Characterization of the Components and Pharmacological Effects of Mountain-Cultivated Ginseng and Garden Ginseng Based on the Integrative Pharmacology Strategy

Sen Li1,2†, Ping Wang3†, Wenzhi Yang4, Chunhui Zhao3, Luoqi Zhang1,2, Jingbo Zhang5, Yuewen Qin3, Haiyu Xu3* and Luqi Huang2*

1College of Chinese Medicinal Materials, Jilin Agricultural University, Changchun, China, 2National Resource Center for Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China, 3Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China, 4Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China, 5College of Pharmacy, Heilongjiang University of Chinese Medicine, Harbin, China

Panax ginseng C. A. Mey (PGCAM) is a herbaceous perennial belonging to the Araliaceae family, mainly including Mountain-Cultivated Ginseng (MCG) and Garden Ginseng (GG) on the market. We aimed to establish a rapid, accurate and effective method to distinguish 15-year-old MCG and GG using ultra-performance liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (UPLC-QTOF-MS/MS), and also explored the pharmacological mechanisms of the main components using the Integrative Pharmacology-based Network Computational Research Platform of Traditional Chinese Medicine (TCMIP V2.0; http://www.tcmip.cn/). Altogether, 23 potential quality markers were characterized to distinguish 15-year-old MCG and GG, including ginsenosides Ra2, Rg1, and Ra1, and malonyl-ginsenoside Ra3, etc. The contents of 19 constituents (mainly protopanaxadiol-type) were higher in MCG compared with that in GG, and four constituents (mainly carbohydrate compounds) were higher in GG. The 105 putative targets corresponding to 23 potential quality markers were mainly involved in 30 pathways, which could be divided into 10 models, such as immune regulation, systems (metabolic, nervous, cardiovascular, reproductive), blood-pressure regulation, as well as antitumor, antiaging, antibacterial and anti-inflammatory effects. Furthermore, the potential quality markers of MCG and GG could inhibit the proliferation of breast cancer by regulating the mRNA expression of PSA, S6K, MDM2, and P53 genes by acting on AR, MTOR, PI3K and other targets. The Integrative Pharmacology Strategy may provide an efficient way to identify chemical constituents and explore the pharmacological actions of TCM formulations.

Keywords: mountain-cultivated ginseng, garden ginseng, UHPLC/IM-QTOF-HDMSE, metabolomics analysis, TCMIP V2.0
INTRODUCTION

Panax ginseng C. A. Mey (PGCAM) is an herbaceous perennial belonging to the Araliaceae family. Its dried root and rhizome (Panax ginseng C. A. Mey. Radix et Rhizoma) have been used as a herbal medicine in China and some Asian countries for thousands of years (Mancuso and Santangelo, 2017), and have immunomodulation, anti-fatigue, anti-aging, and anticancer effects (Shi et al., 2019). PGCAM was firstly recorded in Shennong’s Classic of Materia Medica (Liu et al., 2013), during which all PGCAM grew wild. However, with the development of society, wild-growing PGCAM could not meet human requirements. To solve the problem, farmers began to study and explore suitable cultivation methods for PGCAM. There are four types of PGCAM according to different cultivation methods: wild-growing ginseng, transplanted ginseng, mountain-cultivated ginseng (MCG) and garden ginseng (GG) (Yang et al., 2013). MCG and GG are recorded in the Pharmacopoeia of the People’s Republic of China from 2005 edition. MCG and GG have become the main varieties on the market.

MCG refers to the seeds of PGCAM germinated and grown in tall mountains and dense forests for 10–20 years (Zhu et al., 2018), so also called “Lin-Xia-Shan-Shen”. If MCG grows long enough, its quality and efficacy become almost the same as that of wild PGCAM. GG refers to PGCAM that planted in a garden and harvested after 4–6 years. In general, the quality of MCG is higher than that of GG, which has a stronger pharmacological effect (Kim et al., 2020). The price of PGCAM is directly proportional to the grown period: the longer the period, the higher the price. Therefore, adulteration or falsification of PGCAM has always been a serious problem in the commercial market.

