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Research Article
The Ginsenosides of Black Ginseng Against Prostatic Cancer by Spectrum-Effect and Structure-Effect Relationships

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Abstract
Background and Objective: Black Ginseng (BG) is a new type of processed ginseng and presently, there is almost no anti-cancer comparison among the ginsenosides of BG together. To elucidate the anti-cancer substance of BG and lay a foundation for the development of new drugs. **Materials and Methods:** Firstly, the anti-cancer activities of BG decoction and its separated fractions were compared on prostate cancer cells (DU145) with the serum pharmacology method. And then, the constituents of BG and its separated fractions as well as their serum after BG and its separated fractions administered intragastrically were identified by Ultra-High-Performance Liquid Chromatography-Quadrupole Time-Of-Flight Tandem Mass spectrometry (UPLCQ-TOF-MS/MS). Then, the spectrum-effect relationship was carried out by the analysis of the spectra of 10 batches of BG with High-Performance Liquid Chromatography (HPLC) and Methyl Thiazolyl Tetrazolium (MTT) assay to evaluate the inhibitory rate of BG against DU145. Finally, an anticancer structure-effect relationship of ginsenosides was performed to revalidate the conclusion. **Results:** The results showed that the fractions of Total Saponins (TSF) and 95% Alcohol Eluate (AEF) were the effective fractions for anti-prostate cancer action. There were eight chromatographic peaks (ginsenosides S-Rg2, S-Rg3, R-Rg3, RK1, Rg5, peak number of 17 and 18) which contributed greatly to anti-prostate cancer of black ginseng. The IC50 of ginsenosides Rg5, S-Rh2, R-Rh2, S-Rh1, R-Rh1, RK1, RK3, R-Rg3, PPT and Compound K were less than 50 $\mu mol\ L^{-1}$, which have better anti-prostate cancer activity compared with other ginsenosides. **Conclusion:** The effective components of BG against prostate cancer were its secondary ginsenosides.

Key words: Spectrum-effect relationship, structure-effect relationship, black ginseng, prostate cancer, effective substance, serum pharmacology, alcohol eluate, ginsenosides

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ginseng, the dried roots and rhizomes of *Panax ginseng* C.A. Mey, has been used in China for thousands of years as a restorative and tonic medicine. As usual, processing is an important way for the use of herbal medicines in Traditional Chinese Medicine (TCM)[1]. Traditionally, white, red and sugar ginseng was used in TCM. The white or (sun-dried) ginseng is produced by direct air-drying of the fresh ginseng without steaming, while red ginseng was named from its red appearance by the processing of ginseng after one time steaming and drying[2]. Sugar ginseng is processed by injection of sucrose water to modify its flavour. Presently, white ginseng and red ginseng are generally used and recorded in China's Pharmacopoeia[3].

BG is a newly processed product to produce rare ginsenosides by way of steaming and drying fresh ginseng several times (usually 9 times) and now it was produced industrially in Korea and China, the representative components of BG are ginsenosides Rk1, Rk3, Rg5, Rg3. Compared with ginseng, the processed BG has stronger pharmacological activities of anti-tumour, anti-inflammation, anti-oxidation, lowering blood sugar, enhancing resistance and anti-ageing. However, most studies on BG only focused on its own or single ginsenoside and lacked comparison with separated fractions, ginseng, red ginseng or various ginsenosides or positive drugs[4-8].

Thus, a comparative study should be performed to elucidate the anticancer effective constituents of BG. Recently, we established the local quality standard for industrial processing procedure of BG with Chinese fresh ginseng and compared the ginsenosides, oligosaccharides and amino acids of white ginseng, red ginseng and BG as well as anticancer net pharmacology of ginseng and the network pharmacological analysis of ginseng, red ginseng and BG against cancer was carried out in the early stage, indicating that BG is most sensitive to prostatic cancer[6,9,10].

Therefore, we firstly screened the active fractions of BG against prostate cancer by using the method of serum pharmacology, then, the components of BG and blood after administered intragastrically, with BG were analyzed by UPLCQ-TOF-MS/MS and meanwhile, spectrum-effect and structure-effect relationships of ginsenosides were further performed to elucidate the anticancer substance of BG and lay a foundation for the development of new drugs. It is for the first time that the effective substance of BG against prostatic cancer was elucidated based on spectrum-effect and structure-effect relationships as well as the serum pharmacology.

MATERIALS AND METHODS

Study area: The study was carried out at the Chinese medicine Chemistry Lab, Liaoning University of Traditional Chinese Medicine China from October, 2020-May, 2021.

Materials and reagents: The 10 batches of BG (Liaoning Zhongshu Tang Black Ginseng Co. Ltd.). All the samples were identified by Professor Xu Liang from Liaoning University of Traditional Chinese Medicine. Ginsenosides Rb1, Rd, S-Rg3, R-Rg3, S-Rh2, R-Rh2, Re, Rg1, Ro, S-Rh1, R-Rh1, Rg5, Rk1, Rk3, Compound K (CK), PPT, PPD, S-Rg2, R-Rg2, Rd2 standard products (Sichuan Weikeqi Biotechnology Co. Ltd.), Human prostate cancer cells (Du145) (Kunming Cell Bank of the Academy of Sciences), Methanol (Oceanpak, Sweden), Acetonitrile (Tedia, USA), Methyl thiazolyl tetrazolium (MTT) (Amresco, USA), Dimethyl sulfoxide (DMSO) (SIGMA, USA), RPMI-1640 medium (HyClone, USA), Fetal bovine serum (Gibco, USA), Phosphate-Buffered Saline (PBS) (Solarbio, Beijing). Annexin V-FITC cell apoptosis detection kit and Caspase-3 activity kit (Nanjing Jiancheng Biological Co. Ltd.).

Serum pharmacology

Extracts preparation of BG and its separated fractions: BG powder (80 g) was extracted twice (2 hrs each time) by refluxed with 640 mL of water and freeze-dried (BG). BG was separated into four fractions: Polysaccharides (PF), Oligosaccharides (OF), Total Saponins (TSF) and 95% alcohol eluate (AEF). (Supplementary Materials Fig. S1). The yield of each separated fraction of BG was shown in Supplementary Materials Table S1.

Preparation of rat serum containing drugs: A total of 30 male SD rats (weighing approximately180-220 g) were offered by the Liaoning Changsheng Biotechnology Co. Ltd, Liaoning, China (License Key: SCXK (Liao) 20200001) and maintained under controlled conditions (25$\pm$2°C, 45$\pm$5% relative humidity and 12 hrs light/dark cycle) with free access to standard food and water. Animal research was approved by the Animal Ethical and Welfare Committee of Liaoning University of Traditional Chinese Medicine and the experimental protocols were conducted according to the Guide for Care and Use of Laboratory Animals of Liaoning University of Traditional Chinese Medicine (131/2010).

After one week of adaptation, the rats were randomly divided into 6 groups, every group had five rats. Control groups (CON) (0.5% sodium carboxymethyl cellulose reagent), BG group (3.51 g/kg/day), PF group (1.37 g/kg/day), OF group (1.50 g/kg/day), AEF group (36.9 mg/kg/day) and TSF group

(425 mg kg/day) were administrated intragastrically with the corresponding drugs. Basis of administered concentration was in Supplementary Materials. The drug was given once daily for 7 consecutive days. The serum was obtained by centrifugation at 3500 rpm for 10 min at 4°C and stored at -20°C for later use. RPMI 1640 medium with drug serum-containing of 5, 10, 15 and 20% was prepared, respectively. At the same time, the CON group and Normal Fetal Bovine Serum group (NFBS) were prepared as above.

Detection of cell proliferation by the MTT method: DU145 cells were cultured in RPMI 1640 supplemented with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin. The cells were incubated at 37°C and in 5% CO_2. The cells were seeded in 96-well plates at 1×10^5 cells mL^{-1} in triplicate. After 24 hrs, the cells were treated with the test serum (RPMI 1640 complete culture medium with drug serum-containing of 5, 10, 15 and 20%) and rat control serum, normal fetal bovine serum for 48 hrs. Then 10 μL of the MTT solution (5 mg mL^{-1}) was added to each well and incubated for 4 hrs at 37°C. The supernatant was then removed and the formazan crystals were dissolved with 100 μL of DMSO. The absorbance was measured at 492 nm (reference wavelength was 630 nm) with the enzyme-linked immunosorbent assay plate reader (Shenzhen Caretium Biomedical Electronic Technology Co. Ltd.).

Blood composition analysis of BG and its separated fractions: Prepared a solution of mixed standards (The concentration of Re, Rf, S-Rg2, R-Rg2, F1, Rb2, Rb3, Rd, R-Rg3, Rh4, Rk1, Rg5 was 2.05, 1.96, 2.90, 1.98, 3.45, 4.14, 0.65, 1.88, 0.18, 9.61, 3.67, 0.58 μg mL^{-1}). The BG (81.30 mg), TSF (9.84 mg) and AEF (0.855 mg) were accurately weighed out and added to 60 mL methanol. The serum (200 μL) was extracted with 600 μL of acetonitrile: methanol (1:1, v/v). The sample was swirled for 30 sec and then centrifuged for 5 min (10000 r min^{-1}) at 4°C and 100 μL of supernatant was taken into the sample tube for detection. The prepared samples were performed on the UPLC-QTOF-MS/MS system (Agilent Technology Co. Ltd.), a ZORBAX SB-C18 column (2.1×100 mm, 1.8 μm) from Agilent was used. The samples were separated by using a gradient mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid acetonitrile. The elution condition was as follows: Linear-gradient from 15-30% B (0-3 min), 30-35% B (3-5 min), 35-35% B (5-7.5 min), 35-55% B (7.5-11.5 min), 55-100% B (11.5-18 min), 100-100% B (18-19.5 min). The flow rate was 0.40 mL min^{-1}. The injection volume of samples was 5 μL. The mass spectrometry under negative ion conditions was analyzed under the following conditions: The ion source was ESI source, the capillary voltage was 3.0 kV, the sampling cone voltage was 45 V, the dry gas flow rate was 14 L min^{-1}, sheath gas was 11 L hr^{-1}. The data acquisition rate was set at 1 sec $scan^{-1}$ with a 0.2 sec interscan delay and the mass range was set at 80-1500 m/z.

Identification of BG ingredients: BG freeze-dried powder (81.30 mg) was accurately weighed out and added to 60 mL methanol. The prepared sample was performed on the UPLC-Q-TOF-MS/MS system whose detector was a Diode Array Detector (DAD). An HPH-C18 column (100×3.0 mm, 2.7 μm) from Agilent was used. Chromatographic conditions were in supplementary materials. The effluent was directly transferred to flight mass spectrometry and analyzed with a negative Electrospray Ion Source (ESI). The mass spectrometry conditions were the same as above.

Analysis of the relationship between spectrum and efficiency: Prepare 10 batches of BG to freeze-dry powder solution with methanol, the yield of BG freeze-dry powder and the concentration of the solution was shown in the Supplementary Materials Table S2.

The spectra of 10 batches of samples were obtained by the High-performance Liquid Chromatography (HPLC). The condition of HPLC was the same as that in the identification of BG ingredients. The spectra data of 10 samples were imported into the "Chromatographic Fingerprint Similarity Evaluation System 2012 Edition" issued by the Chinese Pharmacopoeia Commission for analysis and processing.

Total 10 batches of BG were diluted in culture medium to the concentration of 7.5 mg mL^{-1} (The basis of the concentration was in the Supplementary Material). The proliferation inhibition rate of 10 batches of BG against DU145 was determined by the MTT method.

Then we carried on the bivariate correlation analysis and the grey correlation degree analysis to carry on the spectrum efficiency analysis.

