The Anti-RA Activities of a Couplet Medicinals, Gastrodia Elata and Radix Aconitic Lateralis preparata, Explored by Untargeted Metabolomics and Network Pharmacology.

Jie Yang (yangjiee2012@sina.cn)
Bijie medical college
Xiang-Chun SHEN
Guizhou Medical University
Wei-Hong LI
Chengdu University of Traditional Chinese Medicine
Guang-Zhong FAN
Bijie Medical College
Bu-Fa GUO
Bijie Medical College
Qi-Lun PENG
Bijie Medical College
Wei-Yi YAO
Chengdu University of Traditional Chinese Medicine
Di-Hong GONG
Chengdu University of Traditional Chinese Medicine
Wei-Jun DING
Chengdu University of Traditional Chinese Medicine

Research Article

Keywords: couplet Medicinals, Gastrodia elata and Radix aconitic lateralis preparata (GERA), Rheumatoid Arthritis (RA), Network pharmacology, Untargeted metabolomics profile

Posted Date: May 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-29055/v1

Abstract

Background

The couplet Medicinals, *Gastrodia Elata* and *Radix Aconitic Lateralis preparata* (GERA), is an established formula extensively used in Chinese medicine for treating rheumatoid arthritis (RA). However, the anti-RA mechanisms of GERA are still unclear. This paper aims to explore the anti-RA mechanisms of GERA by a combined strategy of untargeted metabolomics and network pharmacology.

Methods

Three water extracts were prepared as propotions: *Gastrodia Elata* compared with *Radix Aconitic Lateralis (w/w)*: 1:1, 3:2, or 2:3. The untargeted metabolomics were executed with UPLC-MS. The metabolites were annotated and identified by Human metabolome database (HMDB) and Lipid maps database. Finally, key genes and pathways related to anti-RA activities of GERA were mined by network pharmacology.

Results

The untargeted metabolomics profile displayed that four differentially expressed metabolites were involved in isoflavonoid biosynthesis and biosynthesis of unsaturated fatty acids ($p < 0.05$). Among these differential metabolites, the essential ingredients of GERA were linoleic acid, daidzein, and daidzin. The principal targets of anti-RA activities of GERA were IL-6, TNF, VEGFA, TP53, CASP3 and PTGS2. Thirty anti-RA targets of GERA were majorly belonged to pathways response for anti-inflammation, endothelial function and apoptosis, suggesting the fundamental process of RA treatment.

Conclusion

The anti-RA activities of GERA were based on the inhibition of inflammation and regulation of endothelial function and apoptosis.

Background

The couplet medicinals is one of the famous features of Chinese medicine which displays marked synengy effects compared with individual herbs. A couplet medicinals, combined by *Gastrodia elata* and *Radix aconitic lateralis preparata* (GERA), has been extensively used in Chinese medicine. 1159 of GERA were observed from the formulae of anicent Chinese medicine[1]. GERA is an establised couplet Medicinals for the treatment of rheumatoid arthritis (RA) [2]. A GERA extract made by Chen et al. [3] displayed significant anti-RA activity, with the cure rate 85.30%. Our former studies indicated that GERA

could efficiently relieve RA, joint swelling and pain syndrome[4]. On the other hand, network pharmacology has become a novel branch of systems biology, particularly used in the identification of efficient targets and their pathways of Chinese formulas[5–6]. Twenty active compounds of a couplet medicinals, *Gastrodia elata* and *Ligusticum chuanxiong Hort*, were annoted to 48 molecular targets which are associated with migraine headaches by network pharmacology[7–8]. The couplet of *Gastrodia elata* and *Ramulus Uncariae* demonstrated multi-target and multi-pathway regulation for the therapy of cerebral ischemia[9]. However, the anti-RA mechanisms of GERA is waiting for further exploration. Therefore we applied the untargeted metabolomics for identifying differentially extracted metabolites of GERA solutions, and mined the hidden KEGG pathways involved in RA therapy by network pharmacological approach.

Methods

Sample preparation

Both herbs used in this study were collected in their genuine area. The *Gastrodia elata* was collected from Dafang county, Guizhou province, China. The *Radix aconitic lateralis preparata* was collected from Jiangyou county, Sichuan province, China. The two herbs were identified by Professor Yun Deng, Chengdu University of Traditional Chinese Medicine. Three kinds of water solutions were prepared as follow propotions: *Gastrodia Elata* compared with *Radix Aconitic Lateralis preparata (*w/w*)*: 1:1, 3:2 or 2:3. Different ratios of medicinal herbs can extract different compounds and, consequently, show distinguish pharmaceutical activities. Ten times of distilled water was added to the raw herbs (w/w), soaked at room temperature for 30 min, boiling for 30 min, filtered and stored the boiled solution. Then added eight times of distilled water into the dregs of decoction (w/w), boiling for 30 min, filtered and mixed the solution to the stored one, and enriched the mixed solution to 1:1.1(raw herb vs extracted solution, w/v). Finally, stored at 4 °C for follow-up analysis.

Untargeted Metabolomics Analysis Integrated With Uplc-ms

The stored solutions of *Gastrodia Elata* (Chinese name, Tian-Ma)and *Radix Aconitic Lateralis* (Chinese name, Fu-Zi) were respectively freeze-dried into powder (100 mg), grounded with liquid nitrogen, homogenated and resuspended with pre-chilled 80% methanol and 0.1% formic acid by vortexing briefly. Three formulae were then composed based on the relative qualities(w/w) of Tian-Ma (T) and Fu-Zi (F) powder: 1:1 (TF11), 3:2 (TF32), or 2:3 (TF32). The samples were incubated on ice for 5 min, centrifuged at 15000 rpm, 4 °C for 5 min. The supernatant was diluted to the final concentration containing 60% methanol in LC-MS grade water. The samples were subsequently transferred to a fresh tube after 0.22 μm filtering, centrifuged at 15000 rpm, 4 °C for 10 min. Finally, the filtrate was injected into the LC-MS system for metabolite analysis.