To solve this problem, researchers have proposed different identification strategies for PGCAM. With the continuous development of new technologies, ultra-performance liquid chromatography-quadrupole time-of-flight-tandem mass spectrometry (UPLC-QTOF-MS/MS) has been widely used in the analysis of chemical components of PGCAM (Wu et al., 2018), multiple compounds have been identified, including ginsenosides, polysaccharides, fatty acids, volatile oils and amino acids (Liang et al., 2016). Ginsenosides, as the main components of PGCAM, show important pharmacological activities during the treatment of cardiovascular diseases (Kim, 2018). In addition, Prof. Xu and his colleague selected 12 chemical components to distinguish GG_{4,7 years} and MCG_{15 years}: ginsenoside Ra3/isomer, gypenoside XVII, quinqueinoside R1, ginsenoside Ra7, notoginsenoside Fo, ginsenoside Ra2, ginsenoside Rs6/Rs7, malonyl ginsenoside Rc, malonyl ginsenoside Rb1, malonyl ginsenoside Rb2, palmitoleic acid, and ethyl linoleate (Xu et al., 2016). However, the characteristic components and pharmacological effects of MCG and GG grown for 15 years have not been reported. Therefore, we aimed to employ the Integrative Pharmacology Strategy to characterize the differential component between the two types of Ginseng and explore their pharmacological effects in vitro. Integrative Pharmacology Strategy (IPS) pays attention to the interactions between Chinese prescriptions and the organism from multiple levels and multiple aspects, its research content mainly includes component analysis, network pharmacology analysis and pharmacological experiment verification (Xu and Yang, 2014). The IPS systematically analyzes the interaction between TCM formulations and the organism from multiple levels and multiple aspects to form a new mode of research of TCM. To practice this strategy better, we established Integrative Pharmacology-based Network Computational Research Platform of Traditional Chinese Medicine (TCMIP V2.0; www.tcmip.cn/), which comprising five databases and seven functional modules (Xu et al., 2019).

Therefore, we aimed to identify potential quality markers to distinguish 15-year-old MCG and GG using the UPLC-QTOF-MS/MS. Then, we employed the TCMIP V2.0 to carry out the network pharmacology analysis of the differential components contained in MCG and GG. Finally, we verified the results of network pharmacology in vitro. Our research will be a good application of Integrative Pharmacology Strategy.

MATERIALS AND METHODS

Chemicals and Reagents

Twelve batches of MCG and GG were collected from the cultivation areas in Huanren County (41.26°N, 125.36°E; Benxi City, Liaoning Province, China) in September 2017, and the voucher specimens were deposited in our lab. Sixty-six standards of PGCAM were purchased from Shanghai Standard Biotech (Shanghai, China) or isolated from the roots of PGCAM and Panax notoginseng C. A. Mey (Yang et al., 2016b). (Supplementary Table S1). HPLC-grade acetonitrile (CH3CN) and methanol were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, United States). Formic acid (FA) was obtained from Sigma-Aldrich (Saint Louis, MO, United States). Ultra-pure water was prepared in-house using the Milli-Q™ system (Millipore, Bedford, MA, United States).

Sample Preparation

First, 50 mg of MCG powder and GG powder were soaked with 3 ml of 70% methanol (v/v), respectively, followed by ultrasound extraction for 60 min at 25°C. Then, the solution was centrifuged at 14,000 rpm for 10 min at room temperature after compensating with 70% methanol for the weight lost. The supernatant was transferred to a 5 ml volumetric flask and diluted to the scale mark. Finally, 1 ml well-mixed liquid was centrifuged for 10 min, and supernatant were transferred to autosampler vials for analyses. Herbal samples were injected randomly. An equal volume of all test solutions was mixed to prepare the quality control (QC) sample, which was used to monitor the stability of the analytical system.

Chromatographic Separation and MS Conditions

UPLC-MS was performed on an Acquity™ UPLC I-Class/Vion Ion Mobility Spectrometry (IMS)-QTOF system (Waters, Milford, MA, United States). The chromatographic separation was carried out on a BEH Shield RP18 column (2.1 × 100 mm,
CH3CN-H2O (v/v) as the purge solvent and 50% CH3CN-H2O as the wash solvent, to minimize the carry-over between injections.

The 20 µm, 1.7 µm; Waters) maintained at 35°C. The mobile phase consisted of 0.1% FA in CH3CN (A) and 0.1% FA in H2O (B). The optimized gradient program was: 0–2 min, 15–20% A; 2–7 min, 20–30% A; 7–17 min, 30–33% A; 17–20 min, 33–60% A; 20–22 min, 60–98% A; 22–24 min, 98–98% A. The flow rate was 0.3 ml/min and the injection volume was 3 µL. A 3-min re-equilibration time was set between successive injections. A “purge–wash–purge” cycle was set on the autosampler, with 10% CH3CN-H2O (v/v) as the purge solvent and 50% CH3CN-H2O as the wash solvent, to minimize the carry-over between injections.