Analysis of the structure-effect relationship

Inhibition of proliferation of different ginsenosides on DU145: The structure of PPT, ginsenosides Rb1, Rd, Rd2, R-Rg3, S-Rg3, CK, S-Rh2, R-Rh2 was shown in Fig. 1a, the structure of PPT, ginsenosides Re, Rg1, S-Rg2, R-Rh2, S-Rh1, R-Rh1 was shown in Fig. 1b, the structure of ginsenoside Ro was shown in Fig. 1c; the structure of ginsenoside Rg5 was shown in Fig. 1d; the structure of ginsenosides Rk1 and Rk3 was shown

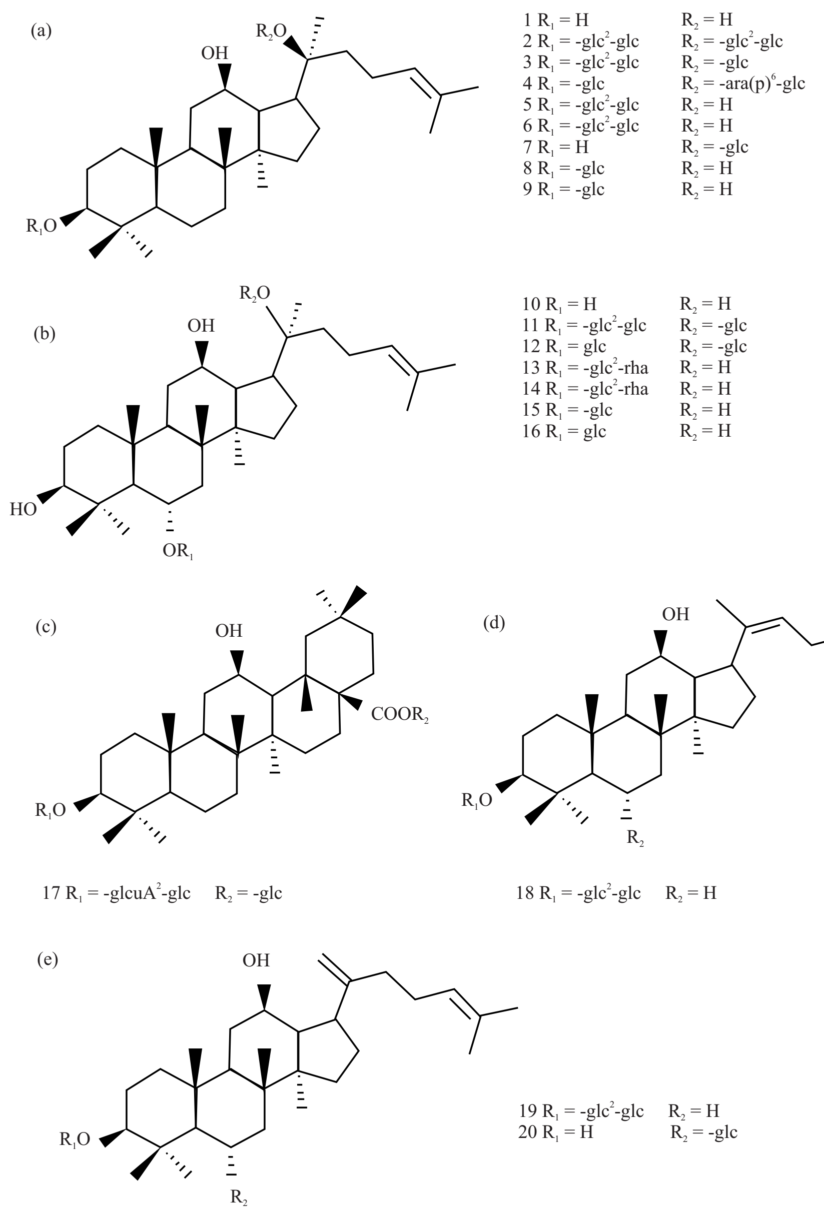

Fig. 1(a-e): Structure diagram of ginsenosides

1: PPT, 2: Ginsenoside Rb1, 3: Ginsenoside Rd, 4: Ginsenoside Rd2, 5: Ginsenoside R-Rg3, 6: Ginsenoside S-Rg3, 7: CK, 8: Ginsenoside S-Rh2, 9: Ginsenoside R-Rh2, 10: PPT, 11: Ginsenoside Re, 12: Ginsenoside Rg1, 13: Ginsenoside S-Rg2, 14: Ginsenoside R-Rg2, 15: Ginsenoside S-Rh1, 16: Ginsenoside R-Rh1, 17: Ginsenoside Ro, 18: Ginsenoside Rg5, 19: Ginsenoside Rk1 and 20: Ginsenoside Rk3

in Fig. 1e and the preparation of the test solution was shown in the Supplementary Materials Table S3. The cells of DU145 were treated with the test compounds with concentrations of 1, 10, 50, 100 μmol L^{-1}.

Then, the cells were treated with the test compounds (ginsenoside Rg5 and ginsenoside Rk1) with concentrations of 10 and 20 μmol L^{-1} for 48 hrs and the positive control group was treated with 10 μmol L^{-1} 5-Fu. Then we carried out experiments according to the instructions of the Annexin V-FITC cell apoptosis detection kit by the flow cytometry was used for detection (excitation wavelength Ex = 488 nm, Em = 530 nm) (BD Company).

Ginsenoside Rg5 (20 μmol L^{-1}) and ginsenoside Rk1 (20 μmol L^{-1}) of Detection of Caspase-3 activity *in vitro* were carried out by Caspase-3 activity kit.

Statistical analysis: SPSS2 5.0 was used for statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA), the results were all expressed as Mean±Standard deviation ($\bar{x}$±s). Grey relational degree analysis was analyzed by using Grey Modeling v3.0 software.

RESULTS

Effects of rat serum (BG and its separated fractions) on the proliferation of DU145 *in vitro:* The inhibitory rates of RPMI 1640 complete culture medium with drug serum-containing of 5, 10, 15 and 20% of BG and its separated fractions (which were PF, OF, TSF and AEF) on proliferation effect of DU145 was conducted and shown in Fig. 2. As compared with CON, 10% drug-containing serum of AEF (p<0.05), BG (p<0.01) and TSF (p<0.01) serum groups could inhibit the proliferation of prostate cancer and the difference exhibited obviously:

$$\text{Inhibitory rate of cell proliferation (\%)} = \frac{\text{1-Experimental group A-Zero group A}}{\text{Control group A-Zero group A}} \times 100$$

Analysis results of blood components of BG and its separated fractions: The total ion chromatogram of BG was in Fig. 3a; the total ion chromatogram of serum of BG was in Fig. 3b; the total ion chromatogram of TSF was in Fig. 3c; the total ion chromatogram of serum of TSF was in Fig. 3d; the total ion chromatogram of AEF was in Fig. 3e, the total ion chromatogram of serum of AEF was in Fig. 3f, the total ion chromatogram of CON serum was in Fig. 3g, the total ion chromatogram of reference solution was in Fig. 3h and the LC-MS data and identification of prototype components and metabolites of BG, TSF and AEF after entering blood were shown in Supplementary Table S4-S6. According to the external standard method, the content of saponins in the above components was simply calculated in Supplementary Table S7-8.

Identification of saponins in BG: By comparing references, the identification and analysis of ginsenosides of BG in ESI negative ion mode were shown in Table 1 by using the UPLC-Q-TOF-MS/MS conditions which were described in the methods. Eleven ginsenosides were identified. The mass spectrogram of ginsenosides in negative ion mode was presented in Supplementary Materials Fig. S2 and the DAD (203 nm) chromatogram as shown in Fig. 4. The mass spectrogram of ginsenoside Rf was in Supplementary Materials Fig. S2a, ginsenoside S-Rg2 was in Supplementary Materials Fig. S2b, ginsenoside R-Rg2 was in Supplementary Materials Fig. S2c, ginsenoside Rk3 was in Supplementary Materials Fig. S2d, ginsenoside Rh4 was in Supplementary Materials Fig. S2e, ginsenoside S-Rg3 was in Supplementary Materials Fig. S2f, ginsenoside R-Rg3 was in Supplementary Materials Fig. S2g, ginsenoside Rk1 was in Supplementary Materials Fig. S2h, ginsenoside Rg5 was in Supplementary Materials Fig. S2i, ginsenoside S-Rh2 was in Supplementary Materials Fig. S2j, ginsenoside R-Rh2 was in Supplementary

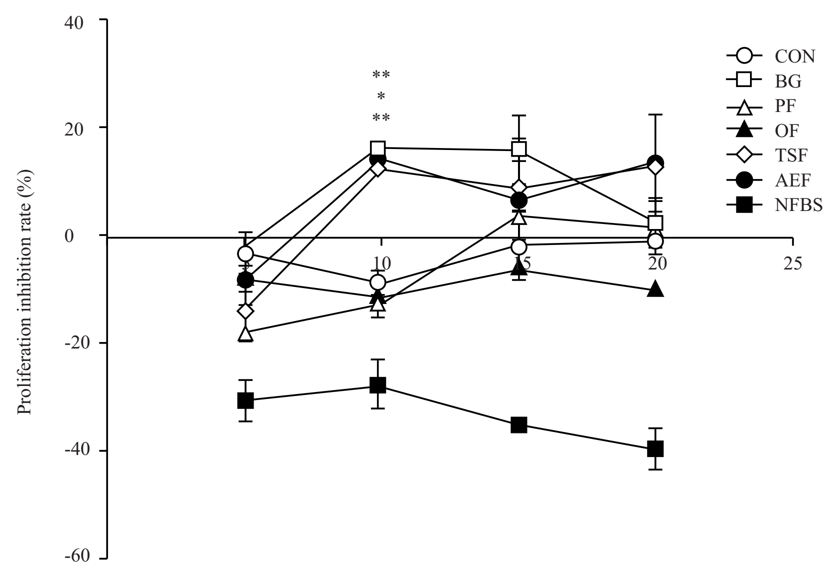

Fig. 2: Effect of drug-containing serum on DU145

Compared with control serum group of rats, *p<0.05, **p<0.01, CON: Control serum group, BG: Black ginseng decoction serum group, PF: Polysaccharide serum group, OF: Oligosaccharides serum group, TSF: Total saponins serum group, AEF: 95% alcohol eluate serum group and NFBS: Normal fetal bovine serum group