LC-MS analyses were performed using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive series mass spectrometer (Thermo Fisher). Samples were injected into a Hyperil Gold column (100 × 2.1 mm, 1.9 μm) using a 16-min linear gradient at a flow rate of 0.2 mL /min. The eluents for the positive polarity mode were eluent A (0.1% formic acid) and eluent B (Methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (Methanol). The solvent gradients were set as follows: 98%A, 2% B, 1.5 min; 100% B, 12.0 min; 100% B, 14.0 min; 98%A, 2% B, 14.1 min; 98%A, 2% B, 16 min. Q Exactive mass spectrometer was operated in positive and negative polarity mode. The ESI optical source was set as follows: spray voltage of 3.2 kV, the capillary temperature of 320 °C, sheath gas flow rate of 35 arb, and aux gas flow rate of 10 arb.

The raw data files generated by UHPLC-MS were processed using Compound Discoverer 3.0 (CD3.0, Thermo Fisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters were set as follows: retention time tolerance, 0.2 minutes; actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; signal/noise ratio, 3; and minimum intensity, 100000. After that, the peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. Then the peaks were matched with the mzCloud (https://www.mzcloud.org/) and ChemSpider (http://www.chemspider.com/) databases to obtain accurate qualitative and relative quantitative results.

These metabolites were annotated using the Human Metabolome Database (HMDB) (http://www.hmdb.ca/) and the Lipidmaps database (http://www.Lipidmaps.org/). Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed at metaX (flexible and comprehensive software for processing metabolomics data). The metabolites with VIP > 1 and P-value < 0.05 and fold change ≥ 2 or FC ≤ 0.5 were considered differential metabolites.

For clustering heat maps, the data were normalized using z-scores of the intensity areas of differential metabolites and were plotted by the Pheatmap package in R language. The functions of these metabolites and metabolic pathways were studied using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The metabolic pathway enrichment of differential metabolites was performed. When the ratio was satisfied by x, n > y N, the metabolic pathway was considered as enrichment, when P-value of metabolic pathway < 0.05, the metabolic pathways was considered as statistically significant enrichment.

Network Pharmacological Analysis

Based on the differential expressed metabolites involved KEGG pathways with P value less than 0.05, we used a Traditional Chinese medicine system pharmacology database and analysis platform (TCMSP) for obtaining these metabolites' targets. RA targets were obtained from the human gene database (https://www.genecards.org). The intersection between GERA and RA targets was produced. Subsequently, we found critical genes related to GERA against RA by the Protein-Protein interaction networks database (String database). The Cytoscape 3.7.1 software was used in constructing a compound-target and target-

pathway network. Finally, functional enrichment analysis of common targets was performed on the DAVID Bioinformatics Resources 6.8 platform database (https://david.ncifcrf.gov).

Statistical analysis

Statistical analyses were performed using the statistical software R (version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). While data were not normally distributed, standard transformations were attempted using of area normalization method. We applied univariate analysis (t-test) to calculate the statistical significance.

Results

Classification annotation of metabolites from GERA

936 negative and 1476 positive metabolites from GERA were identified based on the mzCloud and ChemSpider databases. 200 negative and 314 positive metabolites(200^{-}, 314^{+}) were classified into eleven groups by HMDB. Lipids and lipid-like molecule (63^{-}, 55^{+}), organic acids and derivatives (31^{-}, 74^{+}), benzenoids (20^{-}, 46^{+}) and phenylpropanoids and polyketides (31^{-}, 29^{+}) were the recurrently observed metabolites. Lipid maps is currently the most complete database focused on the lipid structure and biological annotation information. Based on this database, the identified 68 negative and 56 positive metabolites from GERA were classified into 19 groups. Fatty acids and conjugates (12^{-}, 10^{+}), flavonoids (11^{-}, 11^{+}), isoprenoids (13^{-}, 7^{+}) were the frequently observed metabolites. Both databases provide enormous information for further studies of GERA metabolites.

Metabolites Analysis Of The Gera Extract

It is well known that different composition ratios of certain formulae may result in distinctive secondary metabolites. The scatter diagrams of PCA and PLS-DA analysis were displayed in Figs. 1 and 2. PLS-DA analysis is usually used to discriminate the metabolite contents and their distribution pattern; results shown in Fig. 2 indicated that the discriminate model recruited here was not over-fitted.

The negative and positive metabolites differentially isolated between samples TF11and TF23 were 39^{-} and 48^{+}, respectively. As for the other pairs, there were 31^{-} and 45^{+} (TF11vs TF32) and 13^{-} and 24^{+} (TF23 vs TF32) differential metabolites annotated by HMDB database (Fig. 3). Meanwhile, 15^{-} and 13^{+} (TF11 vs TF23), 11^{-} and 12^{+} (TF11 vs TF32) and 9^{-} and 6^{+} (TF23 vs TF32) of differential metabolites annotated by Lipidmaps database (Fig. 4). After merged these differentially expressed metabolites by enrichment analysis, KEGG pathways with P value less than 0.05 were classified into isoflavonoid biosynthesis and biosynthesis of unsaturated fatty acids, which are involved in four metabolites (Table 1).

