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A high-resolution peak fractionation approach for streamlined screening of nuclear-factor-E2-related factor-2 activators in *Salvia miltiorrhiza*

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ABSTRACT

Generation of a high-purity fraction library for efficiently screening active compounds from natural products is challenging because of their chemical diversity and complex matrices. In this work, a strategy combining high-resolution peak fractionation (HRPF) with a cell-based assay was proposed for target screening of bioactive constituents from natural products. In this approach, peak fractionation was conducted under chromatographic conditions optimised for high-resolution separation of the natural product extract. The HRPF approach was automatically performed according to the predefinition of certain peaks based on their retention times from a reference chromatographic profile. The corresponding HRPF database was collected with a parallel mass spectrometer to ensure purity and characterize the structures of compounds in the various fractions. Using this approach, a set of 75 peak fractions on the microgram scale was generated from 4 mg of the extract of *Salvia miltiorrhiza*. After screening by an ARE-luciferase reporter gene assay, 20 diterpene quinones were selected and identified, and 16 of these compounds were reported to possess novel Nrf2 activation activity. Compared with conventional fixed-time interval fractionation, the HRPF approach could significantly improve the efficiency of bioactive compound discovery and facilitate the uncovering of minor active components.

Keywords: Natural products; High-resolution peak fractionation; Cell-based screening; Nrf2 activators; *Salvia miltiorrhiza*
1. Introduction

Natural products (NPs) are recognized to play an invaluable role in drug discovery, and as many as 64% of the small-molecule drugs approved worldwide between 1981 and 2010 are NPs or derived from NPs [1]. Although NPs are a rich source of drug leads, the contribution of NPs to drug discovery has declined in recent years [2]. A major reason for this is the difficulty in accessing NPs in ways that are compatible with modern screening. Efficient discovery of active constituents from natural sources still faces technical challenges due to the chemical diversity and complex matrices of NPs [3,4].

Traditional screening of NPs is performed using crude or roughly fractionated extracts, followed by bioassay-guided fractionation, identification and dereplication of bioactive constituents [5]. Crude extracts are relatively straightforward to prepare, but the highly polar or highly lipophilic components may interfere with the sensitive detection outputs of modern screening, causing false-positive and false-negative results [6,7]. Once a hit extract is identified, the activity-guided isolation process is labor-intensive and time-consuming. An effective strategy to alleviate these types of interferences and shorten the time needed to isolate the active constituents is the use of pre-fractionated libraries or pure natural product libraries [8]. The pre-fractionation approach can remove the majority of the interfering components and improve the quality of samples for screening [9]. However, further purification is required to identify the active constituents (often minor components) from hit fractions. Pure natural product libraries have several advantages in the detection of
quality hits and in moving forward immediately because no further purification is required [10]. However, libraries containing only pure natural compounds are costly and require more time to generate, and some minor or unstable components may be lost in the conventional separation and purification process. A more recent strategy is to directly generate semi-pure fraction libraries from natural product extracts via high-throughput fractionation, which represents an intermediate solution between pre-fractionated libraries and pure natural product libraries [11-15].

In this approach, the subfractions derived from a pre-fractionation step are fractionated into a series of pure or semi-pure fractions by preparative or semi-preparative HPLC. In these approaches, fractions are automatically collected using a fixed-time interval collection trigger mode. Such fractions usually contain more than one compound. After hit notification, the fractions must be further separated into single compounds for determining active compounds and identifying their structures. This massive amount of work remains a significant obstacle to the rapid discovery of active natural compounds.

Here, we described a strategy based on high-resolution peak fractionation (HRPF) to realize accurate isolation, parallel structure identification and high-quality sample preparation for bioactivity screening with one HPLC run. In this approach, peak-fractionation was conducted under the chromatographic conditions optimized for high-resolution separation of the natural product extract. Peak fractions were automatically collected according to the predefinition of certain peaks based on their retention times from a reference chromatographic profile. The corresponding HRPF
database was collected in parallel by a mass spectrometer to ensure purity and characterize the structure of compounds in fractions. Compared with the fixed-time interval fractionation approach, this proposed HRPF strategy with the high accuracy and high resolution of peak-based fractionation, can be expected to create a series of high-purity fractions amenable to screening the active compounds from complex extracts.