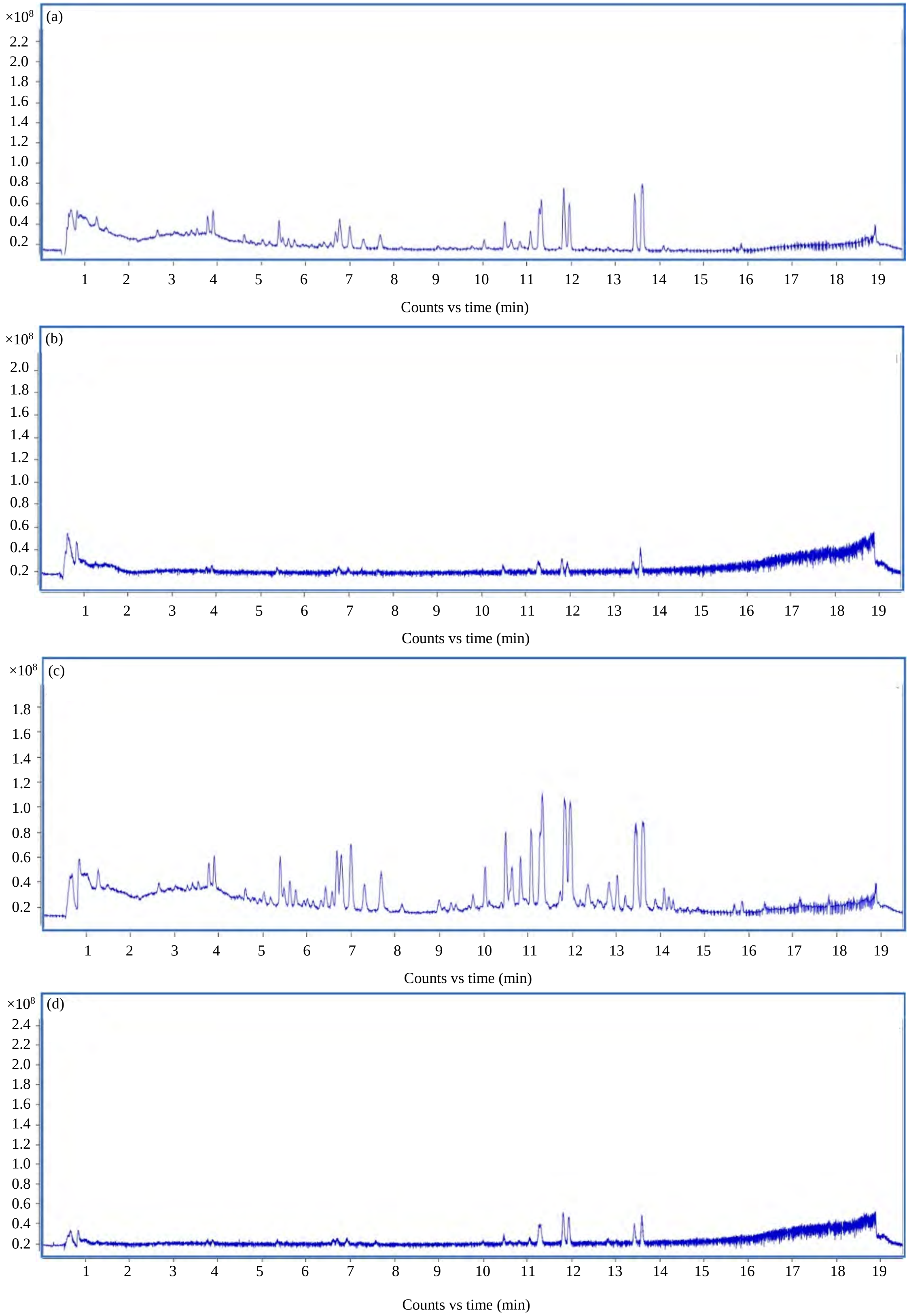

Fig. 3(a-h): Continue

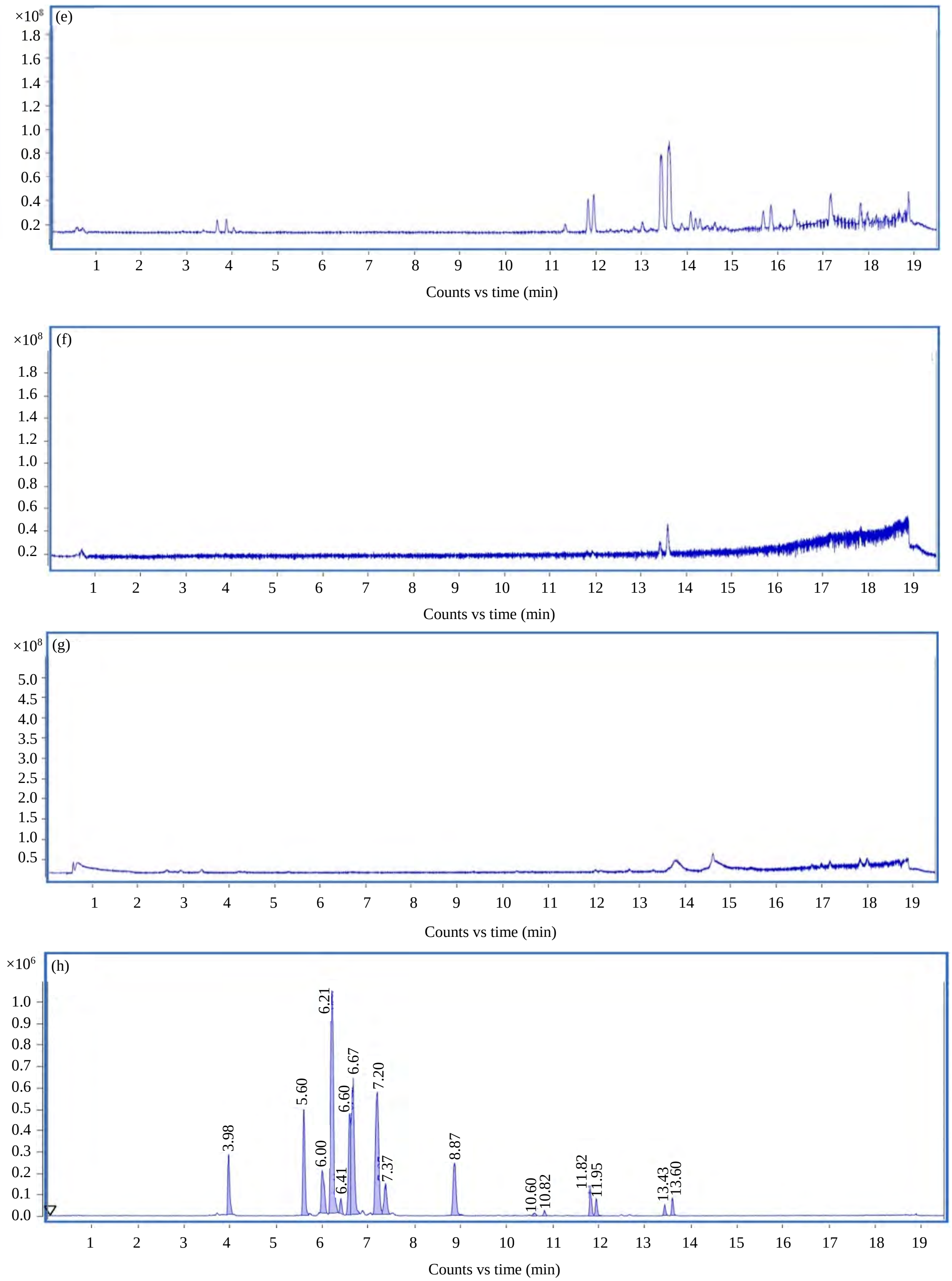

Fig. 3(a-h): Total ion chromatogram of different group, (a) Total ion chromatogram of BG, (b) Total ion chromatogram of serum of BG, (c) Total ion chromatogram of TSF, (d) Total ion chromatogram of serum of TSF, (e) Total ion chromatogram of AEF, (f) Total ion chromatogram of serum of AEF, (g) Total ion chromatogram of CON serum and (h) Total ion chromatogram of reference solution

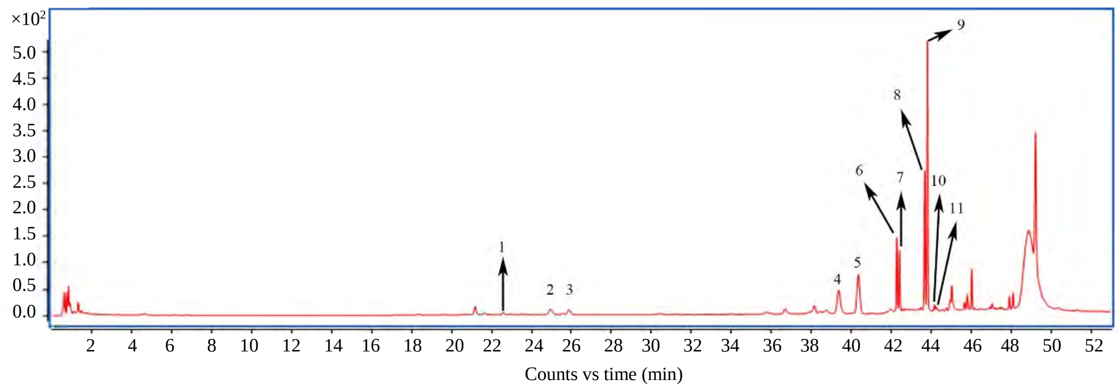

Fig. 4: DAD (203 nm) chromatogram of BG

1: Ginsenoside Rf, 2: Ginsenoside S-Rg2, 3: Ginsenoside R-Rg2, 4: Ginsenoside Rk3, 5: Ginsenoside Rh4, 6: Ginsenoside S-Rg3, 7: Ginsenoside R-Rg3, 8: Ginsenoside Rk1, 9: Ginsenoside Rg5, 10: Ginsenoside S-Rh2 and 11: Ginsenoside R-Rh2

Materials Fig. S2k, PPD was in Supplementary Materials Fig. S2l, PPT was in Supplementary Materials Fig. S2m, ginsenoside Rh3 was in Supplementary Materials Fig. S2n.

Spectrum analysis of different batches of BG: First of all, the results of the methodological investigation showed that the Relative Standard Deviations (RSD %) of each peak relative retention time and the peak area ratio were 0.44-0.56 and 1.43-2.88%, respectively, both of them were less than 3%, it indicated that the precision of the instrument was good. In the stability investigation, the RSD of each peak relative retention time and the peak area ratio were 0.33-0.51 and 1.38-2.56%, respectively, both of them were less than 3%, indicated that the composition of the tested product was stable within 24 hrs. In the reproducibility test, the RSD of each peak relative retention time and the peak area ratio were 0.53-0.92 and 1.88-2.44%, respectively, both of them were less than 3%, indicated that the method had good reproducibility.

The spectra of 10 batches of BG was shown in Fig. 5 and the control spectrum was shown in Fig. 6. The 17 common chromatographic peaks were screened out and the retention times and peak areas of different peaks were obtained. The retention times were 22.553, 23.029, 26.443, 27.434, 38.361, 39.817, 40.977, 41.992, 43.587, 43.719, 45.053, 45.198, 45.984, 46.376, 46.477, 47.104 and 47.258 min for further spectral efficiency analysis.

The IC_{50} value of sample 1 was 7.5 mg mL^{-1}, so it was used as the dose concentration of different batches of BG. The inhibitory effects of different batches of BG on DU145 were shown in Table 2.

Bivariate correlation analysis was performed on the chromatographic peaks and drug efficacy. We adopted pearson correlation analysis because there was a linear correlation between the two research variables and the results were shown in Table 3. The correlation coefficient less than zero indicated that the chromatographic peak had a negative correlation to the pharmacodynamic effect, while the correlation coefficient greater than zero indicated that the chromatographic peaks had a greater contribution to the pharmacodynamic effect and presented a positive promoting effect.

The grey correlation analysis showed that the correlation coefficient between the peak area and the *in vitro* pharmacodynamic evaluation indexes was shown in Table 3. We can see that the 17 main chromatographic peaks had certain differences in their inhibitory effects on prostate cancer. When the drug concentration was 7.5 mg mL^{-1}, the correlation coefficients of the 17 chromatographic peaks were all greater than 0.5. The top-ranking of relevance was the 13th peak.

Comprehensive grey correlation analysis and bivariate correlation analysis results could be speculated that the following eight peaks were the active ingredients of BG against prostate cancer. According to the correlation of grey correlation, the peak order was the peak number of 3>9>10>11>12>14>16>17. According to the identification of BG ingredients above, we identified the peaks 3, 9, 10, 11 and 12 as S-Rg2, S-Rg3, R-Rg3, Rk1 and Rg5, respectively.