Table 1
Differential metabolites involved in KEGG pathways ($P < 0.05$)

Name description	Formula	Molecular weight	Corresponding target numbers	Fold change (FC)		
				TF11 vs TF23	TF23 vs TF32	TF11 vs TF32
behenic acid	$C_{22}H_{44}O_2$	340.33	-	0.28	3.13	-
Linoleic acid	$C_{18}H_{32}O_2$	280.24	15	-	-	2.88
Daidzein	$C_{15}H_{10}O_4$	254.06	70	0.37	-	-
Daidzin	$C_{21}H_{20}O_9$	416.11	14	-	0.47	0.44

Network Pharmacological Analysis Of Metabolites From Different Gera Formulae

Based on differentially isolated metabolites involved in KEGG pathways with $P < 0.05$, we found 15(linoleic acid), 70(daidzein) and 14(daidzin) metabolites' targets from TCMCP, and obtained 3797 targets related to RA from the human gene database. Figure 5 demonstrated genes with connection nodes > 13, according to the relationships between GERA targets and RA. Which indicated that interleukin-6 (IL-6), tumor necrosis factor (TNF), vascular endothelial growth factor A (VEGFA), cellular tumor antigen p53 (TP53), caspase-3 (CASP3), prostaglandin G and H synthase 2 (PTGS2) might be the essential genes response for the anti-RA activities of GERA.

As shown in Fig. 6, the compound-target network consisted of 57 nodes (one disease, one couplet medicinals, three pharmaceutical ingredients and 52 targets) and 120 edges. This network displayed that EIC (linoleic acid), daidzein and daidzin interacted with 2, 26 and 6 targets, respectively. Which preliminarily revealed the interrelationships between GERA compounds and anti-RA targets. On the other hand, PTGS2 was targeted by three active ingredients of GERA, playing an important role in inflammatory modulation.

Results of the target-pathway network analysis were shown in Fig. 7. Of which 30 targets and 12 pathways were obtained, with an average crosstalks of one target related to 6.67 pathways, and one pathway to 10.00 targets. Results displayed that the KEGG pathway, Kaposi sarcoma-associated herpesvirus infection (hsa05167), possess the most crosstalks with anti-RA targets. Pathways of hepatitis B (hsa05161), Epstein-Barr virus infection (hsa05169), human cytomegalovirus infection (hsa05163) are also frequently related with anti-RA targets. RA is a typical autoimmunological

inflammation. Hence inflammatory regulating pathways, such as TNF signaling pathway (hsa04668) and IL-17 signaling pathway (hsa04657) mined from GERA, are the critical anti-RA process. It is note worthy that the enriched pathways, i. e., human cytomegalovirus infection, TNF signaling pathway, arachidonic acid metabolism and Jak/STAT signaling pathway, are all involved in the excess release of proinflammatory cytokines. Therefore, the therapeutical activities of GERA may majorly attribute to tune these proinflammatory pathways.

Discussion

Untargeted metabolomics effectively mined the anti-RA targets of GERA

Tian-Ma pill as a classic anti-RA formula has been extensively applying in Chinese medicine since Ming Dynasty (600 years ago). Tian-Ma pill is composed of ten Chinese herbs and GERA, the couplet Medicinals make up Tian-Ma and Fu-Zi, is the key factor of this effective formula. Accumulate data indicated that different dosage of Tian-Ma and Fu-Zi displayed distinguish pharmaceutical activities. For instance, TF11 and TF32 are widely used for the treatment of arthromyodynia and RA, respectively. However, the underlying mechanisms and targets are open to be explained. Our results of untargeted metabolomics combined with UPLC-MS determined not only the differentially isolated metabolites from blood serum and tissues, but also the GERA extract [10–11]. Figures 3 and 4 showed differentially isolated metabolites between three propositions of GERA, while enrichment analysis of KEGG pathways displayed the overall differential metabolites were majorly involved in isoflavonoid biosynthesis and biosynthesis of unsaturated fatty acids. Both isoflavonoid and unsaturated fatty acids possess broad pharmacological efficacy against RA. Linoleic acid is the major n-6 polyunsaturated fatty acid, its desaturation and elongation gives rise to arachidonic acid and gamma-linolenic acid as a precursor of prostaglandin E_2 [12–13]. Hence, appropriate intake of linoleic acid has effective anti-RA activities [14–16]. Daidzein and daidzin can ameliorate RA symptoms and decrease RA occurrence [17–20]. Our results demonstrated that the content of linoleic acid in TF11 was higher than that of TF32, while the daidzin content of TF32 was higher than either TF11 or TF23 (Table 1). Which help to explain the quantitively anti-RA activities of GERA.

Network Pharmacology Unraveled Crucial Anti-ra Targets Of Gera

Recently, a large amount of clinical cases and research data displayed the anti-RA effects of GERA. However, less work has addressed the overall targets of GERA against RA. Network pharmacology, an integrated platform widely applied to Chinese medicine, can effectively explain the multiple relationships between herbs, ingredients, diseases and their targets [21]. Results of network pharmacological analysis shown that linoleic acid, daidzein and daidzin were the key metabolites response for anti-RA activities of

GERA. Figure 5 displayed that essential genes related to anti-RA ingredients of GERA. Both TNF-α and IL-6 are the major proinflammatory cytokines which modulate the pathologic RA progress [22–24]. COX-2 (PTGS2) is the rate-limiting enzyme of arachidonic acid metabolism, which controls the production of PGE_2, the proinflammatory mediators of autoimmunity activities [25–27]. VEGF is an essential angiogenic marker for RA disorders [28–30]. TP53 and CASP3 regulate the apoptosis of rheumatoid synovial cells [31–32]. On the whole, results of network pharmacological analysis uncovered key anti-RA targets of GERA.