The MS experiment performed on a Vion IMS-QTOF mass spectrometer in the negative electrospray ionization mode (Waters, Corp., Milford, United States). The LockSpray™ ion source was equipped under the following parameters: capillary voltage, 1.0 kV; cone voltage, 20 V; source offset, 80 V; source temperature, 120°C; desolvation gas temperature, 500°C; desolvation gas flow (N2), 800 L/h; cone gas flow (N2), 50 L/h. Default parameters were defined for the traveling-wave IMS separation. HDMSE data covered a m/z of 300–1,500 at 0.3 s per scan. The low collision energy was set at 6 eV and the high-energy ramp was 40–80 eV. Data acquisition was controlled by the UNIFI 1.9.3.0 software (Waters, Corp., Milford, United States). The accuracy error threshold was fixed at 10 ppm.

**Date Processing**

Raw HDMSE data were corrected with reference to m/z 554.2620 in the 12 batches of samples and QC by UNIFI 1.9.3.0. Then, preliminary data were processed automatically by Progenesis QI 2.1 software (Waters, Corp., Milford, CT, United States) [M-H]⁻ and [M + FA-H]⁻ were the main adduct ions in the negative mode. Efficient menu-guided processing, peak alignment and peak selection could generate a data matrix, including retention time (tR), m/z, normalized peak area, and collision cross-sections (CCS). All the ion signals detected in each sample were normalized to the obtained value of the total ion count. The data matrix was filtered based on “80% rule” and “30% variation” (Li J. et al., 2019).

**The Untargeted Metabolomics Analysis Based on Multivariate Statistical Analysis**

Processed data were subjected to principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) using SIMCA-P 14.1 (Umetrics, Umea, Sweden). Potential quality markers were filtered out according to the variable importance for projection (VIP) values (VIP>1.5) and Student’s t-test (p < 0.05), which could be used to distinguish between MCG and GG.

**Prediction of Putative Targets of Potential Quality Markers**

The mol. formats of potential quality markers were uploaded to TCMIPI to predict the putative targets using the TCMIPI prediction and function analysis module (TTFM) of TCMIPI according to the similarity of chemical structures to known drugs on the market. The Tanimoto Score was set at 0.7 (moderate similarity) to select constituent–putative target pairs.

**Pathway Enrichment Analysis**

To further explore the functional biological functions of the putative targets, the pathway–enrichment analyses were undertaken using the database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov). Pathways with p < 0.05 were selected for further analyses.

**Cell Culture**

MCF-7 cell line was kindly provided by Prof. Xiujie Wang (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The MCF-7 cell line was cultured in DMEM high glucose medium (Thermo, Fair Lawn, NJ, United States) with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, United States) and 10% fetal bovine serum (FBS; Sijiqing, Huzhou, Zhejiang, China) at 37°C in humid atmosphere with 5% CO2.

**Cell Proliferation Analysis**

The extraction of MCG and GG was prepared according to Sample Preparation. Then, the methanol was volatilized and the residue was redissolved with sterile water. After filtered by 0.22 µm filter film, the herbal samples were stored in a refrigerator at 4°C. MCF-7 cell line was seeded in 96-well plates (5×10³ cells/well) incubating at 37°C for 24 h. Various concentrations of MCG or GG were added to the wells and then incubated for 48 h. Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK8) assay. The cells treated with various conditions were incubated with 10 µL CCK-8 solution (Dojindo, Kumamoto, Japan) for 90 min. Next the absorbance was measured by a Microplate Reader under 450 nm (Thermo, Fair Lawn, NJ, United States).

All experiments were performed in triplicate and repeated four times. Data were presented as mean ± standard deviation (SD). A One-way ANOVA determined whether the results had statistical significance.

