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Integrated chromatographic approach for the discovery of gingerols antioxidants from *Dracocephalum heterophyllum* and their potential targets

Jun Dang [a], Yue Lv [a, b], Chengzhao Li [b], Yan Fang [b], Gang Li [b,*] and Qilan Wang [a,*]

[a] Qinghai Provincial Key Laboratory of Tibetan Medicine Research, Key Laboratory of Tibetan Medicine Research, Chinese Academy of Sciences, Northwest Institute of Plateau Biology, Xining 810001, P. R. China.

[b] Center for Mitochondria and Healthy Aging, College of Life Sciences, Yantai University, Yantai 264005, P. R. China.

Abbreviations: **ARE**, antioxidant response element; ***D. heterophyllum***, *Dracocephalum heterophyllum*; **HO-1**, oxygenase-1; **HPLC–DPPH**, HPLC–1,1-diphenyl-2-picrylhydrazyl; **Keap1,** kelch-like ECH-associated protein 1; **MDA**, malondialdehyde; **NADPH**, nicotinamide adenine dinucleotide phosphate, **NOX2,** NADPH oxidase 2; **NQO1**, nadph:quinone oxidoreductase-1; **Nrf2**, nuclear factor erythroid 2 related factor 2; **ROS**, reactive oxygen species; **SOD**, superoxide dismutaseheme; **TTM**, traditional Tibetan medicine.

*Corresponding author. Qinghai Provincial Key Laboratory of Tibetan Medicine Research, Key Laboratory of Tibetan Medicine Research, Chinese Academy of Sciences, Northwest Institute of Plateau Biology, Xining 810001, P. R. China.

E-mail addresses: ligang_ytu@126.com (G. Li), wql@nwipb.cas.cn (Q.Wang)

Abstract

As a traditional Tibetan medicine, *Dracocephalum heterophyllum* has many benefits, but due to the complicated procedures of separation and purification of its chemical constituents, there are few reports on the gingerols. In this study, four antioxidative gingerols were isolated from *Dracocephalum heterophyllum* by an integrated chromatographic approach. Antioxidant activity was then determined by *in vitro* experiments and its potential targets of action were investigated. First, the extract was pretreated through silica gel, MCI GEL® CHP20P, and Diol and Spherical medium pressure columns, while the antioxidant peaks were recognized using an online HPLC–1,1-diphenyl-2-picrylhydrazyl system. Then, the antioxidant peaks were directionally separated and purified by high pressure liquid chromatography to obtain four gingerols with purity higher than 95%, namely 5-methoxy-6-gingerol, 6-shogaol, 6-paradol, diacetoxy-6-gingerdiol. Finally, 1,1-diphenyl-2-picrylhydrazyl assays and cellular antioxidant experiments were carried out, and molecular docking were used to explore the potential antioxidant targets. The isolated gingerols upregulated the activity of antioxidant enzymes, including superoxide dismutase (SOD), heme oxygenase-1 (HO-1) and NADPH oxidase 2 (NOX2), while had little effect on that of nadph:quinone oxidoreductase-1 (NQO1). This method can efficiently prepare and isolate antioxidative ginerols from *Dracocephalum heterophyllum*, and can be extended to isolate antioxidants from other natural products.

Keywords: *Dracocephalum heterophyllum*; Preparative HPLC; Gingerols; Antioxidant activity; Molecular docking.

1. Introduction

Under normal circumstances, oxidation and anti-oxidation in the body are in a dynamic equilibrium, but in disease or aging, pathological phenomena occur due to increased levels of free radicals. When the body is attacked by diseases or some exogenous drugs and toxins, free radicals have a strong damaging effect. They damage the biofilm by lipid peroxidation, cause oxidative damage to enzymes, amino acids and proteins and damage the internal organs. The morphological function of the immune system is affected. This leads to the development of diseases related to oxidative damage, such as metabolic syndrome, diabetes, neurodegenerative diseases, and even aging and cancer.[1-6] The addition of exogenous antioxidants can prevent the occurrence of these diseases, but synthetic antioxidants pose safety risks such as toxic side effects. In addition, the shortcomings in the synthesis process, production costs, and environmental protection limit further development of artificial antioxidants, so it makes sense to search for natural antioxidants.[7]

Dracocephalum heterophyllum (*D. heterophyllum*) is a traditional Tibetan medicine (TTM) widely distributed in Xinjiang, Tibet, Qinghai, Gansu and other provinces of China for the treatment of various diseases such as jaundice, liver disease, cough, lymphangitis, oral ulcers and dental disease.[8] At the same time, modern pharmacology has shown that *D. heterophyllum* has antidiabetic, antioxidant and antibacterial activities.[9,10] Flavonoids, alkaloids, triterpenoids, phenylpropane and phenylethanoids have been reported to be isolated from *D. heterophyllum*.[11-13] But gingerol and its derivatives have not yet been isolated. Gingerols possesses diverse biological activities including anti-inflammatory, antioxidant, anticancer, analgesic, gastroprotective, cardiotonic, antipyretic, anti-angiogenic, anti-platelet aggregation effects and anti-hyperglycemia.[14,15] This provides a direction for further exploration of

the pharmacological material basis of *D. heterophyllum*. What's more, the isolation of pure compounds from natural products is frequently necessary for further biological activity investigations. However, one of the main issues inhibiting the exploration of more pharmacodynamic bases of *D. heterophyllum* is the low efficiency ratio and cost associated with the extraction and purification processes used today.

Advances in on-line HPLC–1,1-diphenyl-2-picrylhydrazyl (HPLC–DPPH) screening have greatly facilitated the screening and discovery of antioxidant molecules from natural products. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) is added to the HPLC flow after the column and the antioxidants are detected by absorption reduction at specific visible wavelengths.[16-20] This method can efficiently identify antioxidant gingerols in extracts of various natural products. Traditional approaches for separating complex plant extracts usually require multiple chromatographic steps on silica gel, polyamide, Sephadex LH-20 columns, etc.[21,22] These approaches are usually limited by several shortcomings, such as time-consuming, complicated processes, poor reproducibility, and irreversible adsorption. High-speed counter-current chromatography (HSCCC) has been presented in recent literature as an alternative for the isolation of standard compounds from natural products.[23,24] However, there are still some problems with this method, such as poor separation resolution, the need to determine partition coefficients, and the possibility of isolating only moderately polar compounds. Preparative HPLC is one of the most powerful tools for isolating and purifying individual components from complex samples, including TTM. It uses powerful separation, on-line detection and automated control to efficiently produce target compounds, with many advantages such as high efficiency, high resolution and good reproducibility. However, the main disadvantage is that the stationary phase is easily contaminated, which requires the selection of a suitable sample pretreatment

method to extend the lifetime of the chromatography column.[25-27] Therefore, in combination with preparative HPLC, the online system can quickly and efficiently identify and specifically separate compounds with antioxidant activity.