The root of *Salvia miltiorrhiza* Bunge (Lamiaceae) is one of the most popular traditional Chinese medicine (TCM) remedies, and it is used for the treatment of angina pectoris, hyperlipidemia, atherosclerosis, and acute ischemic stroke [16,17].

This paper describes the strategy of the HRPF approach coupled with a cell-based assay for bioactive compound discovery, and the resulting strategy was applied to screen Nuclear-factor-E2-related factor 2 (Nrf2) activators from *S. miltiorrhiza*.

2. Experimental

2.1. Chemicals and reagents

The dried roots of *S. miltiorrhiza* were obtained from Shangluo (Shanxi, China). HPLC-grade acetonitrile and formic acid were obtained from Merk (Darmstadt, Germany). Deionized water was prepared through a Milli-Q system (Millipore, Bedford, MA, USA). Dimethyl sulfoxide (DMSO) and tert-butylhydroquinone (t-BHQ) were purchased from Sigma (St Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) was supplied by ATCC. Foetalbovine serum (FBS), 0.25% trypsin, and Opti-MEM with Penicillin were obtained from Invitrogen (Carlsbad, CA).
Glolysis buffer and Steady-Glo luciferase Assay system were supplied by Promega (Madison, WI, USA).

2.2. Extraction process

The dried roots of *S. miltiorrhiza* were ground to a homogeneous powder. The powder (25 g) was extracted with 200 mL of ethyl acetate. The crude materials were extracted twice under reflux at 50°C for 2 h. After filtration and removal of the organic solvents by vacuum evaporation at 55°C, the remaining residues were dried by lyophilization and stored at 4°C. The weight of the residue was 4.6007g, and the process showed 18.4% extraction efficiency. Next, the residue was redissolved in 70% alcohol to prepare a stock solution (200 mg/mL). Before fraction preparation, the extract was centrifuged at 13,000 rpm for 10 min, and the supernatant was filtered through 0.22 μm filter units and transferred to auto-sample vials for injection. The injection volume for each HPLC separation was 20 μL.

2.3. High-resolution peak fractionation approach

The HRPF approach was developed to prepare high-purity fractions for bioactive screening, as outlined in Figure 1. First, the HPLC separation condition was optimized for baseline separation of the compounds. Second, the optimized chromatographic profile was used to design the collection program, with the retention time window of each peak entered into the software. Third, the extract was automatically fractionated into a series of peak fractions through a process controlled by the software. After the solvents were rapidly removed from the fractions, a high-purity peak fraction library from the crude extract was generated.
Simultaneously, the liquid flow was split and partly directed to a parallel mass spectrometer system by a diverter valve. The information of the compounds in each peak fraction, including m/z value of protonated molecular ions and fragment ions, molecular formula and retention time, which was collected to establish the HRPF database of the crude extract for compound identification, was synchronously acquired. Our instrumental system for HRPF approach was performed on an Agilent 1200 Series HPLC system and a Collect Pal small volume fraction collection platform (LEAP Technologies, Inc., Carrboro, NC, USA) controlled by LEAP Shell Software, coupled with a parallel time-of-flight mass spectrometer (TOF MS) system (Agilent Technologies, Germany). The separation of the *S. miltiorrhiza* extract was performed using a semi-preparative Zorbax SB-C18 column (9.4 × 250 mm, 5 μm, Agilent, USA) with a flow rate of 2 mL/min. The optimized chromatographic separation and mass spectrometer detection conditions are shown in the Supporting Information. Sample ID (the name of each chromatographic peak), Collection Time (the accurate starting time and ending time of each peak according to the HPLC profile), and Collection Vial (the location) were manually entered into the software. The system delay time calibration was applied for accurate fraction collection. A total of 75 fractions corresponding to the detected peaks in the chromatographic profile of the extract were collected and named P1 to P75.

