7       | 250, 300       | 417.1187        | 415.1040         | ---              | ---              | ---              | C$_{21}$H$_{16}$O$_{9}$ | 415.1040        | 415.1035        | 0.22     | 12                  | Daidzin               |
| P7  | 53.527      | 40.2 ± 18.0       | 286, 313       | 475.1597        | 473.1428         | 473.1428         | 0.42             | 12              | C$_{24}$H$_{16}$O$_{12}$ | 473.1428        | 473.1428        | 0.42     | 12                  | Sophoradise A           |
| P8 a| 56.363      | 47.4 ± 15.3       | 249, 301       | 255.0630        | 253.0498         | 253.0506         | 0.36             | 11              | C$_{26}$H$_{20}$O$_{4}$ | 253.0498        | 253.0506        | 0.36     | 11                  | Daidzein              |
| P9 a| 66.538      | 35.1 ± 13.6       | 259            | 271.0602        | 269.0441         | ---              | ---              | ---              | C$_{17}$H$_{17}$O$_{5}$ | 271.0602        | 271.0601        | 0.37     | 11                  | Genistein             |

Table 1: LC/MS data and assignment of the 11 mitochondria-targeting compounds in the PR extract.
**Table 2**

LC/MS data and assignment of the 12 mitochondria-targeting compounds in the CR extract.

| NO. | tR (min) | ΔP (%) | UV λmax (nm) | [M+H]+ m/z | [M-H]+ m/z | Predicted formula | Meas. (m/z) | Pred. (m/z) | Diff. (ppm) | DBE | Assigned identification |
|-----|----------|--------|-------------|-----------|-------|-----------------|---------|-----------|----------|------|--------------------------|
| C1  | 40.890   | 49.7 ± 15.3 | 212,263,291,350 | —         | —     | —               | —       | —         | —        | —    | —                        |
| C2  | 41.333   | 57.0 ± 18.1 | 231,280,343 | —         | —     | —               | —       | —         | —        | —    | —                        |
| C3  | 54.978   | 40.3 ± 14.7 | 213,266,318 | —         | —     | 203.0712       | C18H12O4 | 203.0712  | 203.0714 | 0.98 | 7                        |
| C4a | 56.783   | 67.7 ± 20.8 | 279         | 193.1221 | —     | 193.1221       | C12H12O4 | 193.1221  | 193.1223 | 1.04 | 5                        |
| C5  | 57.907   | 53.1 ± 18.5 | 228,276     | 207.0996 | —     | 207.0996       | C12H12O4 | 207.0996  | 207.1016 | 9.66 | 6                        |
| C6  | 60.787   | 41.2 ± 17.8 | 220         | 207.1013 | —     | 207.1013       | C12H12O4 | 207.1013  | 207.1016 | 1.45 | 6                        |
| C7a | 61.443   | 68.4 ± 21.0 | 206,281,327 | 191.1059 | —     | 191.1059       | C12H14O4 | 191.1059  | 191.1067 | 4.19 | 6                        |
| C8  | 61.892   | 37.8 ± 15.2 | 210,233,258,312 | 520.3379 | —     | 520.3379       | C12H14N3O6 | 520.3379 | 520.3381 | 0.38 | 8                        |
| C9  | 66.548   | 43.0 ± 15.8 | 256,286,327 | 500.3302 | —     | 500.3302       | C25H32O5 | 500.3302  | 500.3330 | 3.50 | 5                        |
| C10 | 72.693   | 48.1 ± 16.5 | 220,283     | 413.2307 | —     | 413.2307       | C12H12O4 | 413.2307  | 413.2323 | 3.73 | 10                       |
| C11a| 73.945   | 36.6 ± 14.1 | 230,283     | 381.2066 | —     | 381.2066       | C24H28O4 | 381.2066  | 381.2060 | 0.57 | 11                       |
| C12 | 76.663   | 38.4 ± 12.6 | 295         | —         | —     | —               | —       | —         | —        | —    | —                        |

*Comparison with standards.

b ΔP was calculated using the following formula: ΔP = (Pc/P) × 100, where P and Pc are the peak areas in the experiment and control, respectively. Data were obtained from 3 independent experiments and are expressed as the mean ± SD.
Fig. 3. Screening of mitochondria-targeting compounds from CR extract using SM-MBC. HPLC chromatograms of the CR extract (A, 0–50 min; B, 50–85 min) are displayed for the ultrafiltrates, with active mitochondria (black line) and denatured mitochondria (red line) as the control. 12 peaks (C1–C12) showed a significant area enhancement compared with the control. The conditions for detecting the CR extract are available in the Supplementary material.

Fig. 4. Chemical structures of the 17 assigned mitochondria-targeting compounds identified in PR and CR extracts.
mitochondrial functions to exert their pharmacological effects, whilst ligustilide [27] can affect mitochondrial functions, further validating the reliability of the screening results provided by SM-MBC. Nevertheless, whether the other 18 hits can also regulate mitochondrial functions remains unclear. Overall, all of the identified compounds might directly act on mitochondria to protect or damage mitochondrial functions, helping the elucidation of drug action mechanism and the identification of new drugs from TCMs.

3.4. Effects of identified compounds on Ca\(^{2+}\) -induced mPTP opening on isolated myocardial mitochondria

In order to evaluate the capability of identified compounds to regulate mitochondrial functions, a model of Ca\(^{2+}\) -induced mPTP opening on isolated myocardial mitochondria was used. As indicated in Fig. 5, 250 μM CaCl\(_2\), which can open the mPTP [13], induced a notable reduction at A\(_{520}\) in cardiac mitochondrial suspensions. This effect was remarkably inhibited by cyclosporin A (CsA, 10 μM), which can specifically inhibit mPTP opening [13] with a reduction of A\(_{520}\). As for CsA, six hit compounds (P3, P6, P8, P10, C7 and C11) remarkably inhibited the Ca\(^{2+}\) -induced reduction of A\(_{520}\), indicating that they refrained the Ca\(^{2+}\) -induced mPTP opening (i.e., mitochondrial protection) by directly interacting with the mitochondria.