Molecular docking technology is a powerful tool for evaluating the binding efficiency between ligands and protein targets, which strongly supports the screening of the material basis of TTM. Molecular docking mainly determines the degree of binding based on the energy of the interaction between the ligand and the receptor and the amino acid site of the interaction.[28] When oxidative stress occurs, an excess of reactive oxygen species (ROS) is produced in the body. ROS are generated in cells by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system, which is a complex of several membrane-associated and cytosolic components. NADPH oxidase 2 (NOX2) is the main functional subunit of NADPH oxidase in mononuclear cells.[29] Other studies have shown that nadph:quinone oxidoreductase-1 (NQO1) and heme oxygenase-1 (HO-1) are closely related to antioxidant stress,[30,31] these enzyme proteins can be molecularly docked to the isolated gingerols, the mechanism of action can be theoretically analyzed, and the dominant peptides can be specifically identified. Functional prediction of gingerols and screening of potential target proteins of gingerols provide a theoretical basis for subsequent efficacy evaluation.

In this work, an on-line HPLC–DPPH system was used in combination with medium and high pressure chromatography to study the antioxidant activity of gingerols in *Dracocephalum heterophyllum*. These isolation methods were integrated and applied to the actual isolation process to target the rapid isolation of antioxidant compounds in *D. heterophyllum*, which can also be extended to other TTMs or even natural compounds. The extracts of *D. heterophyllum* were successively pretreated with silica gel, MCI GEL® CHP20P, Diol and Spherical medium pressure liquid

chromatography columns. The antioxidant gingerols were then purified from the enriched fraction using a ReproSil-Pur C18 AQ followed by a Kromasil 100-5 Phenyl high pressure liquid chromatography column. Finally, the antioxidant activities of the isolated compounds were verified by *in vitro* experiments and molecular docking experiments to explore potential targets.

2. Materials and methods

2.1 Instrumentation and reagents

A preparative liquid chromatography (Hanbon Science & Technology Co. China) was built from two NP7000 prep-HPLC pumps, a NU3000 UV–Vis detector, 5 mL manual injector, and LC workstation. On-line HPLC–DPPH system has been established using Essentia LC-16 (Shimadzu Instruments, Co. China) and a LC-10AD HPLC devices (Shimadzu Instruments, Co. Japan), each equipped two binary gradient pumps, UV–Vis detector, a column thermostat and a LC workstation. The two HPLCs were merged using a triple valve and the polyether ether ketone reaction coils (18.0 m × 0.25 mm i.d.). The LC-16 was used to carry out the HPLC analysis and the LC-10AD was employed to obtain the DPPH screening chromatogram. A Waters QDa ESI mass spectrometer (Waters Instruments Co. USA) was used to conduct ESI-MS analysis. The 600 MHz Bruker Avance was employed to obtain ^{1}H and ^{13}C NMR spectra (Bruker Instruments Co. Germany) using DMSO-d_6 for the NMR solvent. UV absorbance values were obtained on a Readmax 1900 microplate reader (Flash, Co. China). Flow cytometry was purchased from Hangzhou Aisen Company (Zhejiang, China).

The silica (100-200 mesh) used in a medium pressure column (49 × 460 mm) was obtained from Qingdao Ocean Chemical Corporation (Shandong, China). The MCI GEL®CHP20P (120 μm) separation material was purchased from Mitsubishi Chemical Corporation (Japan). Diol (50 × 500 mm, 25 μm) column was supplied by ACCHROM Corporation (Beijing, China). Spherical C18 (50 × 500 mm, 50 μm) was obtained from SiliCycle (Canada). Two ReproSil-Pur C18 AQ columns (4.6 × 250 mm, 5 μm and 20 × 250 mm, 5 μm) were purchased from Maisch Corporation (Germany). Kromasil 100-5 Phenyl analytical (4.6 × 250 mm, 5 μm) and preparative Kromasil 100-5 Phenyl (20 × 250 mm, 5 μm) columns were obtained by Nouryon Kromasil Corporation (Sweden).

DPPH was purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol, methanol (MeOH), dichloromethane (CH_2Cl_2), acetonitrile (ACN), n-hexane and ethyl acetate in analytical grade were obtained from Kelon Chemical Reagent Factory (Sichuan, China). HPLC grade Ethanol, MeOH and ACN were purchased from Kelon Chemical Reagent Factory (Sichuan, China). HPLC grade H_2O was prepared using a water purifier from Moore (Chongqing, China). The L02 cells used in this work were purchased from BeNa Culture Collection (Beijing, China).

2.2 Plant sample preparation and medium pressure liquid chromatography pretreatment

The whole *D. heterophyllum* was collected from North Mountain in Huzhu, Qinghai (2488 m, N 36°50′, 15", E 101°57′, 06") and validated by Prof. Lijuan Mei of Northwest Institute of Plateau Biology. A sample (nwipb-2016-10-10) was stored in the Qinghai-Tibetan Plateau Museum of Biology. After drying and powdering all *D. heterophyllum* herbs in the shade, 10.0 kg of the sample was extracted three times with 95% v/v ethanol (80.0 L solvent, 12 h for each extraction). The resulting 240.0 L extracting solution was collected, filtered, and concentrated in a rotary evaporator at 40°C. After the volume of concentrated solution was reduced to 5.0 L, it was mixed with 1.5 kg of amorphous silica gel and dried in a 40°C oven. The final dried silica gel mixture (2.8 kg) was pretreated with silica gel medium pressure liquid chromatography and separated with a mobile phase of MeOH and CH_2Cl_2 using the following linear elution gradients: 0-30 min, 0% MeOH; 30-60 min, 0-100% MeOH; 60-90 min, 100% MeOH. The flow rate was kept constant at 57.0 mL/min, and single loading amount was 65 g. Chromatogram was recorded at 210 nm. After repeating 43 times, fraction Fr1 (205.5 g) was obtained as the subsequent separation material.

Fr1 (205.5 g) was dissolved in 2.0 L of methanol, then mixed with 234.0 g of amorphous silica gel and dried in an oven at 40°C. The dry mixture (439.5 g) was pretreated with MCI GEL®CHP20P medium pressure chromatographic column (49 × 460 mm) and eluted with a MeOH/H_2O mobile phase. The elution was carried out according to the following: 0-150 min, 20-100% MeOH; 150-210 min, 100% MeOH. Absorbance was measured at 210 nm with a flow rate of 57.0 mL/min. The single loading amount was 55.0 g. After 8 times repeated, fractions Fr11-Fr15 were obtained and concentrated through drying. Fraction Fr14 (44.2 g) was chosen for subsequent separation.

Fraction Fr14 (44.2 g) was dissolved in 400.0 mL of methanol, then mixed with 74.0 g of amorphous silica gel and dried in an oven at 40°C. The dry mixture (118.2 g) was pretreated with Diol medium pressure chromatographic column (50 × 500 mm) and eluted with N-hexane/ethyl acetate mobile phase. The linear gradient elution involved 0-90 min (0-100% ethyl acetate) at a flow rate of 57.0 mL/min, and the chromatogram was recorded at 280 nm. After two repeated separations, the target fraction (Fr141) was collected, combined and concentrated to yield 9.9 g of enriched sample with 22.4% recovery.