**Quantitative Real-Time PCR Analysis**

The total RNA was isolated from the cultured cells using Trizol (Tiangen, China). Then, cDNA was synthesized using a ReverTra Ace quantitative polymerase chain reaction (PCR) RT Master Mix Kit with gDNA Remover (TOYOBO Co., OSAKA, Japan) according to the manufacturer’s protocol. PCR product amplification was performed with SYBR Green Master Mix (Juhemei, China) on an Agilent Mx 3000P Real-Time PCR System (Applied Biosystems, CA). Primers were 5′-GAATCATCGGACTCTTGAG-3′ and 5′-TGTCTGTCTACTAATTTGTCTCCT-3′ for MDMA2; 5′-CAGCACATGACGGGAGTGTG-3′ and 5′-TATCTCAAAATCTCCACACGC-3′ for P53; 5′-GTGAGGACGGACTAATCAGC-3′ and 5′-GTTCCTGGAACCCTGTCACCC-3′ for PSA; 5′-TTTGAGCTACTTGGGCTACCTTG-3′ and 5′-CGATGAGGGGTGCTTTACTCC-3′ for S6K; 5′-CTGAAGCCGTGAGGGATTAC-3′ and 5′-ATGGAACGACTTCTTACTG-3′ for BET-ACTIN, and 40 PCR cycles (30 s denaturation at 95°C, 20 s annealing at 60°C, 30 s elongation at 72°C) were run. BETA-
ACTIN was used as an internal standard. The relative mRNA levels were determined using the $2^{-\Delta\Delta C_t}$ method.

**Statistical Analyses**
All data were analyzed by SPSS 22.0 software (SPSS Inc., Chicago, IL, United States). Figures in this research were drawn with GraphPad Prism 8.0 software (GraphPad Prism, San Diego, CA, United States). The results were shown as mean ± standard deviation (SD). Significant differences between normally distributed gene expression data were determined by one-way analysis of variance (ANOVA). The significant difference was set at $p < 0.05$.

**RESULTS**

**Differential Components Between MCG and GG Based on Untargeted Metabolomics**
Multi-batch HDMS$^2$ data were processed by Progenesis QI, and generated a list comprising 1,023 ions (Supplementary Table S2). These ions were filtered based on “80% rule” and “30% variation” (Supplementary Table S3). Then, the remained 954 ions were subjected to PCA and OPLS-DA. MCG and GG groups were separated well in PCA score Plots (Figure 1A), indicating the great difference in chemical profiles. To obtain better discrimination, they were subjected to OPLS-DA with R$^2$X at 0.865 and Q$^2$ at 0.897, especially in the component P1+3 direction (Figure 1B). The validity and predictability of OPLS-DA model was evaluated by 200-time permutation test (Figure 1C). The regression lines of R2 and Q2 decreased with decreased in the correlation coefficients between permuted and original response variables. The result indicated that the predictive OPLS-DA models did not overfit. S-plots were obtained to show responsibility of each ion for these variations more showed intuitively the contribution of differential ions on distinguishing between MCG and GG. Most of the ions clustered around the original point (Figure 1D), and only a few of them were scattered in the margin region. Just these few ions that representing chemical constituents contributed to the separation observed in the score plots. The ions that far away from the original point, always have higher value of VIP (Supplementary Figure S1). Therefore, 42 ions with VIP >1.5 and $p < 0.05$ (in the Student’s t-test) were selected as potential quality markers (Supplementary Table S4), 31 (73.8%) of which were higher in MG, and 11 (26.2%) were higher in GG. Nine representative compounds were illustrated in Figures 2A–I.

**Identification of the Potential Quality Markers**
Sixty-six standard compounds and our house library were helped to identify the 23 potential quality markers. The base peak intensity chromatograms of MCG and GG corresponding to negative ion mode were shown in Figure 3. The 23 identified components with numbers on the peaks were classified as protopanaxadiol (PPD)-type sapogenins (M1, M3, M5, M6, M8, M11, M13, M17, M19, M20, M23, M24, M32, M33, M34, M42), Protopanaxatriol (PPT)-type sapogenins (M9, M12, M27,
FIGURE 2 | Box charts illustrating the distribution difference of the nine marker compounds among MCG and GG. (A–F) can be the characteristic components of MCG. (G–I) can be the characteristic components of GG.

FIGURE 3 | UHPLC/IM-QTOF-HDMSE of MCG and GG in the negative ion modes. MCG: the root and rhizome of Mountain-Cultivated Panax ginseng C. A. Mey; GG: the root and rhizome of Garden Panax ginseng C. A. Mey. (CG).
### TABLE 1 | Information of ginsenoside markers for differentiating among MCG and GG.