These data indicated that the tested compounds were potential mitochondria-targeting compounds potentially affecting mitochondrial functionality. In combination with the screening results provided by SM-MBC, the other 17 identified compounds were also potential mitochondria-targeting compounds, which merit further investigations.

Among these 23 hit mitochondria-targeting compounds, puerarin (P3) [13], daidzin (P6) [19,20], daidzein (P8) [21], and formononetin (P10) [25] can interact with mitochondria to exert their pharmacological action, whilst ligustilide (C7) [27] can affect mitochondrial functions, further validating the reliability of the screening results provided by SM-MBC. Nevertheless, whether the other 18 hits can also regulate mitochondrial functions remains unclear. Overall, all of the identified compounds might directly act on mitochondria to protect or damage mitochondrial functions, helping the elucidation of drug action mechanism and the identification of new drugs from TCMs.

[19,20], daidzein [21,22], genistein [23,24] and formononetin [25,26], and one compound (ligustilide [27]) in CR could remedy mitochondrial functions to exert their pharmacological efficacy. However, it is possible that other bioactive constituents present in PR and CR and affecting mitochondrial functions remain unclear, which refrains the interpretation of therapeutic principles of the two TCMs. PR and CR were thus selected to be analyzed in this study. For the first time, SM-MCB was used to identify mitochondria-targeting compounds from PR and CR extracts. Fig. 2 shows the chromatogram of the PR sample analyzed by SM-MCB, with significant area enhancement of 11 peaks (P1–P11) compared with the control containing denatured mitochondria (ΔA > 20%, shown in Table 1), suggesting specific binding with mitochondria. The chemical structure corresponding to each peak was assigned by analyzing UV, MS and MS\(^n\) information obtained from LC/MS (Table 2) and standards. Active compounds were identified as nine isoflavones (P1–P6, P8–P11) and one phenylpropanoid (P7).

CR extract was analyzed using the same method, and the obtained chromatograms (Fig. 3) show remarkable enhancement of 12 peaks (C1–C12) compared with the control (ΔA > 20%, shown in Table 2), indicating specific binding with mitochondria. The characterization of the UV, MS and MS\(^n\) data provided by LC/MS (Table 2) and comparison with previously reported data [33–37] and standards confirmed that the C1–C12 peaks were phthalides (C3–C7) and their dimmers (C10 and C11).

Totally, among these 23 hit compounds, the chemical structures of 17 were identified (Fig. 4). These results further confirmed that SM-MBC could be used as an effective alternative for screening mitochondria-targeting compounds in complex mixtures such as TCMs extracts. The active mitochondria isolated from rat myocardium can recognize target molecules not only through affinity interactions between the molecules and targets on the mitochondrial environments, but also through the electrostatic interaction between them [7], which is helpful for decreasing interference from inactive impurities.
3.5. Effects of identified compounds on cell proliferation

The effects of hits on cell proliferation were investigated for evaluating their potential anti-cancer efficacy. Similar to cisplatin, which is known to act against cancerous proliferation [38], senkyunolide A (C4) significantly inhibited HepG2 cell proliferation with a dose-dependent trend (P < 0.05; Fig. 6). Combining with the screening results, we can speculate that the inhibition of cell proliferation may be attributed to the direct interaction with mitochondria, affecting mitochondrial functionality. This point needs to be further investigated.

3.6. Effects of hits on cellular viability in hypoxia/reoxygenation (H/R)-induced cardiomyocytes

As expected, H/R notably decreased cell viability (~57% decrease in WST-8 reduction; P < 0.01) in cardiomyocytes (Fig. 7). This effect was remarkably inhibited by puerarin (P3: 320 μM), which can specifically inhibit mPTP opening to exert the effective cardioprotection [13,39]. Analogously, 3’-hydroxypuerarin (P2: 140 μM) significantly prevented the loss of cardiomyocyte viability that resulted from H/R induction (Fig. 7; P < 0.05). This effect may be caused by direct binding with mitochondria that further remedies the mitochondrial dysfunctions induced by H/R. In combination with the results of senkyunolide A (C4), the developed screening method could be used as an effective alternative for discovering lead compounds from TCMs extracts.

4. Conclusion

We applied SM-MBC for identifying mitochondria-targeting compounds from TCMs extracts, including PR and CR samples, and 23 mitochondria-targeting compounds were successfully discovered from the two extracts, 17 of which were identified by LC/MS. The direct mitochondria-bound ability of six compounds was validated by pharmacological tests in vitro. Moreover, the inhibition ability of senkyunolide A (C4) and 3’-hydroxypuerarin (P2) on HepG2 cell proliferation and on loss of H/R-induced cardiomyocyte viability were investigated. The results obtained will be helpful for in-depth understanding of therapeutic action of TCMs and developing novel potential mitochondrial modulators from TCMs. SM-MBC was further confirmed to be an efficient proposal for the rapid screening of mitochondria-targeting compounds from complex matrix.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2018.06.001.

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Fig. 7. Effects of 3’-hydroxypuerarin on cellular viability in H/R-induced cardiomyocytes. Puerarin was used as positive control. Data were obtained from 3 independent determinations. * P < 0.05, compared with model group.
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