The enrichment of active components in Fr141 was carried out on the Spherical C18 column. The column was eluted with water/ethanol in gradient mode (55-75% ethanol over 90 minutes). The injection volume was 3.3 g, and flow rate was 57.0 mL/min. The chromatograms were monitored at 210 nm. After three repeated separations, the target fraction (Fr1412) was collected, combined and concentrated to yield 0.9 g of enriched sample with 9.1% recovery.

The chromatographic conditions used in the recognition of antioxidant peaks for Fr14 and Fr141 samples with the on-line DPPH-HPLC system were as follows: The

analytical column: ReproSil-Pur C18 AQ column; mobile phase A: HPLC-grade water, B: Acetonitrile (ACN); gradient: 0-60 min, 40-75% B; monitoring wavelength: 210 nm; flow rate: 1.0 mL/min; column temperature: 30°C. Conditions for DPPH: monitoring wavelength: 517 nm; DPPH solution flow rate: 0.8 mL/min.

2.3 High pressure liquid chromatography separation and purification of antioxidative gingerols from Fr1412

The Fr1412 were further separated using ReproSil-Pur C18 AQ (20 × 250 mm, 5 μm) preparative column. Chromatographic conditions were obtained after linear amplification of analytical chromatographic conditions. The HPLC grade water and ACN were mobile phases A and B, respectively. The elution step of Fr1412 was 55% B isocratic elution for 60 min. Chromatogram was recorded at 210 nm. Then, 101 mg of Fr14123, 78 mg of Fr14124 and 94 mg of Fr14126 were obtained, respectively.

The subsequent separation of Fr14123, Fr14124 and Fr14126 was performed on the Kromasil 100-5 Phenyl (20 × 250 mm, 5 μm) preparative column. Likewise, preparative chromatographic conditions are obtained after linear scaling up of analytical chromatographic conditions. The mobile phase A was HPLC grade water, mobile phases B were acetonitrile (ACN). For Fr14123, Fr14124 and Fr14126, the isocratic elution step was performed 40% ACN for 65 min, 42% ACN for 60 min and 38% ACN for 120 min, respectively. The rate of flow of the eluent was constantly maintained at 19.0 mL/min and the process of elution was tracked at 210 nm. Finally, 7.55 mg of Fr141231, 5.33 mg of Fr141241, 12.55 mg of Fr141261 and 5.70 mg of Fr141262 were obtained, respectively.

2.4 Assessment of purity and activity of the antioxidative gingerols

Evaluation of Fr141231, Fr141241, Fr141261 and Fr141262 purity and activity was conducted using the on-line HPLC–DPPH system. The Kromasil 100-5 Phenyl (4.6

× 250 mm, 5 μm) analytical column was utilized to analyze gingerols antioxidants. HPLC grade water and ACN were used as the mobile phase A and B respectively. The elution conditions were 48% B isocratic elution for 60 minutes at a flow rate of 1.0 mL/min. The absorbance was tracked at 210 nm. The concentration of DPPH was 25 μg/mL, whereas the eluent was allowed to flow at a rate of 0.8 mL/min. At 517 nm, the UV-Vis detector-based chromatograms of the ethanolic solution of DPPH were acquired.

2.5 Antioxidant test *in vitro*

2.5.1 DPPH assays

Determination of the antioxidant activity of gingerol according to the DPPH assay of Sirivibulkovit et al.[32] Each of the isolated antioxidant gingerols was prepared as solutions of different concentrations (0.1, 1, 10, 50, 100, 500 μg/mL). The sample solution and DPPH solution (25 μg/mL) were mixed and incubated in a 96-well plate, and the absorbance of the mixed solution was measured at 517 nm. The experiment was repeated three times. The scavenging rate of DPPH free radicals was calculated as follows.

DPPH inhibition (%) = $[1-(A-A_0)/A_1] \times 100\%$

Where A, A_0 and A_1 were the absorbance of the experimental group, blank group and control group, respectively.

2.5.2 Cellular antioxidant activity assays

L02 cells were cultured in 1640 medium supplemented with 15% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were maintained in a humidified incubator containing atmospheric air and 5% CO_2 at 37°C. For the subculture, the cells were harvested at about 80% confluence and detach by a trypsin-EDTA solution (0.25% trypsin, 0.02% EDTA).

The L02 cells injury model induced by H_2O_2.[33] In the following experiments, the cells were allowed to adhere for 12 h and replaced the cell medium with 20 μM of test compound for another 20 h, then the cells were exposed to H_2O_2 (1 mM) for 4 h.

The cells were cultured in 24-well plates (5×10^4 cells/well) and pre-incubated with the test compound for 20 h and then exposed to H_2O_2 (1 mM) for 4 h. Cell morphology photos were taken by Olympus phase contrast microscope.

Then the cells were collected and lysed. The content of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) were detected according to the manufacturer's instructions of Nanjing Jiancheng Bioengineering Institute.

As for the detection of ROS, the cells were cultured in 12-well plates (1.2×10^5 cells/well) and the cell were treated as previous described. Before the cells were finally collected. The fluorescent probe was further incubated with cells for 30 min. And the fluorescence intensity was determined using flow cytometry. DCFH-DA (10 μM, 470/530 nm (ex/em)) was used for cytoplasmic ROS and Mitosox (5 μM, 510/580 nm (ex/em)) was used for mitochondrial ROS detection.

The cells were cultured in 6-well plates (2×10^5 cells/well) and exposed to H_2O_2 (1 mM) for 3 h. Cells were collected and lysed. Western blot was carried out to analyze the HO-1, NQO1 and NOX2 expression.

2.6 Molecular docking

A molecular docking approach was used to assess theoretical interactions between different molecules.[34] For this study, AutoDock was used to assess potential interactions between four antioxidants (Fr141231, Fr141241, Fr141261 and Fr141262) and the HO-1, NOX2 and NQO1 receptor. The HO-1 crystal structure (PDB ID: 1N3U), NOX2 crystal structure (PDB ID: 2CDU) and NQO1 crystal structure (PDB ID: 1H69) were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/). Water

molecules and ions were removed from the receptor prior to docking, after which polar hydrogen atoms and Coleman charge were added. The AutoGrid program was used to set a grid box. Minimization was conducted with the Lamarckian genetic algorithm and the pseudo-Solis and Wets methods using default parameters. In total, 100 peptide conformations were defined based on dock score values, with the conformation exhibited the minimum binding energy being selected for model development. The results were visualized and analyzed using Discovery Studio 2020.

2.7 Statistical analysis

All of the experiments were performed in triplicate, and data are shown as the mean ± standard deviation. Statistical analysis was performed by one-way ANOVA or Student's t-test using statistical analysis software SPSS version 18.0 (SPSS, Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Sample pretreatment with medium pressure liquid chromatography

Methanol was chosen as the extraction solvent because of its low cost and relatively environmental friendliness. The 1.3 kg of crude sample (calculated by subtracting 1.5 kg of silica from 2.8 kg of silica mixture) was obtained from 10.0 kg air-dried whole herb of *D. heterophyllum*, producing an extraction yield of approximately 13.4%. The crude sample was pretreated with silica medium pressure liquid chromatography to achieve visible separation and eliminate polymers and sugars. The medium pressure liquid chromatography consists of two medium pressure columns of different sizes (49 × 100 mm and 49 × 460 mm) connected to form a column system, thus achieving the purpose of increasing the loading volume and improving the preparation efficiency. The separation chromatogram was shown in Figure 1A. It can be seen that baseline separation can be achieved between the two fractions. After 43 repeated separations, a total of two fractions were produced, with the target fraction Fr1 weighing 205.5 g (recovery 15.8%).