| No | VIP (min) | t<sub>n</sub> | Measured value (m/z) | Theoretical value (m/z) | Error (ppm) | Formula | Adducts | ESI-MS2 | Compound name | References |
|---|---|---|---|---|---|---|---|---|---|---|
| M1<sup>a</sup> | 7.60 | 11.25 | 1,255.6319 | 1,255.6328 | -0.7167 | C<sub>36</sub>H<sub>58</sub>O<sub>26</sub> | +HCOO | 1,209.6271,1077.5858 | Ginsenoside Ra2 | Zuo et al. (2019) |
| M3 | 5.53 | 13.92 | 1,295.6267 | 1,295.6278 | -0.8490 | C<sub>46</sub>H<sub>62</sub>O<sub>29</sub> | -H | 1,251.6307,1209.6275 | Malonyl-ginsenoside Ra1 | Yang et al. (2016a) |
| M5 | 4.23 | 12.00 | 1,295.6267 | 1,295.6278 | -1.0034 | C<sub>61</sub>H<sub>100</sub>O<sub>29</sub> | -H | 1,251.6338,1209.6275 | Malonyl-ginsenoside Ra2 | Yang et al. (2016a) |
| M6<sup>a</sup> | 4.11 | 18.07 | 991.5473 | 991.5483 | -1.0085 | C<sub>48</sub>H<sub>82</sub>O<sub>18</sub> | +HCOO | 945.5399,783.4903 | Notoginsenoside K | Zuo et al. (2019) |
| M7 | 4.11 | 0.78 | 341.1085 | 341.1085 | -1.0000 | C<sub>12</sub>H<sub>22</sub>O<sub>11</sub> | -H | 1,107.5927,1077.5818 | Sucrose or maltose | — |
| M8 | 3.84 | 16.31 | 1,295.6278 | 1,295.6278 | -0.3087 | C<sub>62</sub>H<sub>102</sub>O<sub>30</sub> | -H | 1,107.5964,1077.5855 | Malonyl-ginsenoside Ra3 or isomer | Yang et al. (2016a), Li J. et al. (2019) |
| M9<sup>a</sup> | 3.19 | 9.54 | 845.4896 | 845.4904 | -0.9462 | C<sub>42</sub>H<sub>72</sub>O<sub>14</sub> | +HCOO | 945.5395,783.4899 | Notoginsenoside S or isomer | — |
| M10 | 3.01 | 9.91 | 1,325.6375 | 1,325.6378 | -0.2263 | C<sub>62</sub>H<sub>102</sub>O<sub>30</sub> | -H | 945.5390,783.4896 | Malonyl-ginsenoside Rg1 | Li J. et al. (2019) |
| M11 | 3.00 | 9.62 | 716.3360 | 716.3367 | -0.9772 | C<sub>63</sub>H<sub>106</sub>O<sub>30</sub> | +2HCOO | 1,341.7149,1209.6689,1077.6205,945.5751 | Notoginsenoside S or isomer | — |
| M12<sup>a</sup> | 2.98 | 12.35 | 1,325.6378 | 1,325.6378 | -0.1509 | C<sub>62</sub>H<sub>102</sub>O<sub>30</sub> | -H | 1,107.5964,1077.5855 | Malonyl-ginsenoside Ra3 or isomer | Yang et al. (2016a), Li J. et al. (2019) |
| M13 | 2.97 | 1.35 | 341.1084 | 341.1085 | -0.2932 | C<sub>12</sub>H<sub>22</sub>O<sub>11</sub> | -H | 1,107.5927,1077.5818 | Sucrose or maltose | — |
| M14 | 2.76 | 18.86 | 1,031.5434 | 1,031.5433 | 0.0096 | C<sub>54</sub>H<sub>86</sub>O<sub>24</sub> | -H | 998.4552,945.5363 | Malonyl-floralginnsenoside Rd6 or isomer | Zhang et al. (2019) |
| M15 | 2.55 | 19.03 | 1,117.5429 | 1,117.5432 | -0.2684 | C<sub>62</sub>H<sub>102</sub>O<sub>30</sub> | -H | 998.5576,945.5401 | Malonyl-floralginnsenoside Rd6 or isomer | Zhang et al. (2019) |
| M16 | 2.34 | 19.17 | 1,033.5563 | 1,033.5583 | -1.9351 | C<sub>62</sub>H<sub>102</sub>O<sub>30</sub> | +HCOO | 987.5576,945.5401 | PPD-3GLc-ace | Zhang et al. (2019) |
| M17 | 2.16 | 9.08 | 731.3414 | 731.3420 | -0.8204 | C<sub>40</sub>H<sub>60</sub>O<sub>18</sub> | +HCOO | 945.5376,459.3874 | Notoginsenoside T or isomer | — |