Analysis of the structure-effect relationship: The proliferation inhibition rates of different ginsenosides on DU145 were shown in the Supplementary Materials Table S9 and the IC_{50} values of the proliferation inhibition rate were shown in Fig. 7. The IC_{50} values of ginsenosides Ro, Re, Rd, Rd2,

Table 1: Identification and analysis of ginsenosides in BG decoction in ESI negative ion mode

Peak numbers	TR	Assigned identity	Molecular formula	$[M-H]^-$ m/z $[M-H (or+COOH)]^-$ m/z Mean measured mass (Da)	Theoretical extract mass (Da)	Mass accuracy (ppm)	Production
1	22.65	Ginsenoside Rf	$C_{42}H_{72}O_{14}$	799.4888	799.4848	5	637.4282, 475.3752, 391.2816, 161.0447, 101.0240
2	25.105	Ginsenoside S-Rg2	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	637.4209, 475.3746, 391.2818
3	25.613	Ginsenoside R-Rg2	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	637.4209, 475.3746, 391.2818
4	39.373	Ginsenoside Rk3	$C_{36}H_{60}O_{8}$	619.4252	619.4215	5.97	457.3650, 161.0446
5	40.383	Ginsenoside Rh4	$C_{36}H_{60}O_{8}$	619.4252	619.4215	5.97	457.3650, 161.0446
6	42.266	Ginsenoside S-Rg3	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	621.4313, 459.3803, 375.2874, 221.0646, 161.0445
7	42.406	Ginsenoside R-Rg3	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	621.4313, 459.3803, 375.2874, 221.0646, 161.0445
8	43.659	Ginsenoside Rk1	$C_{42}H_{70}O_{12}$	765.4841	765.4795	6	537.3511, 221.0640, 161.0447
9	43.799	Ginsenoside Rg5	$C_{42}H_{70}O_{12}$	765.4841	765.4795	6	537.3511, 221.0640, 161.0447
10	44.15	Ginsenoside S-Rh2	$C_{36}H_{62}O_{8}$	667.4465	667.4427	5.69	621.9086, 459.3840, 161.8245
11	44.25	Ginsenoside R-Rh2	$C_{36}H_{62}O_{8}$	667.4465	667.4427	5.69	621.9086, 459.3840, 161.8245

Table 2: Inhibition rate of different batches of BG on DU145 ($\bar{x}\pm s$, n = 3)

Samples	Dosing concentration mg mL^{-1}	OD value	Proliferation inhibition rate (%)
1	7.5	0.6638±0.0080	45.63
2	7.5	0.6567±0.0211	46.58
3	7.5	0.7560±0.0193	33.35
4	7.5	0.7597±0.0241	32.86
5	7.5	0.7510±0.0135	34.01
6	7.5	0.7403±0.0139	35.44
7	7.5	0.6820±0.0567	43.21
8	7.5	0.7433±0.0451	35.04
9	7.5	0.7217±0.0189	37.92
10	7.5	0.6797±0.0139	43.52

Table 3: Chromatographic peak area, grey correlation coefficient and correlation coefficient of different batches of BG

Peak numbers	Samples 1	2	3	4	5	6	7	8	9	10	Correlation coefficient	Correlation coefficient pearson
1	167.298	210.109	148.929	249.813	244.647	100.239	128.654	293.658	240.681	218.870	0.7799	-0.2183
2	42.901	53.605	41.589	77.267	63.907	26.348	36.141	73.218	64.766	58.715	0.7748	-0.2981
3	136.251	83.037	36.571	128.367	54.466	42.445	133.853	244.889	169.874	156.325	0.7423	0.1636
4	110.716	96.737	53.603	157.172	93.498	47.825	91.328	197.332	144.262	151.090	0.7476	-0.0122
5	66.186	64.872	36.921	97.831	46.710	21.401	53.190	198.664	124.138	144.348	0.6600	-0.0090
6	84.192	52.342	55.285	85.918	100.005	50.861	127.178	259.118	172.347	133.733	0.7598	-0.1150
7	542.129	425.184	286.127	613.007	387.110	237.385	432.817	1288.459	882.806	888.614	0.7055	-0.0062
8	356.269	329.173	187.711	518.524	423.619	170.975	326.977	1088.421	775.950	795.046	0.6875	-0.0740
9	288.333	88.246	70.268	184.788	86.828	81.851	669.342	971.155	510.776	669.734	0.5954	0.1693
10	103.004	40.099	32.701	84.226	57.097	51.959	249.161	267.114	198.592	371.461	0.6150	0.2830
11	476.810	155.469	121.890	330.234	149.484	173.256	1133.826	1909.867	1177.421	1352.872	0.5798	0.1285
12	711.213	269.150	246.609	571.926	345.023	320.210	1668.980	2866.794	1873.806	2179.536	0.5945	0.1113
13	39.359	57.004	40.856	57.475	49.122	24.268	65.959	126.675	84.984	73.307	0.7875	-0.0397
14	19.049	9.293	7.364	13.597	9.507	8.728	42.238	90.631	54.818	69.224	0.5928	0.0990
15	352.534	530.761	844.36	982.768	598.544	238.72	317.665	757.664	574.782	482.889	0.7188	-0.6030
16	69.800	7.688	6.593	20.024	8.249	15.983	188.611	172.198	131.624	164.602	0.5504	0.3108
17	77.629	12.106	15.760	31.461	19.604	27.623	322.309	317.910	249.737	327.121	0.5257	0.2535

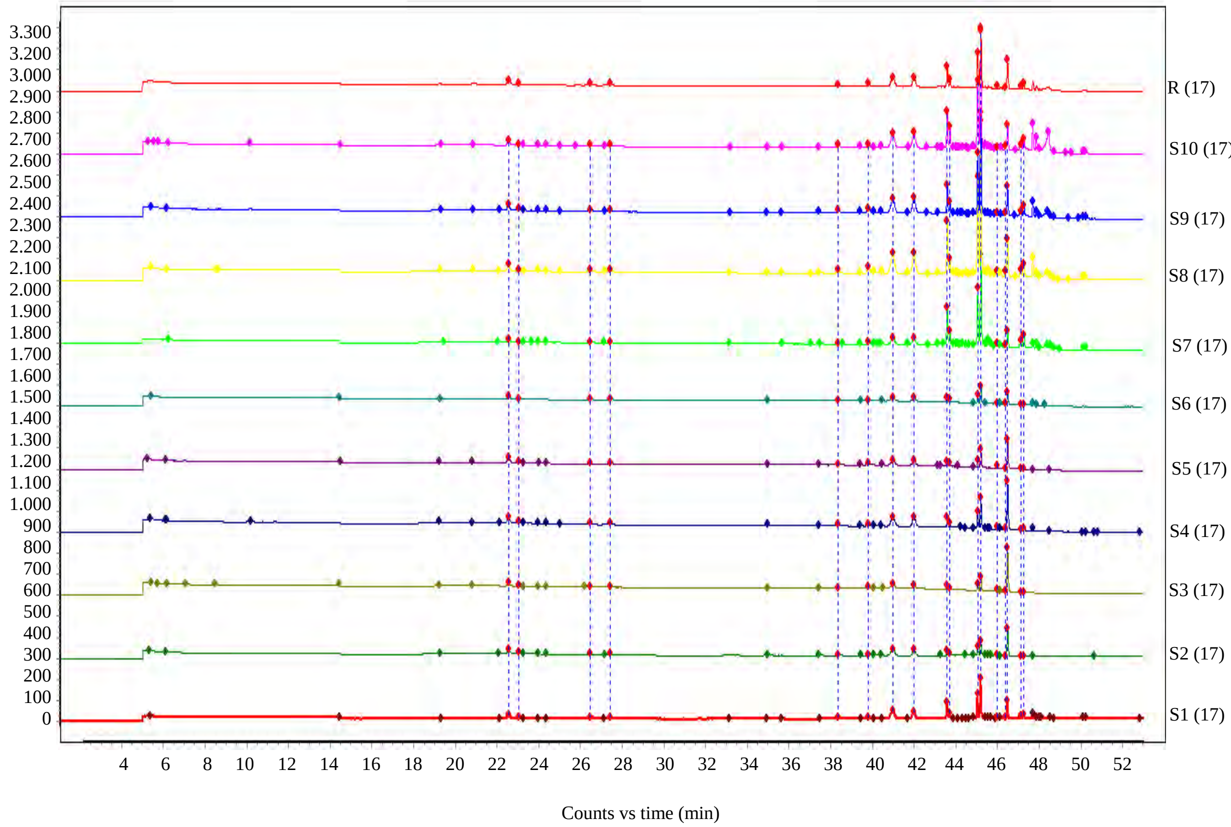

Fig. 5: Spectra of different batches of BG

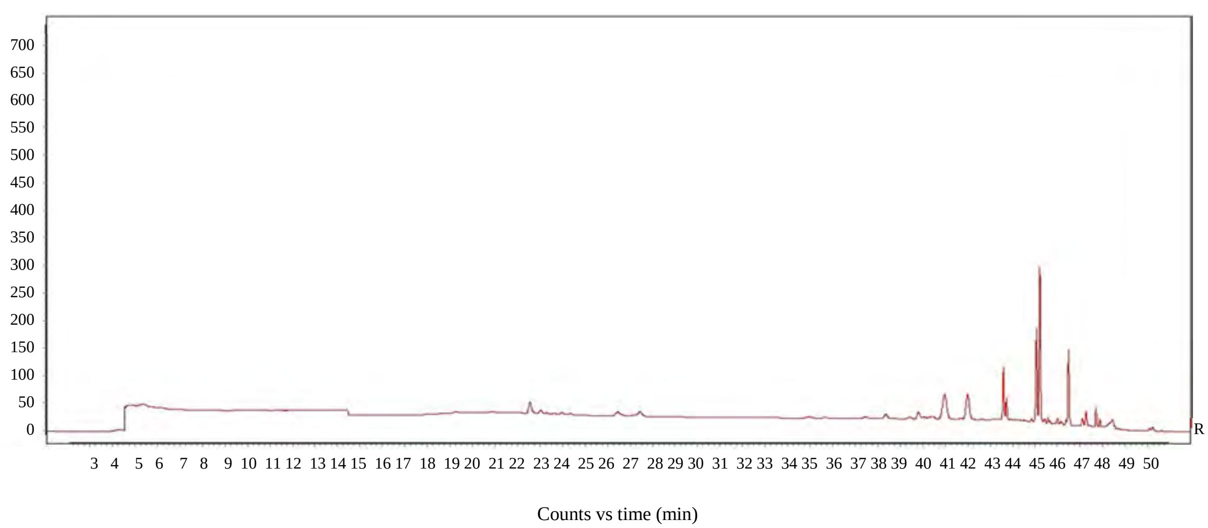

Fig. 6: Comparison of different batches of BG

R-Rg2 and Rb1 were greater than 100 μmol L^{-1} and the IC_{50} values of ginsenosides PPT, S-Rh2, R-Rh2, S-Rh1, R-Rh1, RK1, RK3, R-Rg3, Rg5 and CK, were less than 50 μmol L^{-1}. The IC_{50} value of 5-Fu was the smallest.

Detection of cell apoptosis: The experimental results of ginsenoside Rg5 and ginsenoside Rk1 on prostate cancer cell apoptosis were shown in Fig. 8. The negative control group was in Fig. 8a, 10 μmol L^{-1} 5-Fu group was in Fig. 8b,

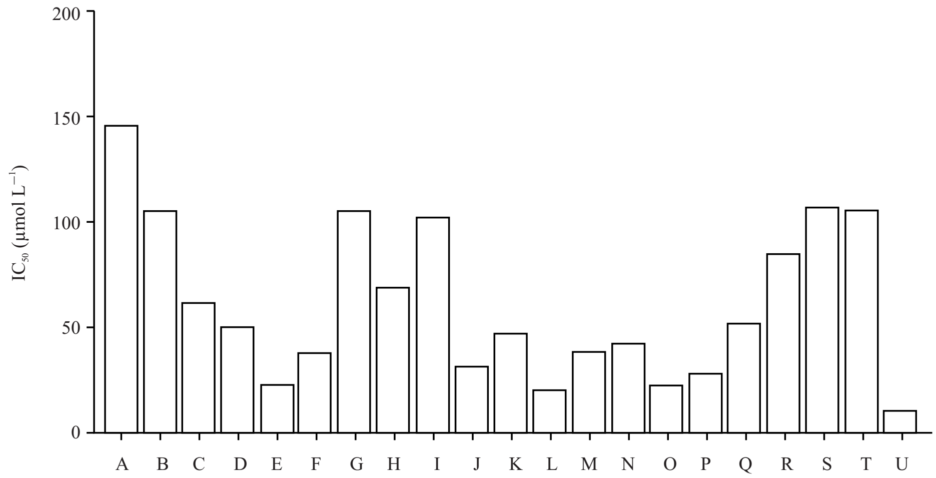

Fig. 7: IC_{50} value of the proliferation inhibition rate of different saponins on DU145