The solvent of the peak fractions was removed by a Genevac EZ-2 evaporator (Tegent technologies Ltd., UK) at 50°C, and the entire drying process of 75 fractions was completed within 5 h. The residue of each fraction was dissolved in 10
μL DMSO and diluted with 1000 μL serum-free DMEM medium as the high-concentration sample. Then, 200 μL of the above solution was combined with 400 μL DMEM medium containing 1% DMSO as the medium concentration sample. After five-fold dilution, the low-concentration sample was prepared in the same manner. The luciferase reporter activity assay was performed at all three concentration levels for each fraction. The remaining samples were store at -20°C for long-term storage.

2.4. Method validation of HRPF

The HRPF approach was validated for purity verification, separation repeatability and preparation recovery. After removing the solvents from seven random selected peak fractions, the residues were redissolved and analyzed on HPLC for their purity verification. In addition, fractions were collected every 1.0 min under the same chromatographic conditions for comparison of fraction purity. Detailed methods were shown in the Supporting Information. The repeatability of the HPLC separation was evaluated by six replicate injections. The software “Similarity Evaluation System for Chromatographic Fingerprint of TCM” by GPC (Version 2004A) was used for similarity analysis. The preparation recovery of each peak fraction was determined from the relative differences of its peak areas between in the redissolved fraction and in the extract, which was used to estimate the loss rate in the process of the peak fraction preparation. The extract sample was run in triplicate for the recovery experiment.
2.5. Luciferase reporter assays

HEK 293T cells were transiently transfected with an expression vector containing the ARE-promoter region of the reporter gene firefly luciferase GL4 [luc2P/NRF2/Hygro] Vector provided by Promega. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, Chicago, IL, USA) according to the manufacturer’s instructions. At 24 h after transfection, 293T cells were incubated with test samples in serum-free medium for 18 h. Subsequently, cells were lysed with Glo Lysis Buffer (100 μL/well) for 15 min. Luciferase activity (mean ± SD) was measured using the Steady-Glo Luciferase Assay system with a fluorescence spectrophotometer (SynergyTM2 SL. BioTeK, USA).

2.6. Activity validation of the screened Nrf2 activators

The relative mRNA levels of heme oxygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM) and Nrf2 were detected by Real-time PCR to validate the Nrf2 activation of the screened compounds in EA.hy926 cells. The detailed procedures and conditions were shown in the Supporting Information. Cell viability was assessed by MTT reduction assay and LDH release assay. The cells were treated with the active compounds at three different concentrations for 24 h, followed by incubation with 120 μg/mL ox-LDL for an additional 12 h. The culture medium was replaced with serum-free medium containing 0.5 mg/mL MTT tetrazolium salt (Biosharp, CA) and incubated at 37 ºC for 4 h. The medium was removed, and the cells were solubilized into DMSO for 10 min before being measured at 490 nm. The level of LDH release in the supernatant
was measured with standard kits (Promega).

2.7. Statistical analysis

Data in this study are expressed as the mean ± SD. Statistical analyses were performed with a one-way ANOVA (analysis of variance) followed by Dunnett’s method (GraphPad Prism 5.0), \( p<0.05 \) was considered a statistically significant difference and \( p<0.01 \) was considered an extremely significant difference.