Since chlorophyll may be dead adsorbed on the stationary phase of the preparative column, it was necessary to remove chlorophyll before further preparative isolation. Therefore, we chose MCI GEL® CHP20P medium pressure column to pretreat Fr1 for the purpose of chlorophyll removal and initial enrichment of fractions. Figure 1B showed the separation chromatogram. After 9 repetitions, five fractions (Fr11, Fr12, Fr13, Fr14 and Fr15) were collected. Here, Fr14 (44.2 g) was chosen as the targeted sample to illustrate the target isolation of antioxidative gingerols.

The fractions Fr14 (100 mg) was solubilized in 0.1 mL of MeOH and filter through a 0.45 mm filter. As shown in Figures 1C and 1D, with optimized chromatographic conditions, Fr14 was re-analyzed using an on-line HPLC–DPPH system on the

ReproSil-Pur C18 AQ analytical column. From Figure 1D, it can be seen that there were many negative peaks at 517 nm in Fr14, indicating that there were many antioxidant peaks in Fr14. At the same time, it can be seen that the composition of Fr14 was complex and it was difficult to separate these antioxidant peaks directly. Further pretreatment by medium pressure liquid chromatography was performed on a Diol column to enrich the active components in Fr14. The results were shown in Figure 1E. Fr14 was divided into five subfractions Fr141-Fr145, and fraction Fr141 (9.9 g) was selected for the next step of separation and purification of antioxidative gingerols. Then, on-line recognition of the antioxidant peak of Fr141 was performed on the HPLC–DPPH system using the ReproSil-Pur C18 analytical column AQ. As shown in Figures 1F and 1G, three negative peaks (peaks I, II and III) appeared at 517 nm between 27 and 40 min, indicating that Fr141 contained at least three antioxidant peaks (heart-shaped peaks 1-3).

However, it can be seen from the Figure 1F that peaks 1-3 were difficult to achieve baseline separation from the adjacent peaks. Next, Fr141 was again enriched for active components using the Spherical C18 medium pressure column. The chromatogram was shown in Figure 1H where Fr141 was subdivided into Fr1411, Fr1412 and Fr1413. Under the same conditions as in Figure 1F, the active peaks in Fr141 were found to be enriched in Fr1412 (923.8 mg), as shown by the red dotted lines in Figure 2A and 2B.

3.2. Target preparation of antioxidative gingerols of Fr1412 with high pressure liquid chromatography

In order to obtain a satisfactory separation profile, the chromatographic condition was optimized in this work based on the analysis conditions in Figure 2B, and Figure 2C was the optimized chromatogram where peaks 1-3 can achieve baseline separation. At the same time, the active peaks of Fr1412 were recognized again on the on-line

HPLC–DPPH system, as shown in Figure 2D, peaks I-III correspond to peaks 1-3 respectively, which were consistent with Figure 1G. Under the optimized conditions, the target preparation was performed on the ReproSil-Pur C18 AQ preparative column. Figure 2E showed the preparative chromatogram of the fraction Fr1412. When compared with Figure 2C and 2E, similar retention times were observed for active chromatographic peaks 1-3 on the preparative column of ReproSil-Pur C18 AQ (Figure 2E) and analytical column of ReproSil-Pur C18 AQ (Figure 2C). Because the packing of the columns is the same and the retention properties of the preparative and analytical columns are the same for the samples, the peak emergence times are similar. After 7 repeated chromatographic separations, the fractions were collected and the solvent was evaporated to yield 101.6 mg of Fr14123, 78.0 mg of Fr14124 and 94.5 mg of Fr14126 with a recovery of 29.7%.

The mechanism of synthesis of Kromasil 100-5 Phenyl is different from that of common C18 reversed-phase column fillers. The re-analysis of Fr14123, Fr14124 and Fr14126 were conducted on the Kromasil 100-5 Phenyl analytical column with isocratic elution. As shown in Figure 2F-2K, peak 1 (Figure 2F correspond to DPPH negative peak I of Figure 2G), peak 2 (Figure 2H correspond to DPPH negative peak II of Figure 2I) and peaks 3 and 4 (Figure 2J correspond to DPPH negative peaks III and IV of Figure 2K) were observed by employing the on-line HPLC–DPPH system using the optimized conditions. It is worth mentioning that peak 3 in Figure 2C was detected with two active negative peaks (peak III and peak IV of Figure 2K) on the Kromasil 100-5 Phenyl analytical column. The results illustrated that Kromasil 100-5 Phenyl column and ReproSil-Pur C18 AQ column have good complementary selectivity. Following linear amplification, 19.0 mL/min was maintained when separating Fr14123, Fr14124 and Fr14126 on the Kromasil 100-5 Phenyl preparative column. Figure 2L,

2M and 2N showed the preparative chromatograms of the Fr14123, Fr14124 and Fr14126. A comparison of Figure 2L and 2F made it evident that peak 1 was found to have nearly identical retention times on the preparative column and the analytical column. The analysis chromatograms and preparative chromatograms of peak 2 in Fr14124 (Figure 2H and 2M) and peaks 3 and 4 in Fr14126 (Figure 2J and 2N) also had nearly identical retention times. After preparative separations, the Fr141231 (peak 1, 7.55 mg, 7.4% recovery), Fr141241 (peak 2, 5.33 mg, 6.8% recovery), Fr141261 and Fr141262 (peaks 3 and 4, 12.55 mg and 5.70 mg with 19.2% recovery) were collected. These results proved the complementary selectivity of ReproSil-Pur C18 AQ and Kromasil 100-5 Phenyl columns for separating peaks 1-4, which could be ascribed to the different polarities of these compounds and their interactions with the stationary phases. The alternate use of stationary phases with different properties can be a good solution to complex separation problems and can be applied to the actual separation process.

3.3. Purity, activity and structural of Fr141231, Fr141241, Fr141261 and Fr141262

The isolated Fr141231, Fr141241, Fr141261 and Fr141262 were re-evaluated for the purity and activity by employing the on-line HPLC–DPPH system with the Kromasil 100-5 Phenyl analytical column. The four antioxidative gingerols were found to have purities well above 95% as illustrated in Figure 3A-3H. In order to elucidate the structure of target compounds, Fr141231, Fr141241, Fr141261 and Fr141262 were identified by comparing their ESI-MS and NMR spectrum data with literature data. Figures S1–S12 in the supplementary material were the full spectrum of the structure identification of the four gingerols in this study. Fr141231, Fr141241, Fr141261 and Fr141262 had NMR and MS data that matched the data for 5-methoxy-6-gingerol, 6-

shogaol, 6-paradol, diacetoxy-6-gingerdiol, respectively. The chemical structures were shown in Figure 3I-3L.