RA is a typical autoimmune disease induced by various risk factors, including Kaposi sarcoma-associated herpesvirus [33–34], Epstein-barr virus [35–36] and human cytomegalovirus infection [37]. Combined analysis on target-pathway network, we believe that KEGG pathways shown in Fig. 7 may monitor the transcription of anti-RA genes involved in inflammation process, vascular endothelial function and apoptosis. First, the activation of TNF [38] and IL-17 signaling pathways [39] indicate the production of TNF-α, IL-6, IL-7 and COX-2, pro-inflammatory cytokines result in the pathological process of RA. Second, PI3K/AKT/mTOR/NF-κB signaling pathway can modulate Chondrocyte proliferation, synovial fibroblasts apoptosis and autophagy that are intensively related to RA [40–41]. Then, suppression of MAPK and FoxO signaling pathways are important mechanisms for ameliorating inflammation and provoking apoptosis in RA patients [42]. Finally, pathways responsible for reducing reactive oxygen species [43] are of vital importance of anti-RA treatment focused on excessive inflammation. These enriched pathways unravel a network characteristic of GERA against RA as a multi-component, multi-target and multi-pathway pattern.

The present work explained the pharmaceutical basis of GERA against RA by an untargeted metabolomics combined with network pharmacological strategy. However, the aetiology of RA includes increasing factors, such as gut microbiota and miRNA regulation. In future researches, we will determine the pharmaceutical activates of linoleic acid, daidzein and daidzin, key anti-RA ingredentis of GERA. The substantial metabolic process of GERA will also be detected by a metagenomic platform.

Conclusion

This work indicates the anti-RA basis of GERA through key metabolites responsible for global regulation of inflammation, endothelial function and apoptosis.

Abbreviations

Gastrodia Elata and *Radix Aconitic Lateralis preparata*
GERA; Human metabolome database:HMDB; Rheumatoid Arthritis:RA; Principal components analysis:PCA; Partial least squares discriminant analysis:PLS-DA; Traditional Chinese medicine system pharmacology database and analysis platform:TCMSP; Interleukin-6:IL-6; Tumor necrosis factor:TNF; Vascular endothelial growth factor A:VEGFA; Cellular tumor antigen p53:TP53; Caspase-3:CASP3; Prostaglandin G and H synthase 2:PTGS2; EIC:linoleic acid.

Declarations

Acknowledgment

We thank Professor Deng Yun for GR identification. We thank Tianjin Novogene Bioinformatics Technological Corporation for their technological support. We are also grateful for the critical review of our peer reviewers.

Author contribution

Yang J was responsible for the necessary experiment process and paper writing. Ding WJ designed the experiment, presided over the necessary experiment process, and revised the paper. Shen XC majorly revised the paper. All authors read and approved the final manuscript.

Funding

The science and Technological fund of Guizhou Province in China financially supported this work (No: [2019]1003).

Availability of data and materials

The research data generated from this study is included within the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yang J, Peng QL, Zhang Y. Research overview of active ingredient of *Gastrodia Elata* BI to dispel wind and dredge meridians[J]. Acta Chin Med Pharmacol (Chin). 2018;46(2):121–3.
2. Dong S. Qi Xiao Liang Fang [M]. People's Publishing House of Nei Meng Gu. 2006;10:40–1.
3. Chen BX, Kang JN. Clinical efficacy analysis of *gastrodia elata* pellets against rheumatoid arthritis[J]. J Chin Pat Med (Chin). 1987;3:17–9.

4. Guo BF, Yang J, Peng QL, et al. The intervention mechanism of *Gastrodia elata* Bl and *Radix Aconiti Lateralis Preparata* herb pairs (GRHP) on rats with rheumatoid arthritis and wind-cold-dampness arthralgia[J]. Nat Prod Res Dev (Chin). 2020;5:1–11.
5. Hua YL, Ma Q, Yuan ZW, et al. A novel approach based on metabolomics coupled with network pharmacology to explain the effect mechanisms of Danggui Buxue Tang in anemia[J]. Chin J Nat Med. 2019;17(4):275–90.
6. Zhang J, Liang R, Wang L, et al. Effects and mechanisms of Danshen-Shanzha herb pair for atherosclerosis treatment using network pharmacology and experimental pharmacology[J]. J Ethnopharmacol. 2019;229:104–14.
7. Li Y, Zhang J, Zhang L, et al. Systems pharmacology to decipher the combinational anti-migraine effects of Tianshu formula[J]. J Ethnopharmacol. 2015;174:45–56.
8. Liu ZK, Ng CF, Shiu HT, et al. A traditional Chinese formula composed of *Chuanxiong rhizoma* and *gastrodiae rhizoma* (Da Chuanxiong Formula) suppresses inflammatory response in LPS -induced RAW 264.7 cells through inhibition of NF-κB pathway[J]. J Ethnopharmacol. 2017;196:20–8.
9. Xian JW, Choi AY, Lau CB, et al. gastrodia and uncaria (Tian ma Gouteng) water extract exerts antioxidative and anti-apoptotic effects against cerebral ischemia in vitro and in vivo[J]. Chin Med. 2016;11:27–33.
10. Shan J, Peng L, Qian W, et al. Integrated serum and fecal metabolomics study of collagen-induced arthritis rats and the therapeutic effects of the Zushima tablet[J]. Front Pharmacol. 2018;9:891–9.
11. Liu Y, Wei M, Yue K, et al. Non-target metabonomic method provided new insights on the therapeutical mechanism of Gancao Fuzi decoction on rheumatoid arthritis rats[J]. J Chromatogr B Analyt Technol Biomed Life Sci. 2019;1105:93–103.
12. Zhao JV, Schooling CM. Role of linoleic acid in autoimmune disorders: a mendelian randomization study[J]. Ann Rheum Dis. 2019;78(5):711–3.
13. Olson JM, Haas AW, Lor J, et al. A comparison of the anti-Inflammatory effects of cis-9, trans-11 conjugated linoleic acid to celecoxib in the collagen-induced arthritis model[J]. Lipids. 2017;52(2):151–9.
14. Hur SJ, Park Y. Effect of conjugated linoleic acid on bone formation and rheumatoid arthritis[J]. Eur J Pharmacol. 2007;568(2):16–24.
15. Namazi MR. The beneficial and detrimental effects of linoleic acid on autoimmune disorders[J]. Autoimmunity. 2004;37(1):73–5.
16. Watkins BA, Seifert MF. Conjugated linoleic acid and bone biology[J]. J Am Coll Nutr. 2000;19(4):478S–486S.
17. Ahmad S, Alam K, Hossain MM, et al. Anti-arthritogenic and cardioprotective action of hesperidin and daidzein in collagen-induced rheumatoid arthritis[J]. Mol Cell Biochem. 2016;423(2):115–27.
18. Mohammadshahi M, Haidari F, Saei AA, et al. Soy protein, genistein, and daidzein improve serum paraoxonase activity and lipid profiles in rheumatoid arthritis in rats[J]. J Med Food. 2013;16(2):147–54.