3. Results and Discussion

3.1. Automated high-resolution peak fractionation of *S. miltiorrhiza* extract

In preliminary experiments, the ethyl acetate extract of *S. miltiorrhiza* which mainly contained lipophilic compounds, exhibited more potent Nrf2 activation activity than the alcoholic extract and the aqueous extract (**Figure S1**). Therefore, the ethyl acetate extract was subjected to the automated HRPF processing and subsequent bioactivity screening assay. To provide adequate chromatographic resolution for peak fractionation, we compared the chromatographic profiles of the ethyl acetate extract on analytical, semi-preparative and preparative columns (**Figure 2**). The loading amount and the separation resolution are two key factors for optimization. The semi-preparative column can offer more than 10-fold injecting amount and exhibited similar separation resolution to the analytical column (**Figure 2B and 2C**). The injecting amount can be further increased on preparative column but the chromatographic resolution was reduced dramatically (**Figure 2A**). Considering both the separation efficiency and the injection amount, the semi-preparative column
was selected for further experimental studies. For the semi-preparative column, when the injection volume increased from 20 µL to 40 µL, the resolution was decreased and more overlapping peaks were observed (data not shown). The fractionation was performed by semi-preparative RP-HPLC, and a typical chromatographic profile of the extract is shown in Figure 3A. Most of the constituents were well separated on the semi-preparative column.

Because the compounds detected in the ethyl acetate extract of *S. miltiorrhiza* have UV absorption, the UV profile was chosen as a reference for defining the collection time window of each peak, and this was confirmed by the total ion current profile provide by TOF MS. To achieve accurate fractionation, the overall run-to-run variation of the HPLC separation was assessed by six replicate injections. Similarity analysis indicated high repeatability of the separation process (Figure S2). The preparative recoveries of the seven fractions, which were higher than 80% with RSD < 5% (n=3) (Table S1), illustrated that the loss in the reformatting process was acceptable and the operational process was reproducible. Based on these predefined positions, the *S. miltiorrhiza* extract was fractionated into 75 peaks (designated P1 to P75) (Table S2). At a flow rate of 2 mL/min, the volumes of collected fractions varied between 0.6 mL to 8.0 mL. Thus, the solvent could easily be removed within 5 hours.

3.2. **Comparison of the HRPF and the fixed-time interval fractionation**

To demonstrate the effectiveness of the HRPF processing, the purities of random selected fractions were investigated. As shown in Figure 4A, seven peak fractions each contained one compound with high purity, and the high-purity peak fractions
would make the subsequent bioassay more direct and structure identification easier.

However, the corresponding fractions prepared by the fixed-time interval fractionation contained one to three compounds (Figure 4B). Another advantage of the HRPF approach was the capability to minimize overlapped fractions. According to the MS information from each fraction, only 8 of all 75 peak fractions prepared by HRPF overlapped (Table S2). Among 145 fractions prepared by the fixed-time interval fractionation method, 49 fractions contained more than one peak. In addition, 65 peaks in the chromatographic profile were collected into two or more adjacent fractions (Table S3). These findings demonstrated the approach using fixed-time interval fractionation had several drawbacks. First, some fractions contain larger quantities of material, while others contain little material. Second, it might be possible to obtain some high-purity fractions, but the recovery is rather low. Third, because one compound may be collected in several adjacent fractions, the overlapped collection may lead to repeated screening. Finally, the hit fractions containing more than one compound must be further separated into single components and rescreened.

Compared with the fixed-time interval fractionation method, the HRPF approach could produce higher purity peak fractions with less duplication.

3.3. Screening of Nrf2 activators in S. miltiorrhiza extract

The Nrf2 signaling pathway plays an important role in the transcriptional activation of phase II detoxifying enzymes and antioxidant genes [18,19]. As a critical regulator of cardiovascular homeostasis through suppression oxidative stress, Nrf2 is thought to be a valuable target for treatment of cardiovascular diseases [20-22]. It was reported
that tanshinone IIA, a major antioxidant constituent in *S. miltiorrhiza*, exhibited Nrf2 signaling pathway activation effect on intracellular redox status in aortic smooth muscle cells [23]. Nrf2 activation activity of *S. miltiorrhiza* was therefore chosen for study in this work. A cell-based luciferase reporter gene system was developed to screen Nrf2 activators from the peak fraction library. 293T cells were transiently transfected with Nrf2/ARE-driven reporter gene construct and then treated with t-BHQ, a well-known Nrf2 activator [24]. As shown in Figure S3, 2×10⁴/well and 18-hour treatment provided optimal activation of the ARE-luciferase construct by t-BHQ. The ARE-luciferase reporter system showed good reproducibility for the determination of Nrf2 activation. In addition, the effect of DMSO on cell viability was investigated. The optimal DMSO concentration for this assay should be kept at or below 1% (Figure S4).