Fr141231: (peak 1, 5-methoxy-6-gingerol, 7.55 mg, yellow oily liquid, ESI-MS *m/z*: 331.28 $[M+Na]^+$). ^1H NMR (600 MHz, DMSO-d_6) δ: 8.65 (1H, s, 4'-OH), 6.75 (1H, d, *J* = 1.9 Hz, 2'-H), 6.64 (1H, d, *J* = 8.0 Hz, 5'-H), 6.56 (1H, dd, *J* = 7.9, 1.9 Hz, 6'-H), 3.73 (3H, s, 3'-OCH_3), 3.57 (1H, m, 5-H), 3.17 (3H, s,5-OCH_3), 2.71 (2H, m, 1-H), 2.62 (1H, dd, *J* = 15.9, 7.2 Hz, 4a-H), 2.46 (1H, dd, *J* = 15.9, 5.2 Hz, 4b-H), 0.85 (3H, t, *J* = 7.0 Hz, 10-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 208.7 (C-3), 147.4 (C-3'), 144.6 (C-4'), 131.9 (C-1'), 120.2 (C-6'), 115.2 (C-5'), 112.5 (C-2'), 76.3 (C-5), 55.9 (C-(3'-OCH_3)), 55.5 (C-(5-OCH_3)), 46.7 (C-4), 44.5 (C-2), 33.2 (C-8), 31.3 (C-6), 28.6 (C-1), 24.1 (C-7), 22.0 (C-9), 13.9 (C-10). These data are consistent with published data for 5-methoxy-6-gingerol. [35]

Fr141241: (peak 2, 6-shogaol, 5.33 mg, yellow oily liquid, ESI-MS *m/z*: 299.21 $[M+Na]^+$). ^1H NMR (600 MHz, DMSO-d_6) δ: 8.65 (1H, s, 4'-OH), 6.85 (1H, dt, *J* = 16.0, 7.0 Hz, 5-H), 6.77 (1H, d, *J* = 1.9 Hz, 2'-H), 6.64 (1H, d, *J* = 7.9 Hz, 5'-H), 6.57 (1H, dd, *J* = 8.0, 1.9 Hz, 6'-H), 6.09 (1H, dt, *J* = 16.0, 1.4 Hz, 4-H), 3.73 (3H, s, 3'-OCH_3), 2.83 (2H, t, *J* = 15.2, 7.2 Hz, 2-H), 2.69 (2H, t, *J* = 15.2, 7.9 Hz, 1-H), 2.17 (2H, m, 6-H), 1.22-1.43 (6H, m, 7, 8 and 9-H), 0.86 (3H, m, 10-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 199.3 (C-3), 147.5 (C-5), 147.4 (C-3'), 144.6 (C-4'), 131.9 (C-1'), 130.2 (C-4), 120.3 (C-6'), 115.2 (C-5'), 112.6 (C-2'), 55.5 (-OCH_3), 41.1 (C-2), 31.7 (C-6), 30.8 (C-8), 29.2 (C-1), 27.2 (C-7), 21.9 (C-9), 13.8 (C-10). The data were in agreement with the literature data for 6-shogaol.[35]

Fr141261: (peak 3, 6-paradol, 12.55 mg, brown black oily liquid, ESI-MS *m/z*: 279.16 $[M-H]^-$). ^1H NMR (600 MHz, DMSO-d_6) δ: 8.65 (1H, s, 4'-OH), 6.74 (1H, d, *J* = 1.8 Hz, 2'-H), 6.64 (1H, d, *J* = 8.0 Hz, 5'-H), 6.55 (1H, dd, *J* = 8.0, 1.8 Hz, 6'-H), 3.73

(3H, s, 3'-OCH3), 2.67 (4H, m, 1 and 2-H), 2.38 (1H, t, J = 7.3 Hz, 4-H), 1.43 (2H, m, 5'-H), 1.13-1.30 (8H, m, 2, 4, 5, 6-H), 0.85 (3H, t, J = 6.9 Hz, 10-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 210.0 (C-3), 147.4 (C-3'), 144.6 (C-4'), 131.9 (C-1'), 120.2 (C-6'), 115.2 (C-5'), 112.5 (C-2'), 55.5 (-OCH_3), 43.7 (C-2), 41.9 (C-4), 31.1 (C-8), 28.8 (C-1), 28.5 (C-6), 28.5 (C-7), 23.2 (C-5), 22.0 (C-9), 13.9 (C-10). The data were in agreement with the literature data for 6-paradol.[35]

Fr141262: (peak 4, diacetoxy-6-gingerdiol, 5.70 mg, brown black oily liquid, ESI-MS m/z: 403.37 $[M+Na]^+$). ^{1}H NMR (600 MHz, DMSO-d_6) δ: 8.65 (1H, s, 4'-OH), 6.71 (1H, d, J = 1.8 Hz, 2'-H), 6.65 (1H, d, J = 8.0 Hz, 5'-H), 6.55 (1H, dd, J = 8.0, 1.8 Hz, 6'-H), 4.80 (2H, m, 3 and 5-H), 3.73 (3H, s, 3'-OCH_3), 2.48 (1H, m, 1a-H), 2.41 (1H, m, 1b-H), 2.00 (3H, s, 2''-H), 1.93 (3H, s, 2'''-H), 1.77 (4H, m, 2 and 4-H), 1.45 (2H, m, 6-H), 1.21 (6H, m, 7, 8 and 9-H), 0.84 (3H, t, J = 6.9 Hz, 10-H). ^{13}C NMR (151 MHz, DMSO-d_6) δ: 170.0 (C-1''), 169.9 (C-1'''), 147.4 (C-3'), 144.5 (C-4'), 131.9 (C-1'), 120.2 (C-6'), 115.3 (C-5'), 112.4 (C-2'), 70.7 (C-3), 70.4 (C-5), 55.5 (-OCH_3), 37.9 (C-4), 35.4 (C-2), 33.5 (C-6), 30.9 (C-1), 30.4 (C-8), 24.1 (C-7), 21.9 (C-9), 20.9 (C-2''), 20.8 (C-2'''), 13.8 (C-10). The above data were in agreement with those of diacetoxy-6-gingerdiol in the literature.[35]

3.4. *In vitro* antioxidant activity analysis of Fr141231, Fr141241, Fr141261 and Fr141262

When H_2O_2-induced damaged L02 cells were treated with isolated gingerols, it can be seen from Figure 4 that the cells in the model group were damaged and the edges shrank, whereas the damaged cells in the four experimental groups all improved in morphology and the cells became rounder and the cell density also increased, but the strength of the antioxidant capacity of the four compounds could not be determined. Therefore, the DPPH assay was used to determine the antioxidant activity of the

gingerols, and the IC_{50} values of Fr141231, Fr141241, Fr141261, and Fr141262 for scavenging DPPH free radicals were 17.18, 18.68, 16.42 and 2.95 μg/mL, respectively. The rate curve was shown in Figure 5A. The lower the IC_{50} value, the higher the antioxidant activity. It can be seen that the antioxidant activity of Fr141262 was the strongest among the four compounds, which may be attributed to the presence of diacetoxy in the structure.