19. Wei G, Liang T, Wei C, et al. Daidzin inhibits RANKL-induced osteoclastogenesis in vitro and prevents LPS-induced bone loss in vivo[J]. J Cell Biochem. 2019;120(4):5304–14.
20. Majid Mohammad-Shahi1. Haidari F, Rashidi B, et al. Comparison of the effects of genistein and daidzein with dexamethasone and soy protein on rheumatoid arthritis in rats[J]. BioImpacts, 2011, 1(3), 161–70.
21. Xiong Y, Hu Y, Chen L, et al. Unveiling active constituents and potential targets related to the hematinic effect of steamed Panax notoginseng using network pharmacology coupled With multivariate data analyses[J]. Front Pharmacol. 2019;9:1514–22.
22. Jin H, Ma N, Li X, et al. Application of GC/MS-based metabonomic profiling in studying the therapeutic effects of *aconitum carmichael* with *ampelopsis japonica* extract on collagen-induced arthritis in rats[J]. Molecules. 2019;24(10):1934–52.
23. Haikal SM, Abdeltawab NF, Rashed LA, et al. Combination therapy of mesenchymal stromal cells and interleukin-4 attenuates rheumatoid arthritis in a collagen-induced murine model[J]. Cells. 2019;8(8):823–39.
24. Jing R, Ban Y, Xu W, et al. Therapeutic effects of the total lignans from Vitex negundo seeds on collagen-induced arthritis in rats[J]. Phytomedicine. 2019;58:152825–35.
25. Li X, Wu Z, He B, et al. Tetrandrine alleviates symptoms of rheumatoid arthritis in rats by regulating the expression of cyclooxygenase-2 and inflammatory factors[J]. Exp Ther Med. 2018;16(3):2670–6.
26. Lai ZZ, Yang HL, Ha SY, et al. Cyclooxygenase-2 in Endometriosis[J]. Int J Biol Sci. 2019;15(13):2783–97.
27. Leng P, Li D, Sun Y, et al. Effects of human cyclooxygenase-2 gene silencing on synovial cells of rheumatoid arthritis mediated by lentivirus[J]. Artif Cells Nanomed Biotechnol. 2018;46(3):274–80.
28. Li Y, Liu Y, Wang C, et al. Succinate induces synovial angiogenesis in rheumatoid arthritis through metabolic remodeling and HIF-1α, VEGF axis[J]. Free Radic Biol Med. 2018;126:1–14.
29. Abdel-Maged AE, Gad AM, Abdel-Aziz AK, et al. Comparative study of anti-VEGF ranibizumab and Interleukin-6 receptor antagonist tocilizumab in adjuvant-induced arthritis[J]. Toxicol Appl Pharmacol. 2018;356:65–75.
30. Chen Z, Wang H, Xia Y, et al. Therapeutic potential of mesenchymal cell-derived miRNA-150-5p-expressing exosomes in rheumatoid arthritis mediated by the modulation of MMP14 and VEGF[J]. J Immunol. 2018;201(8):2472–82.
31. Bergström B, Carlsten H, Ekwall AH, et al. Methotrexate inhibits effects of platelet-derived growth factor and interleukin-1β on rheumatoid arthritis fibroblast-like synoviocytes[J]. Arthritis Res Ther. 2018;20(1):49.
32. Hong SJ, Rim GS, Yang HI, et al. Bee venom induces apoptosis through caspase-3 activation in synovial fibroblasts of patients with rheumatoid arthritis[J]. Toxicon,2005, 46(1):39–45.
33. Ingegnoli F, Tourlaki A, Gualtierotti R. Tocilizumab monotherapy in a patient with rheumatoid arthritis and iatrogenic kaposi sarcoma[J]. Clin Drug Investig. 2014;34(2):159–61.