A: Ginsenoside Rb1, B: Ginsenoside Rd, C: Ginsenoside S-Rg3, D: Ginsenoside R-Rg3, E: Ginsenoside S-Rh2, F: Ginsenoside R-Rh2, G: Ginsenoside Re, H: Ginsenoside Rg1, I: Ginsenoside Ro, J: Ginsenoside S-Rh1, K: Ginsenoside R-Rh1, L: Ginsenoside Rg5, M: Ginsenoside Rk1, N: Ginsenoside Rk3, O: CK, P: PPT, Q: PPD, R: Ginsenoside S-Rg2, S: Ginsenoside R-Rg2, T: Ginsenoside Rd2 and U: 5-FU

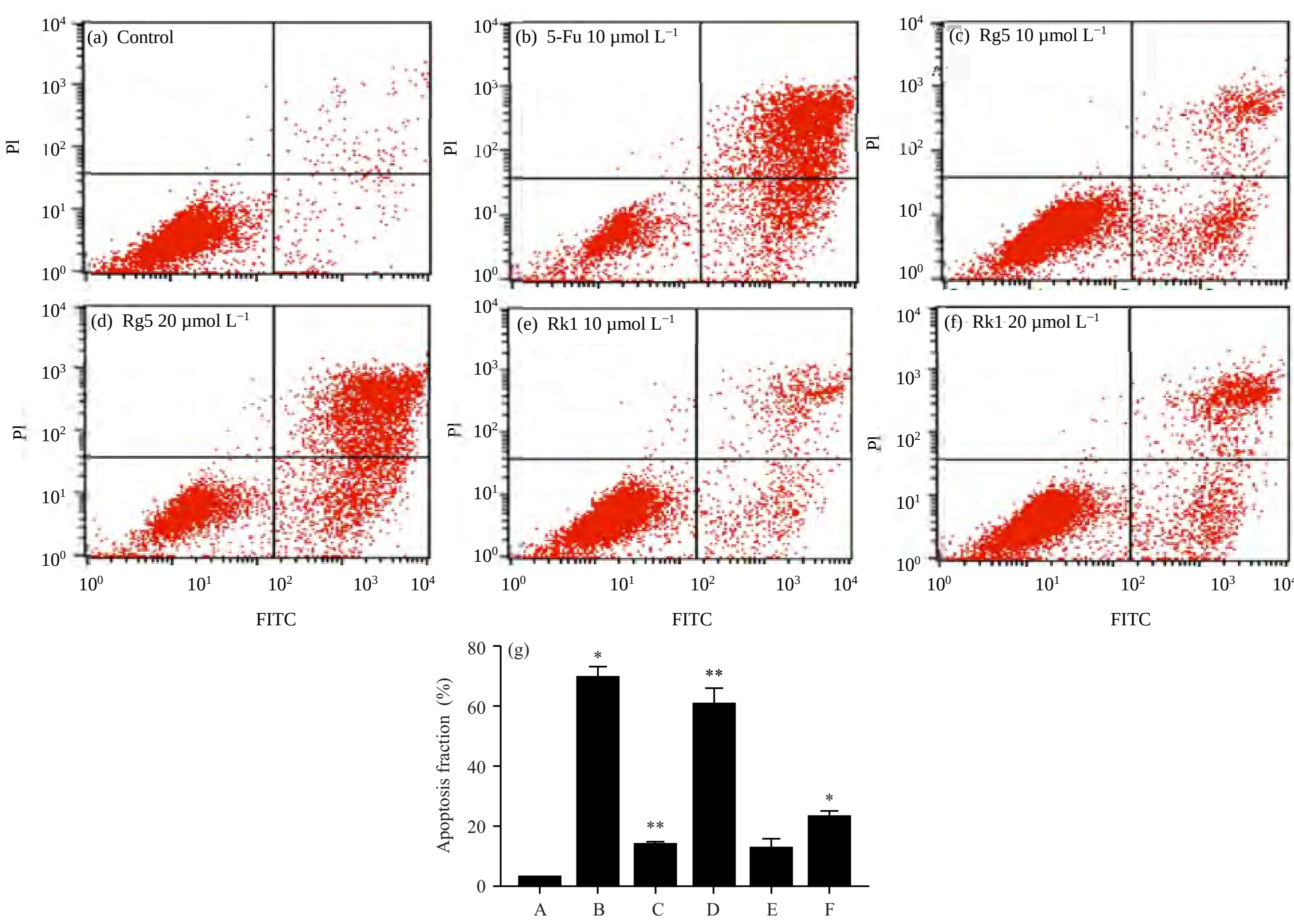

Fig. 8(a-g): Effect of ginsenosides Rg5 and Rk1 on DU145 apoptosis, (a) Negative control group, (b) 10 μmol L^{-1} 5-Fu group, (c) 10 μmol L^{-1} ginsenoside Rg5 group, (d) 20 μmol L^{-1} ginsenoside Rg5, (e) 10 μmol L^{-1} ginsenoside Rk1 group, (f) 20 μmol L^{-1} ginsenoside Rk1 group and (g) Effects of ginsenosides Rg5 and Rk1 on apoptosis of DU145

Compared with control group, *p<0.05, **p<0.01

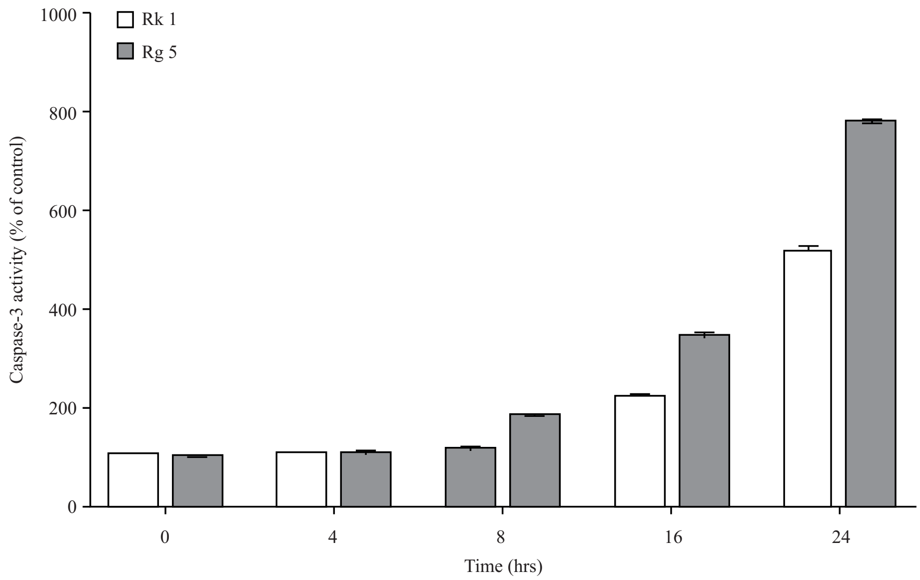

Fig. 9: Activation degree of Caspase-3

10 μmol L^{-1} ginsenosides Rg5 group was in Fig. 8c, 20 μmol L^{-1} ginsenoside Rg5 was in Fig. 8d, 10 μmol L^{-1} ginsenosides Rk1 group was in Fig. 8e, 20 μmol L^{-1} ginsenosides Rk1 group was in Fig. 8f, G. Effects of ginsenosides Rg5 and Rk1 on apoptosis of DU145 was in Fig. 8g.

Ginsenoside Rk1 and Rg5 inhibited the activity of Caspase-3: To investigate whether the DU145 apoptosis by ginsenoside Rk1 and Rg5 were dependent on Caspase-3 activation, we examined the activity of Caspase-3, which were initiating caspases in the mitochondria-mediated apoptosis pathway. As shown in Fig. 9, after treatment of cells with ginsenoside Rk1 and Rg5, after 4 hrs, Caspase-3 was activated in both groups and their activity increased with time, peaking at 24 hrs. The activity of the ginsenoside Rg5 group was enhanced by 7.7-fold and the ginsenoside Rk1 group was enhanced by 5.1-fold as compared to the control group, respectively.

DISCUSSION

The result of serum pharmacological showed that each concentration of drug serum-containing of NFBS group promoted the proliferation of DU145, compared with the CON group of the same concentration, only the BG group had a smaller inhibitory effect in RPMI 1640 medium with 5% drug serum-containing, compared with the CON group of the same concentration, the proliferation inhibition rates of 10% drug serum-containing were BG ($p<0.01$) >AEF ($p<0.05$) >TSF ($p<0.01$), compared with the CON group of the same concentration, BG, TSF, AEF and PF group of 15% the drug-containing serum could inhibit the proliferation of DU145 and the proliferation inhibition rate was BG>TSF>AEF>PF, compared with the same concentration of rat control serum, BG, PF, AEF and TSF group of 20% drug serum-containing could inhibit the proliferation of DU145, the proliferation inhibition rate was AEF>TSF>BG>PF. In summary, TSF and AEF fractions of BG had better anti-prostate cancer activity. Some studies had found that ginsenosides and aglycones have inhibitory effects on prostate cancer. There was no literature available on the serum pharmacology of BG against prostate cancer[11,12].

The result of blood composition analysis showed that TSF mainly included ginsenosides Rf, S-Rg2, R-Rg2, Rk3, Rh4, S-Rg3, R-Rg3, Rk1, Rg5, S-Rh2 and R-Rh2 and AEF mainly included Rk3, Rh4, S-Rg3, R-Rg3, Rk1 and Rg5. The ginsenosides were identified and analyzed according to references[9,13,14].

According to the comparison between the content ratio of saponins in blood and medicinal materials, the serum of BG contains PPT and PPD, it was not found in BG. PPT was speculated to be obtained from the glucose-dropping transformation of ginsenoside Rg3 and ginsenoside Rg2, while PPD was from the glucose-dropping transformation of ginsenoside Rk1 and ginsenoside Rg5. Ginsenoside Rh4 and Rk3 were speculated to be partially obtained from the hydrolysis of ginsenoside Rg2, while the other part was obtained from a prototype blood transfusion. Ginsenoside Rh3 may be derived from ginsenoside Rg5 on the one hand, ginsenoside Rg3 was first transformed into ginsenoside Rg5 and then transformed into ginsenoside Rh3 on the other hand, ginsenosides Rg3, Rk1 and Rg5 in serum of TSF were presumed to be prototyped into blood and the transformation pathways of other ginsenosides Rh4, Rk3, Rh3, PPT and PPD were presumed to be the same as those in serum of BG. The saponins in serum of AEF was the same as that of TSF but the ginsenosides Rk3 and Rh4 were presumed to be completely prototyped into blood and the transformation pathways of other saponins were the same as those of TSF. In conclusion, we speculated that the strong anti-prostate cancer effect of TSF and AEF in BG shown by the serum pharmacological blood result might be the effect of secondary ginsenosides Rg3, Rg5, Rk1 and so on after entering the blood.

Some rare ginsenosides were generated by the dehydration reaction of general primary ginsenosides. For example, the ginsenosides Rg3, F2 and Rh2 in BG were transformed from ginsenosides Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc and Rd by hydrolysis reaction[10,15]. Ginsenoside Rh4 was generated by the dehydration reaction of ginsenoside Rg1, ginsenoside Rg2 was hydrolyzed to produce ginsenosides Rg6, F4, Rk3 and Rh4 and ginsenoside Rg3 was hydrolyzed to produce ginsenoside Rk1 and Rg5[16]. PPT and PPD were the main metabolites of ginsenoside Rg3 in BG[17]. Therefore, in the process of ginseng processing into red ginseng and BG, some secondary ginsenosides were produced and most of them had strong anti-cancer activity.

According to the previous laboratory measurements of oligosaccharide components in ginseng, it can be seen that the oligosaccharide components in BG mainly include fructose, glucose, sucrose, maltose and nystose[10]. Recently research on BG polysaccharides was less, mostly research on ginseng polysaccharides was studying the content, ginseng polysaccharide components mainly including glucose, arabinose, galacturonic acid and galactose, a small amount of rhamnose and some unknown pentose derivatives[18], some changes may occur in the process of processing into BG. And the content of BG polysaccharides needs to be further research.