(Continued on following page)
| No | VIP | $t_r$ (min) | Measured value (m/z) | Theoretical value (m/z) | Error (ppm) | Formula | Adducts | ESI-MS2 | Compound name | References |
|----|-----|-------------|----------------------|------------------------|-------------|---------|---------|---------|--------------|------------|
| M24s | 2.11 | 11.61 | 1,239.6367 | 1,239.6380 | -1.0487 | $\text{C}_{29}\text{H}_{50}\text{O}_{27}$ | -H | 1,107.5968,1077.5858 | Ginsenoside Ra3 | Yang et al. (2013) |
| M27s | 1.93 | 6.90 | 887.4990 | 887.5010 | -2.2535 | $\text{C}_{44}\text{H}_{74}\text{O}_{15}$ | +HCOO | 841.4941,781.4711 | Notoginsenoside Rb | |
| M28 | 1.91 | 10.49 | 887.4999 | 887.5010 | -1.2394 | $\text{C}_{44}\text{H}_{74}\text{O}_{15}$ | +HCOO | 841.4971,781.4917 | Acetyl panajaponol A | Wang et al. (2016) |
| M31s | 1.87 | 6.81 | 1,031.5432 | 1,031.5433 | -0.0969 | $\text{C}_{51}\text{H}_{84}\text{O}_{21}$ | -H | 799.4758,637.4341 | Malonyl floraninsenoside Re1 | Zuo et al. (2019) |
| M32 | 1.78 | 16.74 | 1,295.6266 | 1,295.6278 | -0.9262 | $\text{C}_{51}\text{H}_{84}\text{O}_{21}$ | -H | 1,107.5887,1077.5786 | PPD-20-GlcXyXyl-3-Glc-Glc-malonyl | Yang et al. (2012) |
| M33s | 1.77 | 13.12 | 1,255.6302 | 1,255.6328 | -2.0707 | $\text{C}_{51}\text{H}_{84}\text{O}_{21}$ | +HCOO | 1,209.6275,1077.5862 | Ginsenoside Ra1 | Zuo et al. (2019) |
| M34s | 1.73 | 18.82 | 1,031.5434 | 1,031.5433 | 0.0969 | $\text{C}_{51}\text{H}_{84}\text{O}_{21}$ | -H | 1,077.5992,945.5440 | Malonyl-floraninsenoside Rd5 | Zuo et al. (2019) |
| M42 | 1.51 | 7.38 | 971.4854 | 971.4857 | -0.3088 | $\text{C}_{48}\text{H}_{72}\text{O}_{20}$ | -H | 799.4721,637.4406 | (US-5)-glc-glurA-glc | Yang et al. (2012) |

s, Ginsenosides identified by comparing with reference standards.
M28, M31) and carbohydrate compounds (M7, M15). The detailed information of the 23 components was listed in Table 1.

Characteristic MS/MS features of the ginsenoside Ra2 were observed in the MS² spectrum, including m/z 1209.6271 ([M-H]⁻), 1077.5858 ([M-H-Xyl]⁻), 945.5432 ([M-H-Xyl-Ara]⁻), 783.4898 ([M-H-Xyl-Ara-Glc]⁻), 621.4375 ([M-H-Xyl-Ara-2Glc]⁻), and 459.3874 ([PPD-H]⁻) (Figure 4). M1 (ginsenoside Ra2, tR, 11.25 min, chemical formula, C₅₈H₉₈O₂₆), M6 (notoginsenoside K, tR, 18.07 min, chemical formula, C₄₈H₈₂O₁₈), M24 (ginsenoside Ra3, tR, 11.61 min, chemical formula, C₅₉H₁₀₀O₂₇), M33 (ginsenoside Ra1, tR, 13.12 min, chemical formula, C₅₉H₉₈O₂₆), M34 (malonyl-floralginsenoside Rd5, tR, 18.82 min, chemical formula, C₅₃H₈₄O₂₁) were identified as PPD-type sapogenins compared with the reference compounds (Supplementary Table S1).

Taking ginsenoside Ra2 as an example, the fragmentation pattern of panaxadiol saponins was analyzed. Taking ginsenoside Rg1 as an example, the fragmentation pattern of panaxatriol saponins was analyzed. Characteristic MS/MS features of ginsenoside Rg1 were observed in the MS² spectrum, including m/z 799.4859 ([M-H]⁻), 637.4334 ([M-H-Glc]⁻), 475.3788 ([M-H-2Glc]⁻), and 391.2841 ([M-H-2Glc-C₆H₁₂]⁻) (Figure 4). M9 (ginsenoside Rg1, tR, 9.54 min, chemical formula, C₄₂H₇₂O₁₄), M12 (notoginsenoside S, tR, 9.62 min, chemical formula, C₄₀H₁₀₆O₂₀), M27 (notoginsenoside Rt, tR, 6.90 min, chemical formula, C₄₃H₇₂O₁₃), M31 (malonyl-floralginsenoside R1, tR, 6.81 min, chemical formula, C₅₁H₈₆O₂₁) were identified as PPT-type sapogenins compared with the reference compounds (Supplementary Table S1).