34. Jacobs SA, Vidnovic N, Patel H, et al. Durable remission of HIV-negative, kaposi's sarcoma herpes virus-associated multicentric castleman disease in patient with rheumatoid arthritis treated with methotrexate[J]. Clin Rheumatol. 2007;26(7):1148–50.
35. Balandraud N, Roudier J. Epstein-Barr Virus and Rheumatoid Arthritis[J]. Joint Bone Spine. 2018;85(2):165–70.
36. Masuoka S, Kusunoki N, Takamatsu R ,et al. Epstein-Barr virus infection and variants of epstein-barr nuclear antigen-1 in synovial tissues of rheumatoid arthritis[J]. PLoS One. 2018;13(12):e0208957.
37. Gardiner BJ, Haas EM, Bailey RC, et al. Reactivation of latent cytomegalovirus infection in patients with rheumatologic disease: a case-control study[J]. Rheumatol Int. 2019;39(7):1229–40.
38. Park JS, Yoo SH, Lim MA, et al. A bispecific soluble receptor fusion protein that targets TNF-α and IL-21 for synergistic therapy in inflammatory arthritis[J]. Faseb J. 2020;34(1):248–62.
39. Kostareva OS, Gabdulkhakov AG, Kolyadenko IA, et al. Interleukin-17: functional and structural Features, application as a therapeutic Target[J]. Biochemistry. 2019;84(1):193–205.
40. Feng FB, Qiu HY. Effects of artesunate on chondrocyte proliferation, apoptosis, and autophagy through the PI3K/AKT/mTOR signaling pathway in rat models with rheumatoid arthritis[J]. Biomed Pharmacother. 2018;102:1209–20.
41. Wang SG, Wang L, Wu CS, et al. E2F2 directly regulates the STAT1 and PI3K/AKT/NF-κB pathways to exacerbate the inflammatory phenotype in rheumatoid arthritis synovial fibroblasts and mouse embryonic fibroblasts[J]. Arthritis Res Ther. 2018;20(1):225–37.
42. Yang GL, Chang CC, Yang YW, et al. Resveratrol alleviates rheumatoid arthritis via rReducing ROS and inflammation, inhibiting MAPK signaling pathways, and suppressing angiogenesis[J]. J Agric Food Chem. 2018;66(49):12953–60.
43. Sire RD, Rizzatti G, Ingravalle F, et al. Skeletal muscle-gut Axis: emerging mechanisms of sarcopenia for intestinal and extra intestinal diseases[J]. Minerva Gastroenterol Dietol,2018,64 (4): 351–362.

Figures

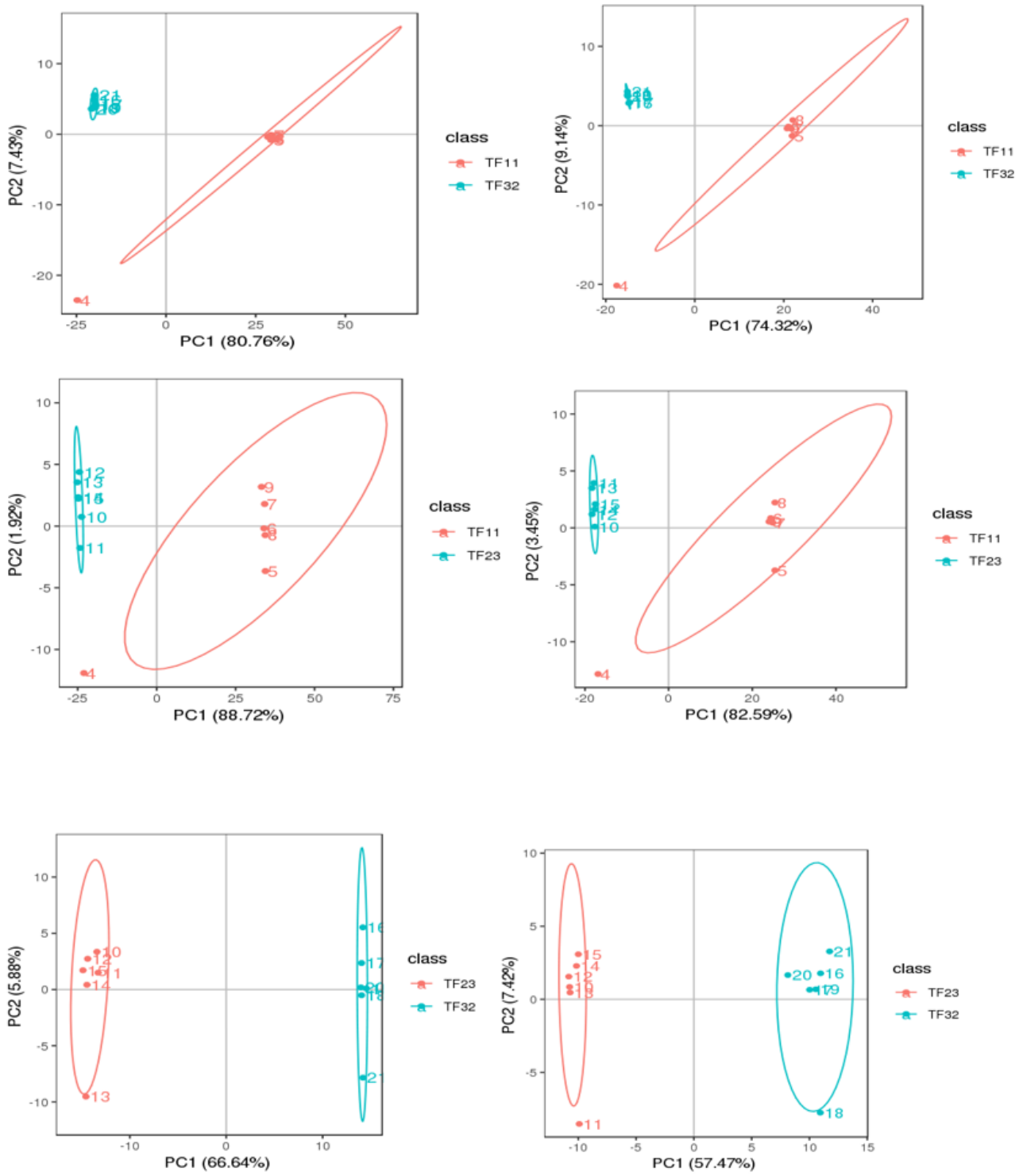

Figure 1

PCA scored plots between three proportions of GERA. Three formulae were composed on the relative wieght of Tian-Ma (T) and Fu-Zi (F) powder: 1:1 (TF11), 3:2 (TF32), or 2:3 (TF23). PC is the principal component. The X-axis is the score of PC1 in the sample, Y-axis is the score of PC2. Left, positive ion mode. Right, negative ion mode.