Two methods of grey correlation analysis and bivariate correlation analysis were used respectively. Based on two different analysis methods of spectrum efficiency, the saponin components of BG with strong anti-prostate cancer activity were selected as ginsenosides S-Rg2, S-Rg3, R-Rg3, RK1, Rg5, the peak number of 16, the peak number of 17.

The results of the DU145 structure-effect experiment showed that the IC_{50} of ginsenosides Ro, Re, Rd, Rd2, R-Rg2 and Rb1 were greater than 100 μmol L^{-1}, they had little anti-cancer activity, it was speculated that because these ginsenosides contained 3 or 4 sugar molecules besides ginsenoside R-Rg2. In general, the number of sugar molecules in ginsenosides was negatively correlated with the anti-cancer activity, however, there were some exceptions for DU145, such as ginsenoside R-Rg2. The IC_{50} of ginsenosides CK, PPT, S-Rh2, R-Rh2, S-Rh1, R-Rh1, RK1, RK3, R-Rg3 and Rg5 were less than 50 μmol L^{-1}, in which PPT did not contain sugar molecules, ginsenosides Rg5, Rg3, Rk1 contained two sugar molecules and the other saponins contained one sugar molecule. Therefore, these saponins had strong anti-cancer activity because of their fewer sugar molecules. Ginsenosides Rg5, S-Rh2 and CK which had one or two sugar molecules belong to protopanaxadiol, had stronger anti-prostate cancer activity than PPD. Therefore, protopanaxadiol secondary saponins containing one or two sugar molecules had stronger anti-prostate cancer activity and the anti-cancer activity of saponins was better than that of aglycones in DU145, which was different from the previous studies. And study had shown that the anticancer activities of ginsenosides were inversely proportional to the number of sugar units in the molecule[19,20].

Through the structure-effect relationship verification, the saponins (S-Rg2, S-Rg3, R-Rg3, RK1 and Rg5) with strong anti-prostate cancer activity were obtained from the spectrum-effect relationship were the active components of anti-prostate cancer in BG. According to the IC_{50} value, the activity intensity was Rg5>Rk1>R-Rg3>S-Rg3>S-Rg2. The peak number of 16 and the peak number of 17 need further study.

The first two ginsenosides Rg5 and Rk1, which were active components of anti-prostate cancer in BG, were selected by combining spectrum-effect relationship and structure-effect relationship. Cell apoptosis was detected by Annexin V-PI double staining method. The higher the amount of cell apoptosis, the stronger the inhibitory effect of the drug on cancer cells. The results showed that after being treated with 10 and 20 μmol L^{-1} ginsenoside Rg5 for 48 hrs, the apoptosis rates were (14.03 ± 0.65) and (60.73 ± 5.14)%, respectively (compared with the negative control group, $p<0.01$). After being treated with 20 μmol L^{-1} ginsenoside Rk1 and 10 μmol L^{-1} 5-Fu for 48 hrs, the apoptosis rates of the cancer cells were 23.03 ± 2.37 and 69.55 ± 3.96%, respectively (compared with the negative control group, $p<0.05$). The ability to induce apoptosis of cancer cells was gradually enhanced, both of which had an obvious anti-cancer effect. At the same concentration, ginsenoside Rg5 had a stronger ability to induce apoptosis than ginsenoside Rk1, it indicated that ginsenoside Rg5 had a stronger anti-cancer effect than ginsenoside Rk1. Studies showed that the microwave-irradiated processed ginseng had a higher content of ginsenosides Rg3, Rg5 and Rk1, which can inhibit the growth of human prostate cancer cells[21].

The result of the detection of Caspase-3 activity showed that ginsenosides Rk1 and Rg5 increased Caspase-3 activity. These suggested that apoptosis of DU145 by ginsenosides Rk1 and Rg5 involved mitochondria-mediated pathways and Caspase-3 may be the direct target of ginsenoside Rk1 and Rg5 on cell apoptosis. Caspase-3 was an apoptotic effector and one of the important targets for the treatment of some cancers[22].

The research indicated that the rare ginsenosides with changes in the side-chain of the structure of ginsenosides showed stronger anti-prostate cancer activity than the general primary ginsenosides, such as ginsenoside Rg5 was stronger than ginsenoside Rg3. In addition, this result was not correspondent with our previous research in which aglycones showed stronger anti-cancer activity than saponin, such as protopanaxadiol was stronger than ginsenoside Rg3. It is for the first time that the effective material basis of BG for anti-prostate cancer was studied by serum pharmacology, spectrum-effect and structure-effect relationship method. This research could provide a basis for the development of new anti-prostate cancer drugs and the application of BG in the treatment of prostate cancer. However, several active ingredients discovered in this study still need to be further studied by an *in vivo* tumour experiment on animals to validate their anti-cancer activity.

CONCLUSION

In summary, the active components of BG against prostate cancer were mainly ginsenosides Rg5, S-Rg3, R-Rg3, RK1 and S-Rg2. The effective components of BG against prostate cancer were its secondary ginsenosides. It will provide a direction for further research on the treatment of cancer and provide a pharmacodynamic material basis for the research and development of new drugs.

SIGNIFICANCE STATEMENT

This study discovers the active components of BG against prostate cancer were mainly ginsenosides Rg5, S-Rg3, R-Rg3, RK1 and S-Rg2 and the effective components of BG against prostate cancer were its secondary ginsenosides. This study will help the researcher to uncover the critical area of the effective substance of BG against prostatic cancer that many researchers were not able to explore. Thus, a new theory on the treatment of cancer and provide a pharmacodynamic material basis for the research and development of new drugs may be arrived at.

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Table S1: Yield of each fractionated component of BG

Groups	Weight (g)	Yield (%)
PF	21.150	21.15
OF	23.203	23.20
TSF	6.561	6.56
AEF	0.565	0.57
BG	43.36	54.20

PF: Polysaccharide fraction, OF: Oligosaccharides fraction, TSF: Total saponins fraction, AEF: 95% Alcohol eluate fraction, BG: Black ginseng

Table S2: Concentration of test solution

Samples	Weight (g)	Yield (%)	Concentration (g mL^{-1})
1	5.0029	46.8	0.625
2	5.0027	47.5	0.588
3	5.0017	49.4	0.625
4	5.0030	52.1	0.625
5	5.0027	48.9	0.625
6	5.0016	49.3	0.588
7	5.0022	50.3	0.555
8	5.0003	53.2	0.588
9	5.0009	46.9	0.555
10	5.0007	49.9	0.555

Table S3: Preparation of drug solution to be tested

Compounds	Molecular weight	Weight (mg)	DMSO (µL)	Concentration (mmol L^{-1})
Ginsenoside Rb1	1109.29	27.73	250	99.99
Ginsenoside Rd	947.15	23.76	250	100.34
Ginsenoside S-Rg3	785.02	2.87	50	73.12
Ginsenoside R-Rg3	785.02	2.24	50	57.07
Ginsenoside S-Rh2	622.87	2.14	50	68.71
Ginsenoside R-Rh2	622.87	1.90	50	61.01
Ginsenoside Re	947.14	23.58	250	99.58
Ginsenoside Rg1	801.01	3.78	50	94.38
Ginsenoside Ro	957.11	2.36	50	49.32
Ginsenoside S-Rh1	638.87	2.42	50	75.76
Ginsenoside R-Rh1	638.87	2.68	50	83.9
Ginsenoside Rg5	767.01	2.68	50	69.88
Ginsenoside Rk1	767.01	1.99	50	51.89
Ginsenoside Rk3	620.86	2.06	50	66.36
Ginsenoside CK	622.88	1.92	50	61.65
PPT	462.70	2.22	50	95.95
PPD	460.73	2.21	50	95.93
Ginsenoside S-Rg2	785.01	2.48	50	63.18
Ginsenoside R-Rg2	785.01	2.36	50	60.13
Ginsenoside Rd2	915.13	2.38	50	52.01

DMSO: Dimethyl sulfoxide

Table S4: LC-MS data and identification of prototype components and metabolites of BG in blood

				$[M-H]^-$ m/z $[M-H (or+COOH)]^-$ m/z				
Numbers	TR	Assigned identity	Molecular formula	Mean measured mass (Da)	Theoretical extract mass (Da)	Mass accuracy (ppm)	Production	Note
1	11.32	Rk3	$C_{36}H_{60}O_8$	619.4252	619.4215	5.97	457.3650, 161.0446	Prototype component, metabolite
2	11.81	Rh4	$C_{36}H_{60}O_8$	619.4252	619.4215	5.97	457.3650, 161.0446	Prototype component, metabolite
3	11.945	PPD	$C_{30}H_{52}O_3$	459.3841	459.3844	-0.65	459.2702, 423.0816	Metabolite
4	16.12	PPT	$C_{30}H_{52}O_4$	475.379	475.3793	-0.63	405.3536, 441.9932	Metabolite
5	18.647	Rh3	$C_{36}H_{60}O_7$	603.4289	603.4266	3.81	603.3689	Metabolite

Table S5: LC-MS data and identification of prototype components and metabolites of TSF in blood

				$[M-H]^-$ m/z $[M-H (or+COOH)]^-$ m/z				
Numbers	TR	Assigned identity	Molecular formula	Mean measured mass (Da)	Theoretical extract mass (Da)	Mass accuracy (ppm)	Production	Note
1	11.32	Rk3	$C_{36}H_{60}O_8$	619.4252	619.4215	5.97	457.3650, 161.0446	Prototype component, metabolite
2	11.81	Rh4	$C_{36}H_{60}O_8$	619.4252	619.4215	5.97	457.3650, 161.0446	Prototype component, metabolite
3	11.83	S-Rg3	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	621.4313, 459.3803, 375.2874, 221.0646, 161.0445	Prototype component
4	11.93	R-Rg3	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	621.4313, 459.3803, 375.2874, 221.0646, 161.0445	Prototype component
5	11.945	PPD	$C_{30}H_{52}O_3$	459.3841	459.3844	-0.65	459.2702; 423.0816	Metabolite
6	13.43	Rk1	$C_{42}H_{70}O_{12}$	765.4841	765.4795	6	537.3511, 221.0640, 161.0447	Prototype component
7	13.6	Rg5	$C_{42}H_{70}O_{12}$	765.4841	765.4795	6	537.3511, 221.0640, 161.0447	Prototype component
8	16.12	PPT	$C_{30}H_{52}O_4$	475.379	475.3793	-0.63	405.3532; 441.9932	Metabolite
9	18.647	Rh3	$C_{36}H_{60}O_7$	603.4289	603.4266	3.81	603.3689	Metabolite

Table S6: LC-MS data and identification of prototype components and metabolites of AEF in blood

Number	TR	Assigned identity	Molecular formula	$[M-H]^-$ m/z $[M-H (or+COOH)]^-$ m/z: Mean measured mass (Da)	Theoretical extract mass (Da)	Mass accuracy (ppm)	Product ion	Note
1	11.32	Rk3	$C_{36}H_{60}O_8$	619.4252	619.4215	5.97	457.3650, 161.0446	Prototype component
2	11.81	Rh4	$C_{36}H_{60}O_8$	619.4252	619.4215	5.97	457.3650, 161.0446	Prototype component
3	11.83	S-Rg3	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	621.4313, 459.3803, 375.2874, 221.0646, 161.0445	Prototype component
4	11.93	R-Rg3	$C_{42}H_{72}O_{13}$	783.4945	783.49	5.74	621.4313, 459.3803, 375.2874, 221.0646, 161.0445	Prototype component
5	11.945	PPD	$C_{30}H_{52}O_3$	459.3841	459.3844	-0.65	459.2702, 423.0816	Metabolite
6	13.43	Rk1	$C_{42}H_{70}O_{12}$	765.4841	765.4795	6	537.3511, 221.0640, 161.0447	Prototype component
7	13.6	Rg5	$C_{42}H_{70}O_{12}$	765.4841	765.4795	6	537.3511, 221.0640, 161.0447	Prototype component
8	16.12	PPT	$C_{30}H_{52}O_4$	475.379	475.3793	-0.63	405.3536; 441.9932	Metabolite
9	18.647	Rh3	$C_{36}H_{60}O_7$	603.4289	603.4266	3.81	603.3689	Metabolite