Functional Analysis of Potential Quality Markers

A total of 105 putative targets were predicated based on 23 primarily identified compounds (Tanimoto score ≥0.7) using TTFM of TCMIP v2.0 (Supplementary Table S5). Which were mainly involved in 30 pathways after functional analysis DAVID 6.8 (Supplementary Table S6). The network of interactions between 23 components, the corresponding 105 putative targets and 30 pathways was visualized using Cytoscape v3.7.1 (Boston, MA, United States) as show in Figure 5. The 30 pathways were related to 10 function modules, such as metabolic system, nervous system, cardiovascular system, immune-regulation system and reproductive system. Among them, there were 12 immune pathways (fc epsilon RI signaling pathway; leukocyte transendothelial migration; non-small cell lung cancer; pathways in cancer; acute myeloid leukemia; toll-like receptor signaling pathway; small cell lung cancer; apoptosis; prostate cancer; natural killer cell mediated cytotoxicity; glioma; mammalian target of rapamycin signaling pathway (mTOR) signaling pathway), nine metabolic pathways (starch and sucrose metabolism; oxidative phosphorylation; type II diabetes mellitus (DM); insulin signaling pathway; galactose metabolism; tricarboxylic acid cycle (TCA cycle); glycolysis/
FIGURE 5 | Illustration of the relevance among marker compounds for distinguishing MCG and GG, their target, pathways and therapeutic effects. Blue nodes represent characteristic constituents of MCG. Green nodes represent characteristic constituents of GG. Orange nodes refer to the targets; Purple nodes represent the pathways; Yellow nodes refer to therapeutic effects. Red nodes represent the effect of ginseng.

FIGURE 6 | In vitro pharmacodynamics experiments and RT-PCR detection of key effector genes in MCG and GG. (A): Results of in vitro pharmacodynamics experiments of MCG and GG. (B–F): Results of RT-PCR detection of key effector genes in MCG and GG. **: $p < 0.05$; ****: $p < 0.0001$. (F): Mechanism of anti-breast cancer effect of quality markers of MCG.
Inhibitory Effect of MCG and GG on MCF-7 Cell Line by Regulating the PSA, MDM2, P53, and S6K genes

The enrichment analysis above showed that 23 components played more important roles in tumor pathway, which indicated that the anticancer effect of MCG was stronger than GG. Therefore, we selected MCF-7 cell line to verify the results. The CCK-8 assay showed that MCG or GG did not significantly inhibit the proliferation of MCF-7 cell line when the concentration was lower than 4 mg/ml. When the concentration of MCG or GG was 8 mg/ml, the survival rate of MCG group was only 11.8%, and GG group was 21.99%, which indicated that the concentration of 4–8 mg/ml was the range of MCG and GG to inhibit the proliferation of MCF-7 cell line (Supplementary Figure S2). Thus, the concentrations of 4, 5, 6, 7, and 8 mg/ml were set to explore the inhibitory effect of MCG and GG on MCF-7 cell line. When the extract concentration was 4 mg/ml, the survival rate of MCF-7 cell line was significantly inhibited by MCG compared with GG (Figure 6A).

Therefore, MG and GG at 4 mg/ml were selected to subject to RT-PCR detection. The mRNA expression of PSA, MDM2 and S6K genes in the group of MCG was significantly down-regulated compared with that of control group, and the degree of down regulation was stronger than that of GG. Conversely, the mRNA expression of P53 gene was significantly up-regulated, and the degree stronger than that of GG (Figures 6B–E).