We performed a titration-based screening [25] in which samples from each peak fraction can be rapidly tested at three concentrations (high, medium and low), and the dose-effect relationship was analyzed. The ARE-luciferase activities of 75 peak fractions at high concentration are shown in Figure 3B. It was shown that 20 peak fractions including P25, P30, P35, P36, P39-P44, P50, P52, P56-P59, P62-P65 exhibited the luciferase activities larger than 2.5-fold compared to the control. Among these fractions, 8 peak fractions (P25, P35, P36, P39, P42, P43, P44 and P64) showed high efficacy (above 5-fold signal induction). The luciferase activities of these 20 active fractions at three different concentrations were shown in Table 1. Fractions P25, P36, P39-P44 and P64 induced luciferase activities in a
dose-dependent manner. The luciferase activities of some fractions, such as P50, P52, P56, P59, P63 and P65, were not observed to have a dose-effect relationship, which might be caused by increased cytotoxicity in 293T cells at high concentrations.

3.4. Identification and semi-quantitative analysis of hit compounds in *S. miltiorrhiza* extract

After the hit fractions were found, the rapid structural identification of the active components was based on the MS data in the HRPF database. The majority of components in hit fractions were identified as diterpene quinones, an important class of components in *S. miltiorrhiza* [26]. Among total 20 active fractions, 16 components were unequivocally identified by matching of retention times and accurate mass measurements of extracted molecular ions compared with those of reference compounds. The other four active peak fractions (P30, P35, P40 and P44) were tentatively identified by HPLC-Q/TOF-MSMS based on matches between their characteristic fragment ions and the literature [27]. The major fragmentation pathways of these four possible structures are outlined in the Figure S5. Their intense \([\text{M+H}]^+\) molecular ions, molecular formulas and compound names are listed in Table 1, and their structural formulas are shown in Figure S6. Among the Nrf2 activators screened from *S. miltiorrhiza*, tanshinone IIA has been reported in a previous study [23]. In addition, three other tanshinones (tanshinone I, dihydrotanshinone and cryptotanshinone) were recently identified as novel Nrf2 activators [28]. The Nrf2 activation activities of the other 16 compounds (P25, P30, P35, P36, P39, P40, P41, P42, P43, P44, P50, P52, P58, P59, P64 and P65) are reported here for the first time.
To estimate the absolute quantities of the compounds prepared by the HRPF approach, the internal standard method was used to determine multiple components in the active extract [29]. The calibration curve of crytotanshinone was prepared, and its absolute content in the extract was determined. Then crytotanshinone served as the internal standard to simultaneously determine the other diterpene quinone analogues in the extract. Due to their structural similarity to crytotanshinone, the relative correction factor of each component was set as 1 for semi-quantitative analysis. As shown in Table 1, the approximate amounts of 20 active components produced by the HRPF method ranged from 2.8 μg to 153.6 μg. After peak-based fractionation on a semi-preparative scale, most of the 75 peak fractions prepared from 4 mg of the extract were on the microgram scale. The fractions that were dried and redissolved in 10 μL DMSO were present in quantities sufficient for cell-based screening at three different concentrations. Although they were presented in the extract at low contents (about 0.1%), P25, P30, P35, P40 and P58 showed increased ARE-luciferase activities in the screening assay. These results demonstrated the feasibility of screening minor components from the extract by the HRPF approach coupled with bioassay. One important factor in this success was that the HRPF approach allowed the enrichment of minor components prior to cell-based screening.