The four antioxidative gingerols were all aryldecane compounds. However, due to the different substituents at the C3 and C5 positions, the IC_{50} values for DPPH assays were not the same. In Fr141231, Fr141241 and Fr141261 all C3 substitutions were carbonyl groups, the difference was C5 substitutions. When C5 had no substituent (Fr141261) or the substituent was a methoxy group (Fr141231), the IC_{50} values were similar but both lower than for Fr141241 (double bond substitution of C5). Compared with the structures of these three compounds and Fr141262, when the methyl ester group was substituted at both the C3 and C5 positions, the compound had the strongest ability to scavenge DPPH free radicals, and the IC_{50} value was the lowest (2.95 μg/mL).

SOD is an important antioxidant enzyme that scavenges free radicals from superoxide anions *in vivo* and can protect cells from oxygen free radical damage. MDA is one of the products formed in the reaction between lipids and oxygen free radicals, and its content indicates the degree of lipid peroxidation. SOD and MDA are important indicators for assessing antioxidant capacity and oxidative capacity during oxidative stress. They can be used as markers to evaluate oxidative stress in organisms and can be used for disease pathogenesis and drug discovery.[36] In this work, the SOD activity was decreased and the MDA content was increased in the model group compared with the control group (Figure 5B). After treatment with gingerols (experimental group), SOD activity increased to some extent. There was a significant difference between

Fr141261 and Fr141262 ($p < 0.05$). At the same time, these two compounds could also significantly reduce the content of MDA ($p < 0.01$). In addition, Fr141241 could significantly reduce the MDA content ($p < 0.05$). Fr141231 could also improve the changes of these two indicators, but there was no significant difference. This result may indicate that the four isolated gingerols have different effects against oxidative damage.

ROS are very important signal transduction factors, and an increase in ROS can lead to oxidative stress in cells. In this study, DCFH2-DA and Mitosox were used to detect the production of cytoplasmic ROS and mitochondrial ROS, respectively. As shown in Figures 5C and 5D, the ROS content in the cytoplasm and mitochondria of L02 cells was significantly increased after H_2O_2 induction compared with controls. Compared with the model group, Fr141241, Fr141261 and Fr141262 treatments significantly inhibited H_2O_2-induced ROS production in the cytoplasm of L02 cells, whereas H_2O_2-induced mitochondrial ROS production in L02 cells was significantly inhibited by Fr141261 and Fr141262 treatment groups. ROS is a highly active molecule that can balance cellular homeostasis without stimulation, but an excess of ROS can cause metabolic disorders and inflammatory diseases in the body. Mitochondria are the main source of production of ROS. The above experimental results show that the isolated gingerols can reduce ROS in cells and mitochondria to some extent, reduce the body damage caused by excess of ROS, and play an antioxidant role.

Compound Fr141241 is formed by demethoxylation (CH_3O-) of compound Fr141231 and further dehydrogenation at the C4/C5 position. When the methoxy group was lost to increase the double bond, Fr141241 can significantly reduce intracellular ROS, decrease MDA content and improve antioxidant activity. While the change of SOD was not significantly affected *in vitro*, it can be concluded that the appearance of double bonds plays a role in the change of intracellular ROS. Comparing the structure

of compound Fr141231 and compound Fr141261, it could be seen that the antioxidant activity of the compound was significantly increased when no methoxy group was present in the structure, and the antioxidant indicators show a good improvement effect. Similarly, when comparing compound Fr141231 and compound Fr141262, the presence of a methyl ester group significantly improved the antioxidant activity of the compound, and it was the compound with the strongest antioxidant activity among the four compounds.

The above results of *in vitro* antioxidant experiments proved that the isolated gingerols exhibit certain antioxidant activities at both chemical and biological levels. Structural differences also lead to differences in their antioxidant capacity. To further explore the possible pathways of gingerols in their antioxidant effects, molecular docking and western blot experiments analysis were used to investigate potential targets.

3.5. Potential targets research by molecular docking and western blot

The kelch-like ECH-associated protein 1 (Keap1) - nuclear factor erythroid 2 related factor 2 (Nrf2) - antioxidant response element (ARE) signaling pathway is an important pathway in the human body's defense mechanism against oxidative stress. Under physiological conditions, Nrf2 mainly binds to cytoplasmic Keap1 and keeps its transcriptional activity low. However, when cells are stimulated by oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, and interacts with the antioxidant response element ARE in the nucleus. This binding initiates the transcription of downstream antioxidant proteins such as HO-1, NQO1 and NOX2, which play a role in combating oxidative damage.[37-39] In this work, the downstream antioxidant proteins HO-1, NQO1 and NOX2 were used as receptors for molecular docking experiments and western blot experiments with isolated gingerol to predict the

possible target proteins that these compounds may exert in exerting their antioxidant activities.

HO-1 is an enzyme with inducible isoform that controls the response to stress conditions such as oxidative stress, hypoxia, cytokines, heavy metals, etc.[40] When oxidative stress occurs in the body, ROS itself can activate NOX2 through the intracellular signaling pathway, reducing ROS in the body and decreasing oxidative stress.[41] In addition, NQO1 is a quinone oxidoreductase and a metabolic enzyme in the electrophilic stress process. It plays a detoxifying role in the cytotoxic effect of quinones and their derivatives (structural damage such as DNA and proteins) and can also reduce oxidative stress.[42] To investigate the potential target proteins of isolated gingerols for their antioxidant effects, HO-1, NOX2 and NQO1 were selected for molecular docking and western blot in this study.

The results of molecular docking were shown in Table 1. The binding energies of Fr141231, Fr141241, Fr141261, and Fr141262 to HO-1 (PDB ID: 1N3U) [43] were -5.63, -6.08, -5.40, and -5.84 Kcal/mol, respectively. The amino acid residues of leucine (Leu), alanine (Ala), aspartic acid (Asp), phenylalanine (Phe), methionine (Met), threonine (Thr), asparagine (Asn), and arginine (Arg) were combined with the compounds in different bonding modes (conventional hydrogen bonding, pi-sigma, pi-alkyl, alkyl, hydrocarbon bonding, pi-cation, pi-anion), and the combined 3D patterns were shown in Figures 6A-D. Due to the existence of direct double bonds between C4 and C5 in Fr141241, more amino acid residues were bound to it through π bonds, resulting in the lowest binding energy. When the compound docked to NOX2 (PDB ID: 2CDU),[44] the binding energies of Fr141231, Fr141241, Fr141261 and Fr141262 to NOX2 were -6.36, -7.04, -6.54 and -6.65 Kcal/mol, respectively. Alanine (Ala), phenylalanine (Phe), serine (Ser), isoleucine (Ile), cysteine (Cys), glutamate (Glu),