Table S7: Content of different saponins in each component (mg g^{-1} the amount of crude drug)

Compounds	RT (min)	BG	TSF	AEF
Ginsenoside Rf	5.6	0.2	0.26	
Ginsenoside S-Rg2	6.41	0.16	0.23	
Ginsenoside R-Rg2	6.55	0.16	0.23	
Ginsenoside Rk3	11.32	1.47	3.62	0.022
Ginsenoside Rh4	11.81	1.56	4.23	0.12
Ginsenoside S-Rg3	11.83	3.11	7.36	0.107
Ginsenoside R-Rg3	11.93	2.77	6.45	1.21
Ginsenoside Rk1	13.43	1.95	4.25	0.395
Ginsenoside Rg5	13.6	4.39	6.36	0.946
Ginsenoside S-Rh2	14.09	0.14	0.34	
Ginsenoside R-Rh2	14.23	0.07	0.11	

TSF: Total saponins fraction, BG: Black ginseng, AEF: Alcohol eluate fraction and RT: Retention time

Table S8: Content of different saponins in serum (ng mL^{-1})

Compounds	RT (min)	BG (blood)	TSF (blood)	AEF (blood)
Ginsenoside Rk3	11.32	0.62	2.72	0.6
Ginsenoside Rh4	11.81	0.22	0.38	0.44
Ginsenoside S-Rg3	11.83		6.45	7.44
Ginsenoside R-Rg3	11.93		4.33	3.78
PPD	11.945	0.18	0.17	0.24
Ginsenoside Rk1	13.43		1.61	5.06
Ginsenoside Rg5	13.6		2.73	5.6
PPT	16.12	0.44	0.65	1.2
Ginsenoside Rh3	18.647	0.17	1.12	0.04

TSF: Total saponins fraction, BG: Black ginseng, AEF: Alcohol eluate fraction and RT: Retention time

Table S9: Proliferation inhibition rates of different ginsenosides on DU145 ($\bar{x}\pm s$, n = 3)

Groups	Concentration (μmol L^{-1})	OD value	Inhibition rate (%)
Control		0.775±0.011	0.00
Ginsenoside Rb1	1	0.977±0.011	4.52
	10	0.977±0.019	4.45
	50	0.979±0.008	4.19
	100	0.968±0.007	5.63
Ginsenoside Rd	1	0.950±0.008	8.06
	10	0.911±0.008	13.16
	50	0.892±0.009	15.65
	100	0.823±0.010	24.62
Ginsenoside S-Rg3	1	0.885±0.014	16.48
	10	0.784±0.004	29.73
	50	0.754±0.006	33.66
	100	0.622±0.003	50.97
Ginsenoside R-Rg3	1	0.876±0.006	17.68
	10	0.757±0.011	33.27
	50	0.691±0.009	41.89
	100	0.519±0.002	64.42
Ginsenoside S-Rh2	1	0.730±0.028	36.80
	10	0.661±0.021	45.84
	50	0.275±0.007	96.40
	100	0.249±0.001	99.87
Ginsenoside R-Rh2	1	0.786±0.021	29.54
	10	0.671±0.001	44.53
	50	0.648±0.021	47.61
	100	0.450±0.016	73.54
Ginsenoside Re	1	0.943±0.019	8.84
	10	0.903±0.001	14.15
	50	0.888±0.009	16.18
	100	0.838±0.005	22.72
Ginsenoside Rg1	1	0.876±0.003	17.74
	10	0.844±0.019	21.81
	50	0.726±0.011	37.33
	100	0.703±0.001	40.34
Ginsenoside Ro	1	0.977±0.002	4.49
	10	0.896±0.006	15.00
	50	0.876±0.015	17.62
	100	0.789±0.008	29.01
Ginsenoside S-Rh1	1	0.725±0.007	37.46
	10	0.661±0.011	45.78
	50	0.568±0.010	58.00
	100	0.417±0.004	77.73
Ginsenoside R-Rh1	1	0.795±0.007	28.29
	10	0.749±0.005	34.25
	50	0.705±0.006	40.08
	100	0.528±0.025	63.25
Ginsenoside Rg5	1	0.722±0.004	37.85
	10	0.647±0.010	47.68
	50	0.250±0.001	99.64
	100	0.254±0.007	99.15
Ginsenoside Rk1	1	0.790±0.003	28.95
	10	0.750±0.001	34.18
	50	0.695±0.021	41.39
	100	0.349±0.010	86.71
Ginsenoside Rk3	1	0.824±0.005	24.45
	10	0.756±0.001	33.40
	50	0.744±0.006	34.97
	100	0.377±0.014	83.04
Ginsenoside CK	1	0.781±0.008	30.14
	10	0.703±0.001	40.40

Table S9: Continue

Groups	Concentration (μmol L^{-1})	OD value	Inhibition rate (%)
	50	0.298±0.001	93.44
	100	0.260±0.013	98.40
PPT	1	0.708±0.021	39.62
	10	0.672±0.028	44.40
	50	0.566±0.006	58.27
	100	0.307±0.016	92.16
PPD	1	0.763±0.016	32.48
	10	0.738±0.016	35.76
	50	0.686±0.010	42.57
	100	0.671±0.019	44.60
Ginsenoside S-Rg2	1	0.974±0.006	4.91
	10	0.881±0.004	16.99
	50	0.785±0.001	29.60
	100	0.716±0.010	38.64
Ginsenoside R-Rg2	1	0.980±0.010	4.99
	10	0.905±0.012	14.94
	50	0.867±0.007	19.46
	100	0.830±0.004	23.31
Ginsenoside Rd2	1	0.966±0.012	5.86
	10	0.882±0.007	16.89
	50	0.872±0.006	18.17
	100	0.849±0.004	21.22
5-FU	1	0.436±0.027	75.37
	10	0.317±0.009	90.83
	50	0.252±0.002	99.37
	100	0.250±0.001	99.74