3.5. Evaluation of Hit Compounds as Potent Nrf2 Activators in Endothelial Cells

To confirm the screening results, ten available reference compounds (CN-50, CN-52, CN-56, CN-57, CN-58, CN-59, CN-62, CN-63, CN-64 and CN-65)
corresponding to each peak fraction among the hits, were tested for their effects on 
ARE-luciferase activities. As shown in Figure 5, the Nrf2 activation effects of these 
compounds were also observed. Most of them induced ARE-luciferase activity in a 
dose-dependent manner. CN-65, in particular, produced a maximum fold-activation 
higher than that of the classical Nrf2 activator t-BHQ.

To confirm Nrf2 activation by these hits, we used real-time PCR (RT-PCR) to 
investigate the effects of the hits on the expression of Nrf2 target genes in the human 
umbilical vein endothelial cell line EA.hy926. The relative mRNA levels of heme 
xygenase-1 (HO-1), glutamate-cysteine ligase catalytic subunit (GCLC), 
glutamate-cysteine ligase modifier subunit (GCLM) and Nrf2 were determined 
(Figure 6). Among the ten compounds tested, CN-50, CN-56, CN-62 and CN-65 
induced the expression of HO-1 in a dose-dependent manner, with over a 6-fold 
increase at high doses. These four compounds significantly increased mRNA 
expression of GCLM and GCLC in EA.hy926 cells compared to the control, even at 
the low dose of 1 μM. They also significantly increased mRNA expression of Nrf2 to 
a limited extent (less than 3-fold induction) at some doses. These results indicated 
that CN-50, CN-56, CN-62 and CN-65 had the potential to increase the endogenous 
mRNA expression of Nrf2-regulated antioxidant genes and Nrf2.

To further explore the biological activity of the four Nrf2 activators, we tested 
the effects of these compounds on oxidized low-density lipoprotein (ox-LDL) induced 
injury in the endothelial cell line EA.hy926 [30]. The MTT assay and LDH release 
were used to measure cell viability. As shown in Figure S7, ox-LDL induced a
dose-dependent decrease in cell viability in EA.hy926 cells. Pretreatment with CN-50, CN-56, CN-62 or CN-65 resulted in a significant increase in cell viability in ox-LDL-treated cells at high doses (Figure S8). Exposure to ox-LDL caused a six-fold increase in LDH release compared with unexposed cells, indicating that ox-LDL induced cell damage. Pretreatment with each compound significantly inhibited LDH release in a dose-effect relationship (Figure S8). These results indicated that these four Nrf2 activators could protect EA.hy926 endothelial cells from injury caused by ox-LDL, and suggested that they might be responsible for the endothelial protective activity of the roots of S. miltiorrhiza.

Our results showed that the strategy of HRPF coupled with cell-based assay achieved the streamlined connection of automated fractionation to modern bioactivity screening. Although the concept of “peak fractionation” has previously existed and has been applied to the isolation and purification of compounds or proteins by chromatographic techniques, few applications of peak-fractionation-based sample preparation for screening NPs have been reported in detail. Because the high resolution of the fraction preparation was ensured by the HRPF approach, most of the peak fractions contained only one main compound each. Therefore, the subsequent work of isolation, purification and identification of the hits was significantly reduced and simplified. In addition, our results demonstrated that the HRPF approach can provide adequate sample quantities for cell-based assays.

4. Conclusions
In the present study, a strategy of HRPF coupled with a cell-based assay was described for rapidly and efficiently screening active compounds from complex NPs. This strategy was applied for screening the activators of the Nrf2-ARE pathway from the roots of *S. miltiorrhiza*. Based on this strategy, 20 Nrf2 activators were isolated and identified and 16 of those activators were reported to possess that activity for the first time. In addition to reducing follow-up work on screening and identification, such a high-purity fractionation approach can enhance the possibility of uncovering minor active components. The overall results indicated that the strategy of combining HRPF with bioassays could significantly improve the resolution, sensitivity and efficiency for the discovery of bioactive compounds from NPs. This strategy is expected to serve as a universal and practical means to facilitate the discovery of potential drug leads from NPs.