histidine (His), and valic acid (Val) amino acid residues as shown in Figure 6E-H, these amino acid residues and compounds were bound by conventional hydrogen bonds, pi-alkyl, alkyl, carbon-hydrogen bonds, pi-sigma, pi-lone pair, and pi-sulfur. Comparing the amino acid residues binding to the compounds, we found that the amino acid residues around Fr141231, Fr141261 and Fr141262 were essentially the same, and it can be assumed that the three compounds act on NOX2 in the same domain. It is possible, that the double bond in the structure of Fr141241 has altered the distribution of the surrounding electron cloud, while the other three compounds do not have double bonds in the structure and the distribution of the surrounding electron cloud is similar, resulting in the amino acid residues bound to them also being similar. Figure 6I-L showed the binding modes of the four gingerols to NQO1 (PDB ID: 1H69).[45] The amino acid residues on the NQO1 protein bind to the compounds via conventional hydrogen bonds, pi-alkyl, alkyl, pi-anion, and pi-sulfur with binding energies of -4.25, -5.01, -4.44 and -3.56 Kcal/mol, respectively. In general, the binding energy is below -5 Kcal/mol, and we assume that this compound has good binding ability to proteins.[46] Comparing the binding energies of the four gingerols with HO-1, NOX2 and NQO1, the binding energy of NOX2 protein was all below -6 Kcal/mol, which means good binding ability, while the binding energy of NQO1 protein was very high, which can be considered as general binding ability. During the actual effect, it may not have any effect on NQO1.

To test the reliability of the theoretical results of molecular docking, we performed western blot assays with these three proteins. From Figure 7, it can be seen that when L02 cells were damaged by H_2O_2, the expression levels of HO-1 and NOX2 were significantly lower in the model group compared with the control group. But the expression level of NQO1 hardly changed, and there was no significant difference.

After the cells were treated with the isolated gingerols, Fr141241 and Fr141261 significantly increased the expression of HO-1 (Figure 7A), whereas Fr141261 could significantly increase the expression of NOX2 (Figure 7B), and the NQO1 protein level basically showed no change (Figure 7C). When no methoxy group was present in the structure of gingerols, the antioxidant activity of the compound becomes stronger, and the presence of a double bond (Fr141241) and a carboxyl group (Fr141262) in the structure significantly increased the expression of HO-1 protein. Only when the C3 carbonyl group was substituted (Fr141261), the expression of NOX2 was significantly increased, exerting the antioxidant capacity.

For the HO-1 protein, the molecular docking results were completely consistent with the results of the western blot experiment. The strongest antioxidant activity was Fr141241, followed by Fr141262, Fr141261 was the third, and Fr141231 had the worst activity. Molecular docking experiments showed that the binding energy of Fr141241 to NOX2 protein was the lowest, followed by Fr141262, Fr141261 and Fr141231. The results of western blot experiments showed that Fr141261 could significantly increase the expression of NOX2 and had the best antioxidant activity. The two experimental results cannot be completely matched one-to-one. The molecular docking experiment predicts the binding ability with the target protein only theoretically and provides clues for biological verification. The final biological experimental results can prove the reliability of the molecular docking experiment. It is also worth mentioning that the results of DPPH assays at the chemical level showed that the antioxidant activity of Fr141262 was the strongest (lowest IC_{50} value), but the effect of Fr141262 at the cellular level was not the strongest, indicating that the results at the chemical level can be used as a reference, but there are many factors that affect the effect of the compound when actually used in cells or even *in vivo*, and the inconsistency with the chemical

results is worth further exploration. Although there are differences, overall the antioxidant tendencies of the compounds are the same. Regarding NQO1 protein, the results of molecular docking experiments and western blot experiments showed that the four gingerols had no significant effect on NQO1, suggesting that NQO1 is not the potential target protein of the four gingerols to exert antioxidant activity. In-depth mechanistic experiments may rule out this protein. Thus, we can conclude that HO-1 and NOX2 may be potential targets of gingerols in restoring oxidative damage. However, the detailed mechanism needs to be verified in detail by further knockdown experiments.

4. Conclusions

In this study, we used an integrated chromatographic approach to recognize, isolate, and purify the antioxidant gingerols from *Dracocephalum heterophyllum*. Four gingerols with purity higher than 95% were obtained, namely 5-methoxy-6-gingerol, 6-shogaol, 6-paradol and diacetoxy-6-gingerol. To evaluate the antioxidant capacity of these compounds, we performed DPPH assays, cellular antioxidant experiments, molecular docking prediction experiments and western blot verification. It was finally concluded that these compounds have good antioxidant capacity. In addition, the 6-shogaol (Fr141241) and diacetoxy-6-gingerol (Fr141262) could increase the expression of HO-1. The 6-paradol (Fr141261) can increase the expression of NOX2. It can be concluded that the isolated antioxidative gingerols are very likely to act on the two antioxidant enzymes HO-1 and NOX2 in the antioxidant process. The mechanism of action still needs to be explored in more in-depth experiments, but from what we know, the described technique lays the foundation for the extraction of antioxidants with good activity from various natural products.

Author Contributions

Jun Dang: conceptualization, methodology, writing—original draft, supervision, funding acquisition. Yue Lv: methodology, writing—original draft. Chenzhao Li: software, writing—review & editing. Yan Fang: investigation. Gang Li: formal analysis, writing—review & editing, data curation, resources. Qilan Wang: conceptualization, methodology, project administration, validation, supervision, funding acquisition.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Figure Captions

Figure 1. *D. heterophyllum* extract separation chromatogram (A) with silica gel medium-pressure liquid chromatography system and the pretreatment chromatogram (B) of Fr1 from *D. heterophyllum* with MCI GEL®CHP20P medium pressure liquid chromatography. The on-line HPLC-DPPH activity screening profiles of Fr14 (C, D). The pretreatment chromatogram (E) of Fr14 with Diol medium pressure liquid chromatography. The on-line HPLC-DPPH activity screening profiles of Fr141 (F, G). The pretreatment chromatogram (H) of Fr141 with Spherical medium pressure liquid chromatography.

Figure 2. Analytical chromatograms of fractions Fr141 (A) and Fr1412 (B). On-line HPLC-DPPH activity screening profiles of Fr1412 (C, D). Preparative chromatograms of Fr1412 (E). On-line HPLC-DPPH activity screening profiles of Fr14123, Fr14124 and Fr14126 (F-K); Preparative chromatograms (L-N) of Fr14123, Fr14124 and Fr14126.

Figure 3. Purity and DPPH inhibitory activity verification chromatogram of the isolated Fr141231 (A, B), Fr141241 (C, D), Fr141261 (E, F), Fr141262 (G, H). Chemical structures of Fr141231 (I), Fr141241 (J), Fr141261 (K) and Fr141262 (L).

Figure 4. Effects of Fr141231, Fr141241, Fr141261 and Fr141262 on H_2O_2-induced L02 cells morphology.