DISCUSSION

PGCAM as a herbal nutritional supplement is well known for its extensive pharmacological effects (Nguyen and Nguyen, 2019). MCG and GG are the main PGCAM varieties on the market to meet the huge demand of people (Yang et al., 2013) (Shen et al., 2019). At present, ginsenoside is the main marker for identification of different species of MCG and GG. For example, ginsenosides can be used to distinguish different planting methods and different years of ginseng (Cui et al., 2013; Ruan et al., 2018). As natural plants, the environment and duration of growth affect the quality and price of PGCAM directly. (Xu et al., 2016; Zhu et al., 2018). Many studies have been undertaken to characterize the chemical profiles of PGCAM cultivated in different environments for various durations, which could help distinguish diverse samples (Chang et al., 2016; Chang et al., 2017). Hence, we aimed to employ integrative pharmacology strategy to characterize the differential components between the two types of Ginseng and explore their pharmacological effects by network pharmacology and verified in vitro.

Altogether, 23 chemical components were identified to distinguish the two sets of samples, and nine of them were identified according to use of reference compounds. The characteristic fragments of PPD-saponins were m/z 459.38 and m/z 375.29. These data could be used to identify unknown marker compounds belonging to this type, including M14, M16, M18, M26, M29, M30, M36, M39, and M41. Ginsenosides were the main characteristic components of the MCG group. Carbohydrates were the main characteristic components of the GG group. The peak area of PPD-type saponins in the MCG group was higher than that of GG group, and the peak area of carbohydrate compounds was higher in the GG group. The results showed that ginsenoside Rg2 and sucrose could not only distinguish 15 years old MCG and GG, but also 10–20 years old MCG and 4–6 years old GG (Xu et al., 2016). Ginsenoside Rg1 can not only distinguish the 15-year-old forest ginseng and garden ginseng, but also distinguish the ginseng of different ages, different planting sites and different slope directions (Zhu et al., 2021). In conclusion, some of the quality markers in the same region can distinguish ginseng under different conditions. It shows that our research results have certain universality.

To explore further the pharmacological activities of these marker components, components–targets–pathways network was constructed. We found that the marker components could regulate immune, metabolic, nervous–system, cardiovascular–system and reproductive–system pathways. Among them, immunomodulatory and metabolic regulatory pathways were the main pathways regulated by marker components during the treatment of cancer and diabetes mellitus.

The pharmacological effects of marker components were investigated further. Ginsenoside Rg1 has been found to: 1) protect the heart from cardiovascular diseases (Xu et al., 2018); 2) impact the neuroendocrine system for treatment of depression (Mou et al., 2017); 3) inhibit inflammation and apoptosis (Guo et al., 2019); 4) possess neuroprotective properties (Li G. et al., 2019); 5) enhance gene expression and oxidative muscle metabolism in muscles (Jeong et al., 2019); 6) protect against diabetic nephropathy (DN) by reducing oxidative stress (Du et al., 2018). Hence, Rg1 can regulate the cardiovascular system, nervous system and immune system. Sucrose can reduce procedural pain from single events, including heel lancing, venipuncture and intramuscular injection, which shows that sucrose can regulate the nervous system without side effects. (Stevens et al., 2016). Ginsenoside Ra1 is the main active component of ginseng used for immune regulation (Liu et al., 2019). Hence, ginsenosides Ra1 can affect the cardiovascular system and regulate the immune system. Notoginsenoside K has been shown to exhibit immunologic-adjuvant properties in the humoral immune responses of ICR mice against ovalbumin (Sun et al., 2005; Qin et al., 2006). Ginsenosides Ra1, notoginsenoside K, and ginsenoside Rg1 can affect the cardiovascular system, immune regulation, nervous system, and have anti-inflammatory and antioxidant effects. Sucrose has a protective effect upon HepG2 cells and
ampli
downstream targets 40 S ribosomal S6 kinases (S6K).

cancer. mTORC1 mediates its function via its

tumor or cancer (Balk et al., 2003). The RT-PCR data showed that

tumor or cancer (Konopleva et al., 2020)and suggests that sucrose has an

MCF-7 cell samples treated by MCG and GG showed that there

pharmacological activities of potential quality markers were

P53 activity in some human tumors (Konopleva et al., 2020)and

notoginsenoside Rt, notoginsenoside S, notoginsenoside T,

P53 in order to treat cancer (Gupta et al., 2019). Moreover,

MTOR pathway plays an important role in the treatment of

PROSTATE SPECIFIC ANTIQUATION. AR is a serine protease produced by prostate epithelial cells and prostate cancer (PCA), which can be regulated by AR. The increase of PSA level can be used as a diagnostic marker of

pharmacological actions of TCM formulations.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the

AUTHOR CONTRIBUTIONS

HX and LH designed the study. SL and PW drafted the

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**SUPPLEMENTARY MATERIAL**

The Supplemental Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.659954/full#supplementary-material.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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