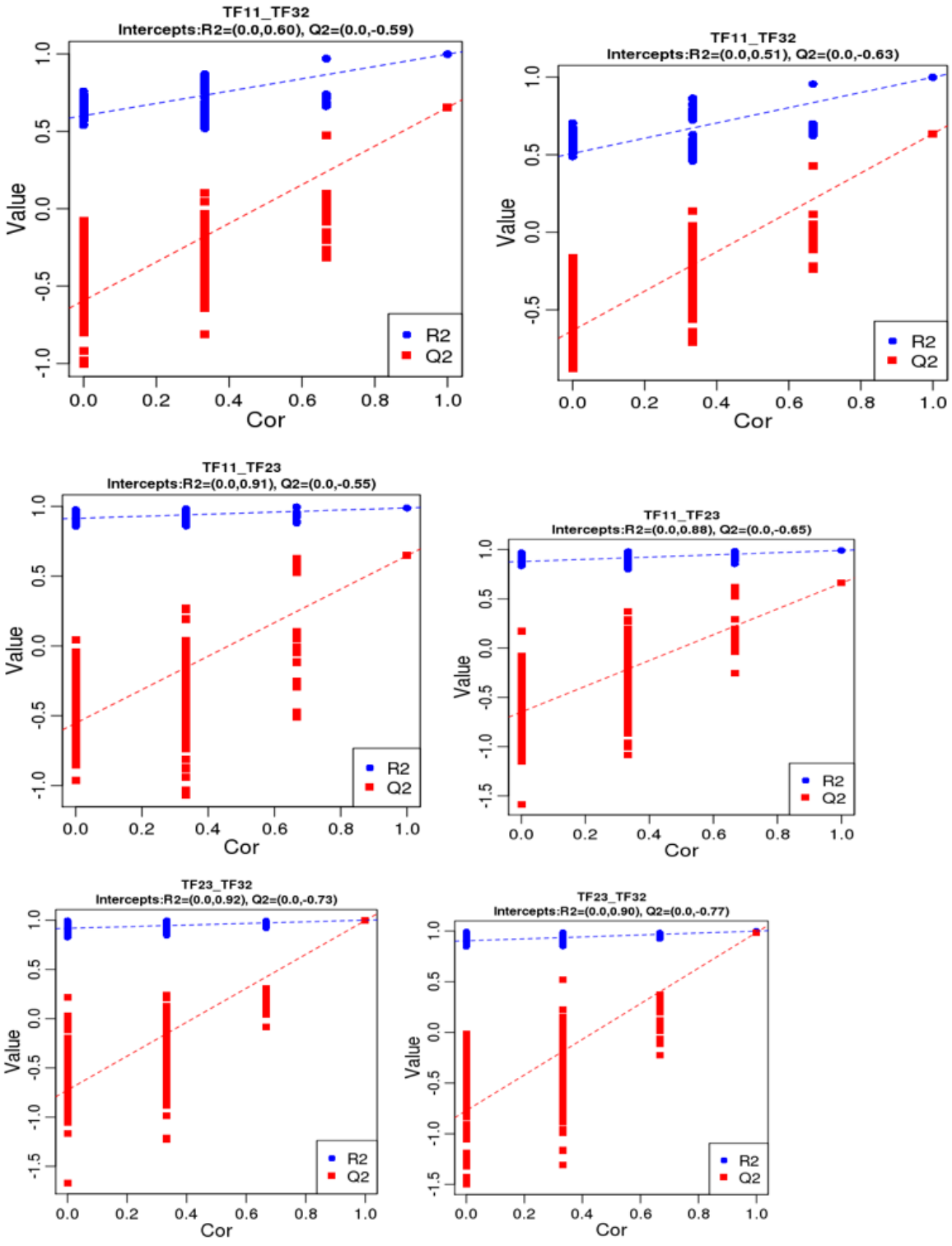

Figure 2

Precedence ordering plot of PLS-DA from three GERA proportions. Three formulae were composed on the proportion (w/w) of Tian-Ma (T) and Fu-Zi (F): 1:1 (TF11), 3:2 (TF32), or 2:3 (TF23). PC: the principal component. X-axis is the relative random group with the primary group. Y-axis is the score of R2 or Q2. R2 represents for fitting ability. Q2 represents for forecasting ability. Left, positive ion mode. Right, negative ion mode.