Figure 5. Fr141231, Fr141241, Fr141261 and Fr141262 to DPPH free radical scavenging rate spectrum (A). Effects of Fr141231, Fr141241, Fr141261 and Fr141262 on H_2O_2-induced L02 cells SOD activities and MDA contents (B). Effects of Fr141231, Fr141241, Fr141261 and Fr141262 on intracytoplasmic and mitochondrial ROS (C and D). #$p < 0.05$ and ##$p < 0.01$ in comparison to normal cells, *$p < 0.05$ and **$p < 0.01$ in comparison to H_2O_2-induced cells. Duplicate samples were assessed in a minimum

of three independent experiments.

Figure 6. Molecular docking analysis of HO-1, NOX2 and NQO1 binding to Fr141231, Fr141241, Fr141261 and Fr141261, respectively.

Figure 7. Effects of Fr141231, Fr141241, Fr141262 and Fr141262 on H_2O_2-induced L02 cells HO-1, NOX2 and NQO1 expression. #$p < 0.05$ and ##$p < 0.01$ in comparison to normal cells, *$p < 0.05$ and **$p < 0.01$ in comparison to H_2O_2-induced cells. Duplicate samples were assessed in a minimum of three independent experiments.

738 **Table 1** Intermolecular interactions of isolated gingerols with HO-1, NOX2 and NQO1.

Compound	Binding Energy (Kcal/mol)	Ligand Interactions
HO-1		
Fr141231 (5-methoxy-6-gingerol)	-5.63	**LEU147** (Pi-Sigma, Pi-Alkyl, Alkyl); **MET34 (**Alkyl); **PHE214** (Alkyl); **ALA28** (Alkyl); **ASN210** (Carbon Hydrogen Bond); **ASP140** (Conventional Hydrogen Bond, Carbon Hydrogen Bond, Pi-cation, Pi-Anion); **ARG136** (Conventional Hydrogen Bond, Pi-cation, Pi-Anion), **THR135** (Carbon Hydrogen Bond).
Fr141241 (6-shogaol)	-6.08	**PHE167** (Pi-Alkyl, Alkyl); **PHE166** (Pi-Alkyl, Alkyl); **LEU147** (Pi-Alkyl, Alkyl); **ASP140** (Pi-Anion); **ARG136** (Conventional Hydrogen Bond); **MET34** (Pi-Sigma); **LEU54** (Pi-Alkyl, Alkyl); **VAL50** (Pi-Alkyl, Alkyl); **ASN210** (Conventional Hydrogen Bond); **PHE214** (Pi-Alkyl, Alkyl, Pi-Pi T-shaped); **LEU213** (Pi-Alkyl, Alkyl).
Fr141261 (6-paradol)	-5.40	**HIS25** (Conventional Hydrogen Bond, Pi-cation); **ALA28** (Pi-Alkyl, Alkyl); **MET34** (Pi-Alkyl, Alkyl, Pi-Sulfur); **PHE214** (Pi-Alkyl, Alkyl); **LEU213** (Pi-Alkyl, Alkyl); **VAL50** (Pi-Alkyl, Alkyl); **LEU54** (Pi-Alkyl, Alkyl).
Fr141262 (diacetoxy-6-gingerol)	-5.84	**ARG136** (Conventional Hydrogen Bond); **LEU54** (Pi-Alkyl, Alkyl); **VAL50** (Pi-Alkyl, Alkyl); **LEU147** (Pi-Alkyl, Alkyl); **LEU146** (Pi-Alkyl, Alkyl); **GLN38** (Conventional Hydrogen Bond); **MET34** (Pi-Sulfur); **LEU213** (Pi-Alkyl, Alkyl); **PHE214** (Pi-Alkyl, Alkyl).
NOX2		
Fr141231 (5-methoxy-6-gingerol)	-6.36	**ALA300** (Pi-Alkyl, Alkyl); **LEU299** (Pi-Alkyl, Alkyl); **PHE425** (Carbon Hydrogen Bond); **PHE245** (Pi-Alkyl, Alkyl) **SER41** (Carbon Hydrogen Bond); **LYS134** (Pi-Alkyl, Alkyl); **ILE160** (Pi-Alkyl, Alkyl); **CYS133** (Pi-Alkyl, Alkyl); **ILE44** (Conventional Hydrogen Bond, Pi-Alkyl, Alkyl); **ALA45** (Conventional Hydrogen Bond).
Fr141241 (6-shogaol)	-7.04	**GLU250** (Conventional Hydrogen Bond); **LEU251** (Conventional Hydrogen Bond, Alkyl, Pi-Sigma); **VAL81** (Conventional Hydrogen

		Bond); **MET33** (Alkyl).
Fr141261 (6-paradol)	-6.54	**ALA303** (Pi-Alkyl, Alkyl); **SER41** (Pi-Lone Pair); **LYS134** (Conventional Hydrogen Bond); **CYS133** (Pi-Sulfur); **ILE160** (Pi-Alkyl, Alkyl); **ILE44** (Pi-Alkyl, Alkyl); **ALA45** (Conventional Hydrogen Bond); **GLU163** (Carbon Hydrogen Bond).
Fr141262 (diacetoxy-6-gingerol)	-6.65	**ALA11** (Pi-Alkyl, Alkyl); **ALA303** (Pi-Alkyl, Alkyl); **HIS10** (Carbon Hydrogen Bond); **LEU299** (Pi-Alkyl, Alkyl); **ALA300** (Pi-Alkyl, Alkyl); **PHE245** (Pi-Sigma); **LYS134** (Conventional Hydrogen Bond) **SER41** (Conventional Hydrogen Bond); **ILE160** (Pi-Alkyl, Alkyl); **GLU163** (Conventional Hydrogen Bond, Carbon Hydrogen Bond).
NQO1		
Fr141231 (5-methoxy-6-gingerol)	-4.25	**LYS270** (Pi-cation); **ARG210** (Pi-cation); **PRO186** (Conventional Hydrogen Bond); **GLN187** (Conventional Hydrogen Bond); **LEU188** (Conventional Hydrogen Bond, Alkyl); **ILE203** (Alkyl).
Fr141241 (6-shogaol)	-5.01	**ARG14** (Conventional Hydrogen Bond, Pi-Alkyl, Alkyl); **ALA43** (Pi-Alkyl, Alkyl, Carbon Hydrogen Bond); **MET44** (Pi-Alkyl, Alkyl); **TYR19** (Pi-Alkyl, Alkyl); **ASP40** (Conventional Hydrogen Bond, Pi-Anion).
Fr141261 (6-paradol)	-4.44	**ARG14** (Conventional Hydrogen Bond, Pi-Alkyl, Alkyl); **ALA43** (Pi-Alkyl, Alkyl); **MET44** (Pi-Alkyl, Alkyl, Pi-Sulfur); **TYR19** (Pi-Alkyl, Alkyl); **ASP40** (Pi-Anion).
Fr141262 (diacetoxy-6-gingerol)	-3.56	**ARG14** (Conventional Hydrogen Bond, Pi-Alkyl, Alkyl); **ALA43** (Pi-Alkyl, Alkyl); **MET44** (Pi-Alkyl, Alkyl); **TYR19** (Pi-Alkyl, Alkyl); **LYS90** (Conventional Hydrogen Bond).

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