Compared with control, *p<0.05, **p<0.01, OD: Optimal density

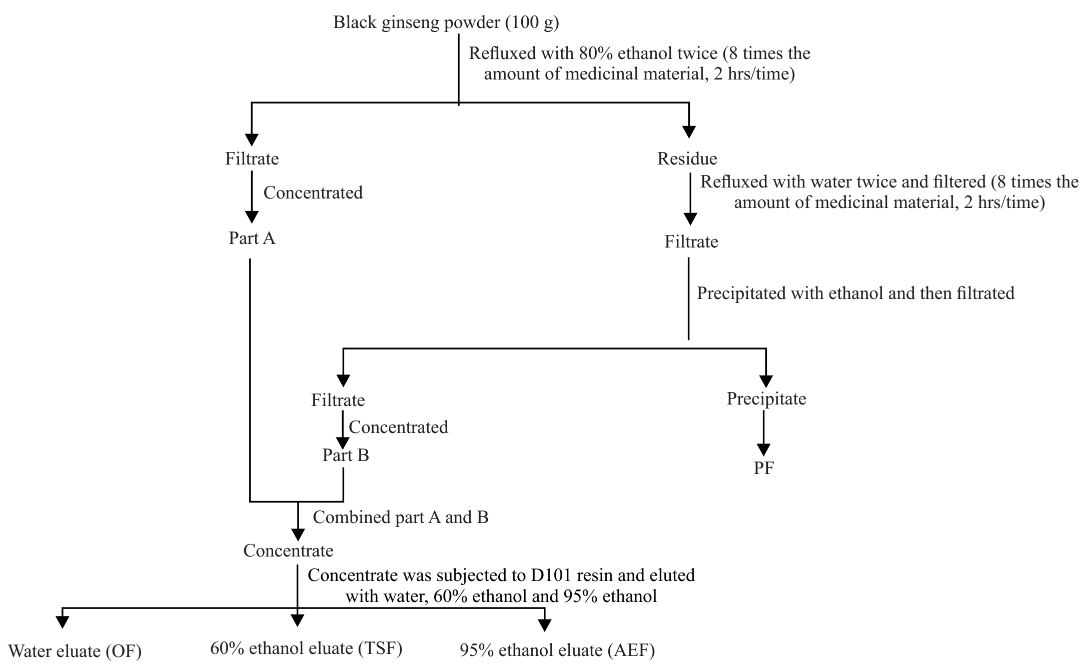


Fig. S1: Extraction process of fractionated components of BG

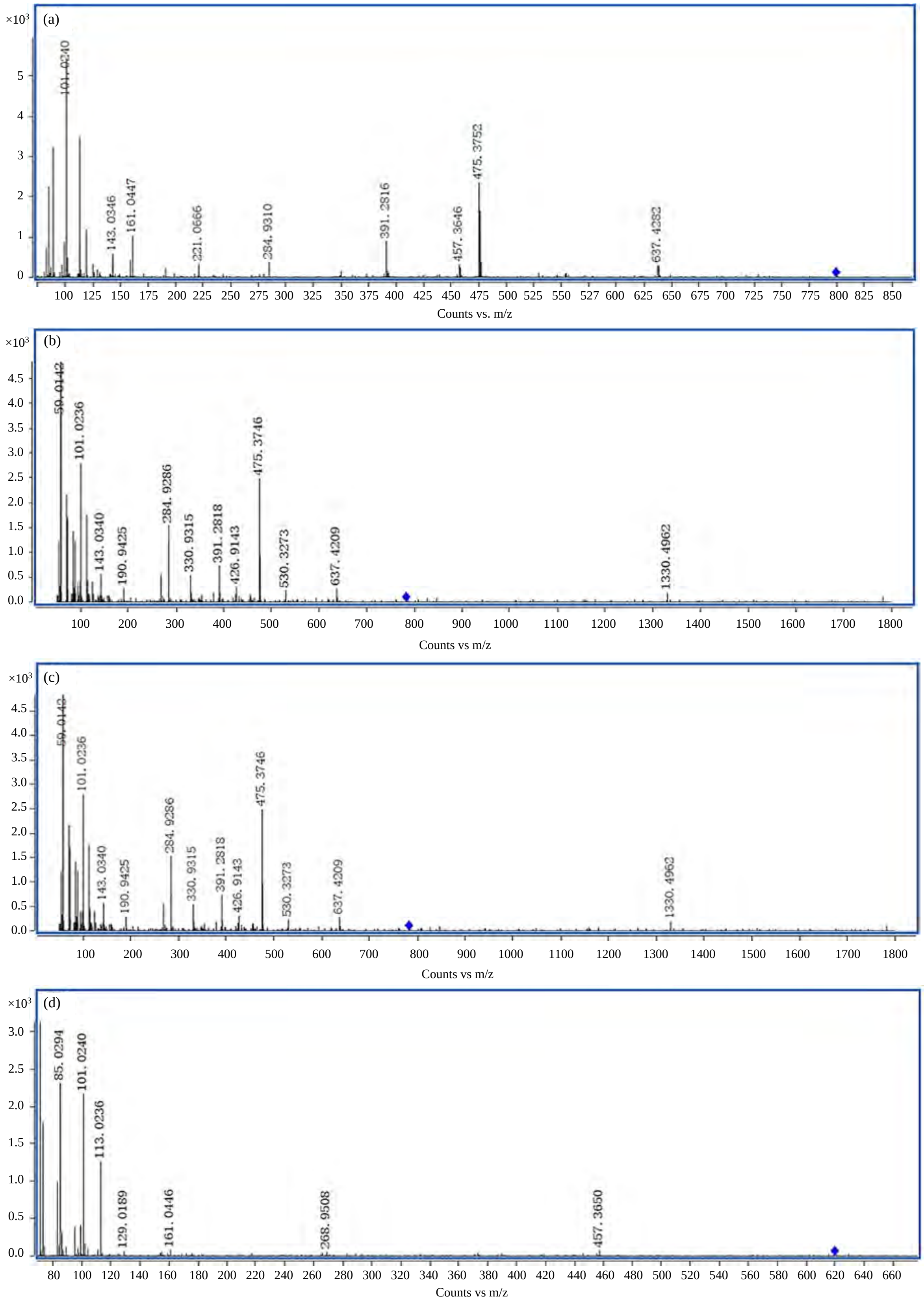
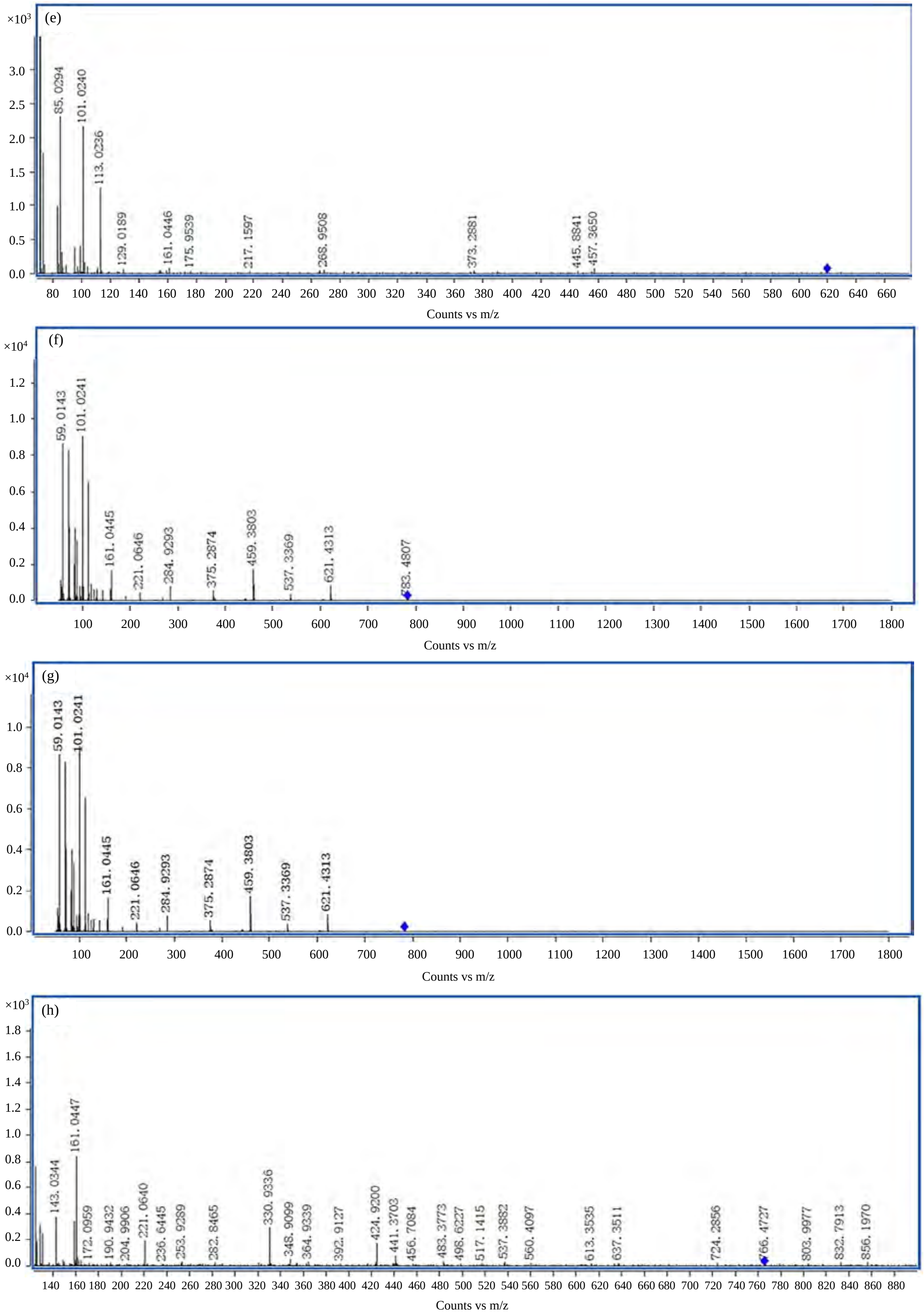


Fig. S2(a-n): Continue



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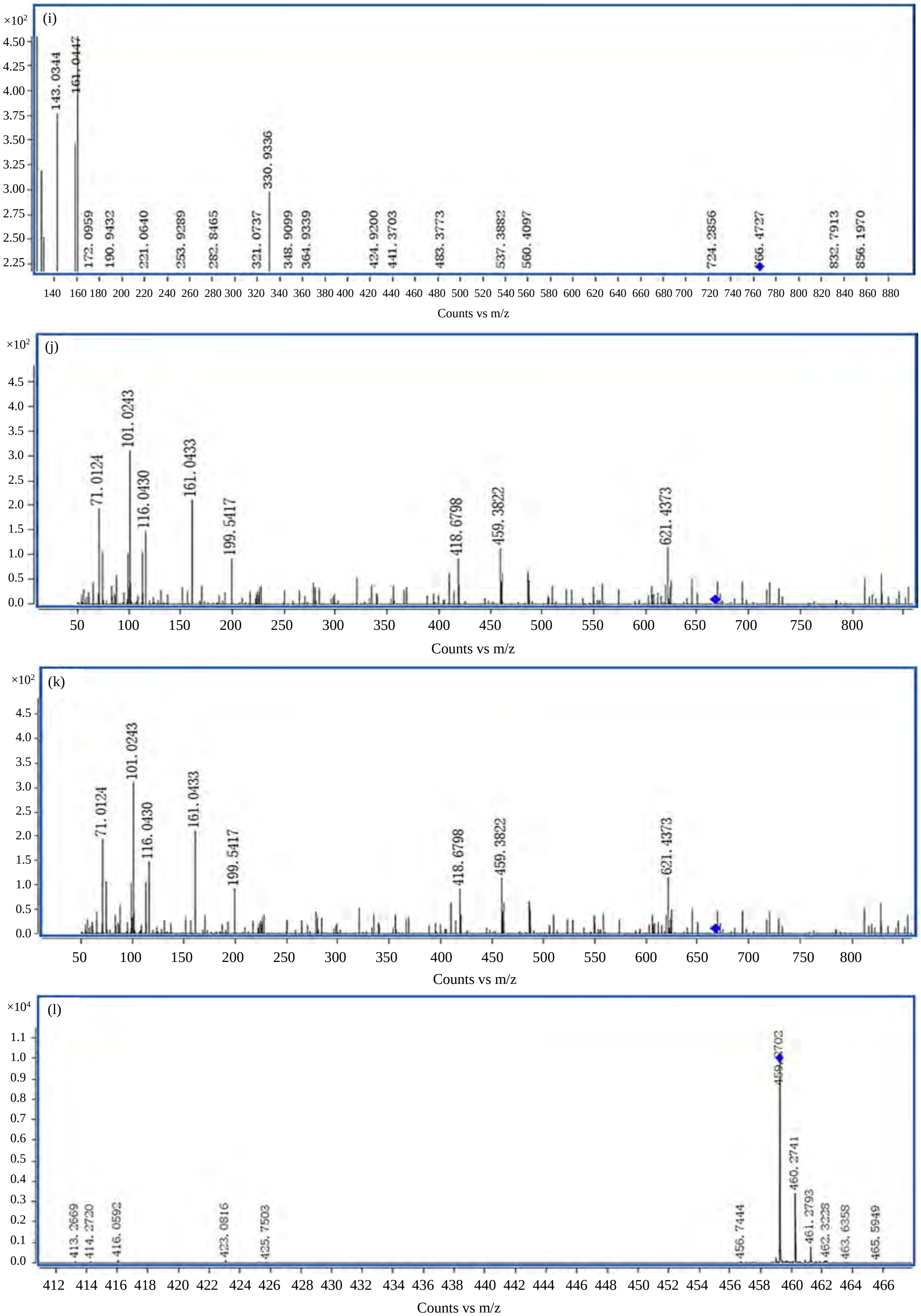

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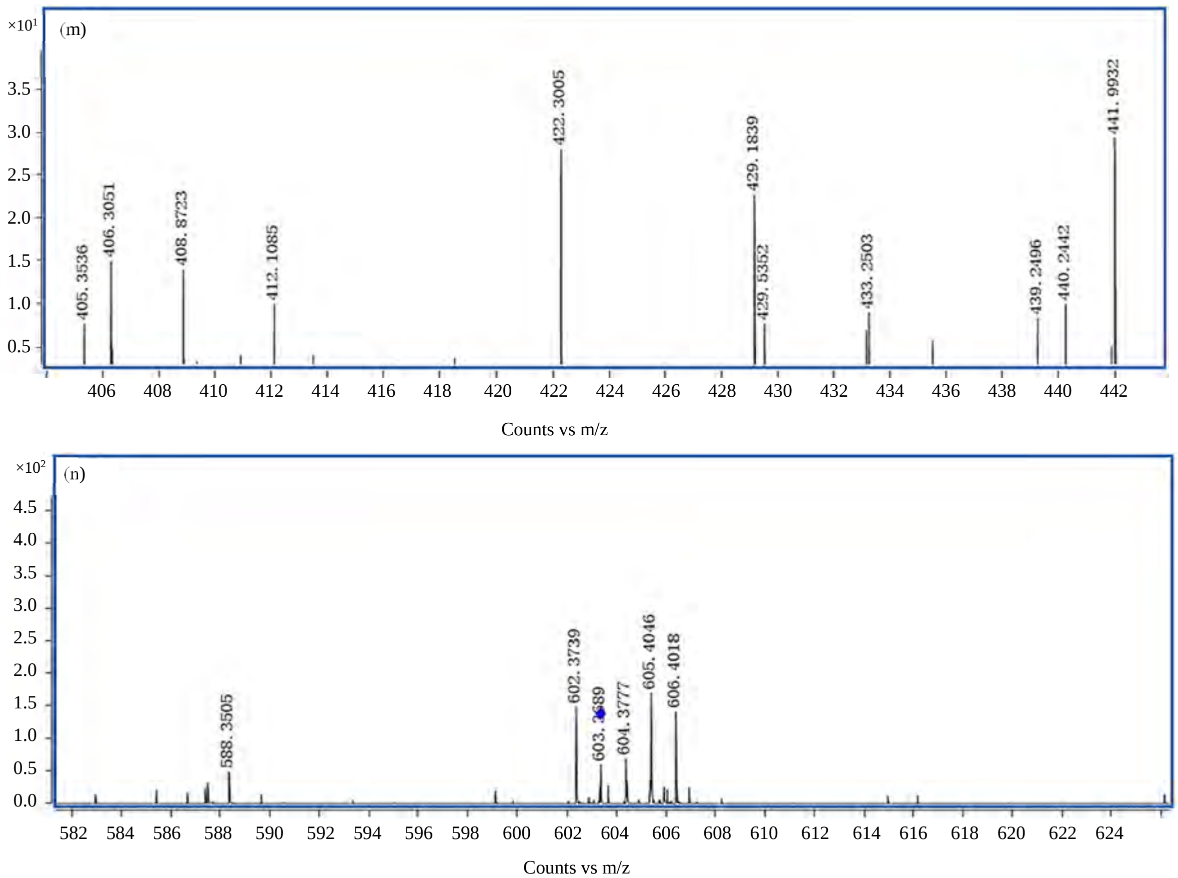

Fig. S2(a-n): Mass spectrogram of ginsenosides in negative ion mode, (a) Ginsenoside Rf, (b) Ginsenoside S-Rg2, (c) Ginsenoside R-Rg2, (d) Ginsenoside Rk3, (e) Ginsenoside Rh4, (f) Ginsenoside S-Rg3, (g) Ginsenoside R-Rg3, (h) Ginsenoside Rk1, (i) Ginsenoside Rg5, (j) Ginsenoside S-Rh2, (k) Ginsenoside R-Rh2, (l) PPD, (m) PPT and (n) Ginsenoside Rh3