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**REFERENCE**


**Figure Captions**

**Figure 1.** Schematic representation of high-resolution peak fractionation (HRPF) strategy.

**Figure 2.** The chromatographic profiles performed on three different columns. (A) Preparative HPLC (Shim-pack PREP-ODS, 20.0 mm ID × 250 mm, 15 μm), injection volume: 120 μL; flow rate: 10.0 mL/min. (B) Semi-preparative HPLC (Agilent ZorBax SB-C18, 9.4 mm ID × 250 mm, 5 μm), injection volume: 20 μL; flow rate: 2.0 mL/min. (C) Analytical HPLC (Agilent ZorBax SB-C18, 4.6 mm ID × 250 mm, 5 μm), injection volume: 2 μL; flow rate: 0.5 mL/min.

**Figure 3.** (A) The chromatographic profile of *S. miltiorrhiza* extract detected at 281 nm. The profile was separated into 75 fractions according to the chromatographic peaks (P1-P75). (B) The ARE-luciferase activities of 75 peak fractions at high concentrations. The value of luciferase activity of cells treated with DMSO was set at 1. Luciferase activity was expressed as fold to control. Each treatment had at least three replicates. Points, mean-fold increase relative to control value; bars, SD.

**Figure 4.** Purity verification of fractions collected by HRPF and by the fixed-time interval fractionation approach. (A) The HPLC chromatograms of seven random selected peak fractions (P7, P25, P34, P45, P57, P59 and P63) prepared by HRPF.
approach. (B) The HPLC chromatograms of the corresponding fractions (F9, F29, F42, F68, F100, F102 and F120) prepared by the fixed-time interval fractionation approach at the same retention times. By the fixed-time interval fractionation approach, 145 fractions were collected with 1.0 min per fraction.

**Figure 5.** Validation of the ARE-luciferase activity of ten screened compounds. (A) The ARE-luciferase activities of ten screened compounds on 293T cells using the standard substances at different concentrations. (B) The effects of ten screened compounds on cell viability of 293T cells by MTT assay. * Indicated significant difference to the control \((p < 0.05)\); ** Indicated highly significant difference to the control \((p < 0.01)\).

**Figure 6.** The effects of four screened compounds on the expression of HO-1, GCLC, GCLM and Nrf2 in EA.hy926 cells by RT-PCR. The mRNA levels were compared to the untreated control group (data were mean ± SD of three independent experiments); * Indicated significant difference to the control \((p < 0.05)\); ** Indicated highly significant difference to the control \((p < 0.01)\).
Highlights

1. A universal HRPF strategy was developed for discovery of bioactive compounds.

2. This strategy realized high-purity peak fraction preparation in one HPLC run.

3. HRPF combined with a luciferase reporter gene assay to screen Nrf2 activators.

4. 20 diterpene quinones were screened out and unambiguously identified.

5. 16 compounds were reported to possess novel Nrf2 activation effect.
Step 1
Preparation of peak fractions

A series of high-purity peak fractions

Herbal medicine (HM)

Chromatographic profile

Parallel TOF MS acquisition

Comp. 1 2 3 4 5 6

Step 2
Screening of peak fractions

Activity test at three diluted concentrations

Step 3
Validation of the active compounds

Bioactivity analysis
(A) Preparative HPLC

(B) Semi-preparative HPLC

(C) Analytical HPLC
Table 1. The Structural Information, Content and Luciferase Activities of 20 Bioactive Compounds

<table>
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<tr>
<th>Peak No.</th>
<th>[M+H]^+ (m/z)</th>
<th>Formula</th>
<th>Compound Name</th>
<th>Content (%) a</th>
<th>Quantity applied by one-time HRPF (μg)</th>
<th>Luciferase Activity (fold to control)</th>
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<td></td>
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a The contents of 20 compounds were semi-quantified using cryptotanshinone as internal standard calibration curve y=17641x+9.34.