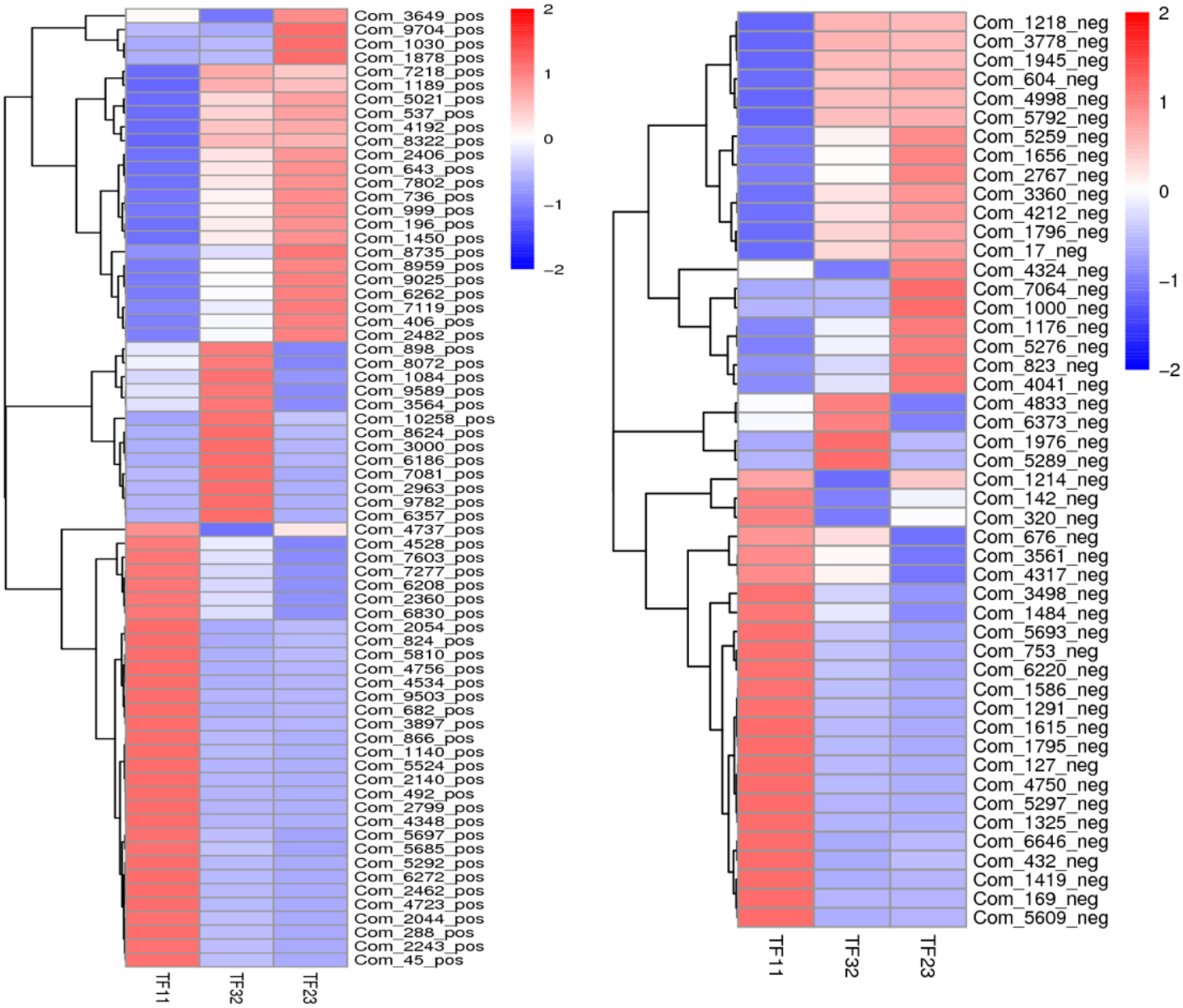

Figure 3

The heatmap of differentially isolated metabolites annotated by HMDB. Three formulae were composed based on the relative wieght of Tian-Ma (T) and Fu-Zi (F) powder: 1:1 (TF11), 3:2 (TF32), or 2:3 (TF23). Red represents up-regulated metabolites, blue represents down-regulated metabolites, and white represents non-significantly expressed metabolites. Left, positive ion mode. Right, negative ion mode. HMDB: the Human Metabolome Database.

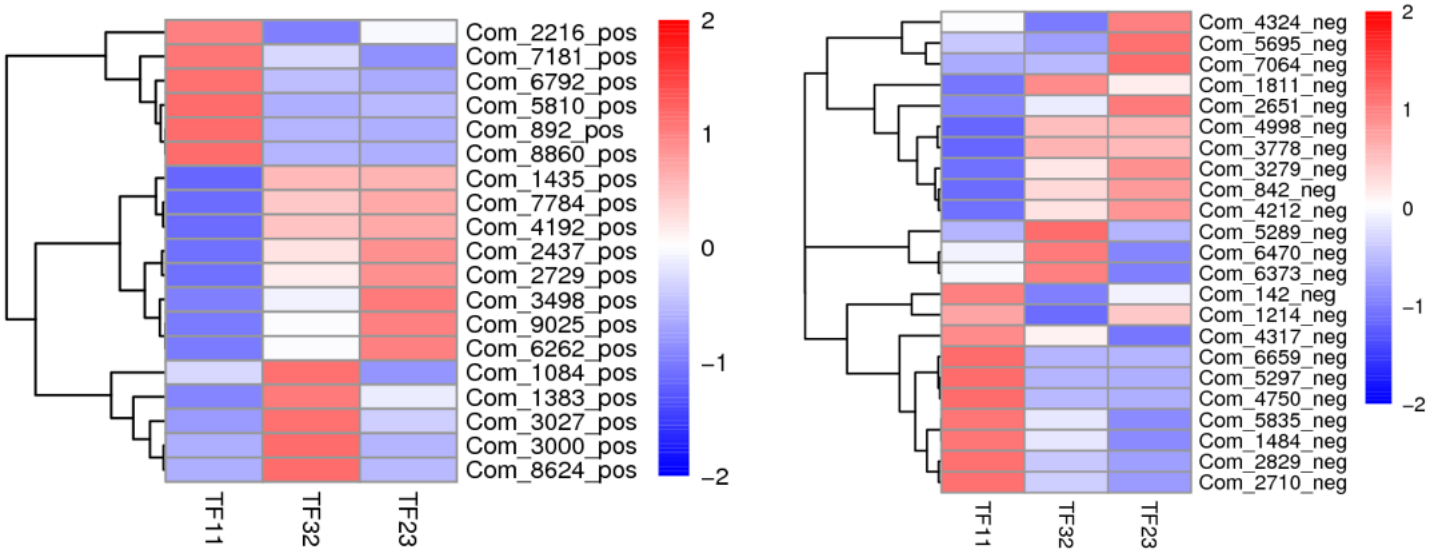

Figure 4

The heatmap of differential metabolites annotated by Lipidmaps. Three formulae were composed based on the relative weight of Tian-Ma (T) and Fu-Zi (F) powder: 1:1 (TF11), 3:2 (TF32), or 2:3 (TF23). Red represents up-regulated levels, blue represents down-regulated levels, and white represents not-expressed levels. Left, positive ion mode. Right, negative ion mode. The Lipidmaps (http://www.lipidmaps.org/) was found and executed by Professor Michael Wakelam.

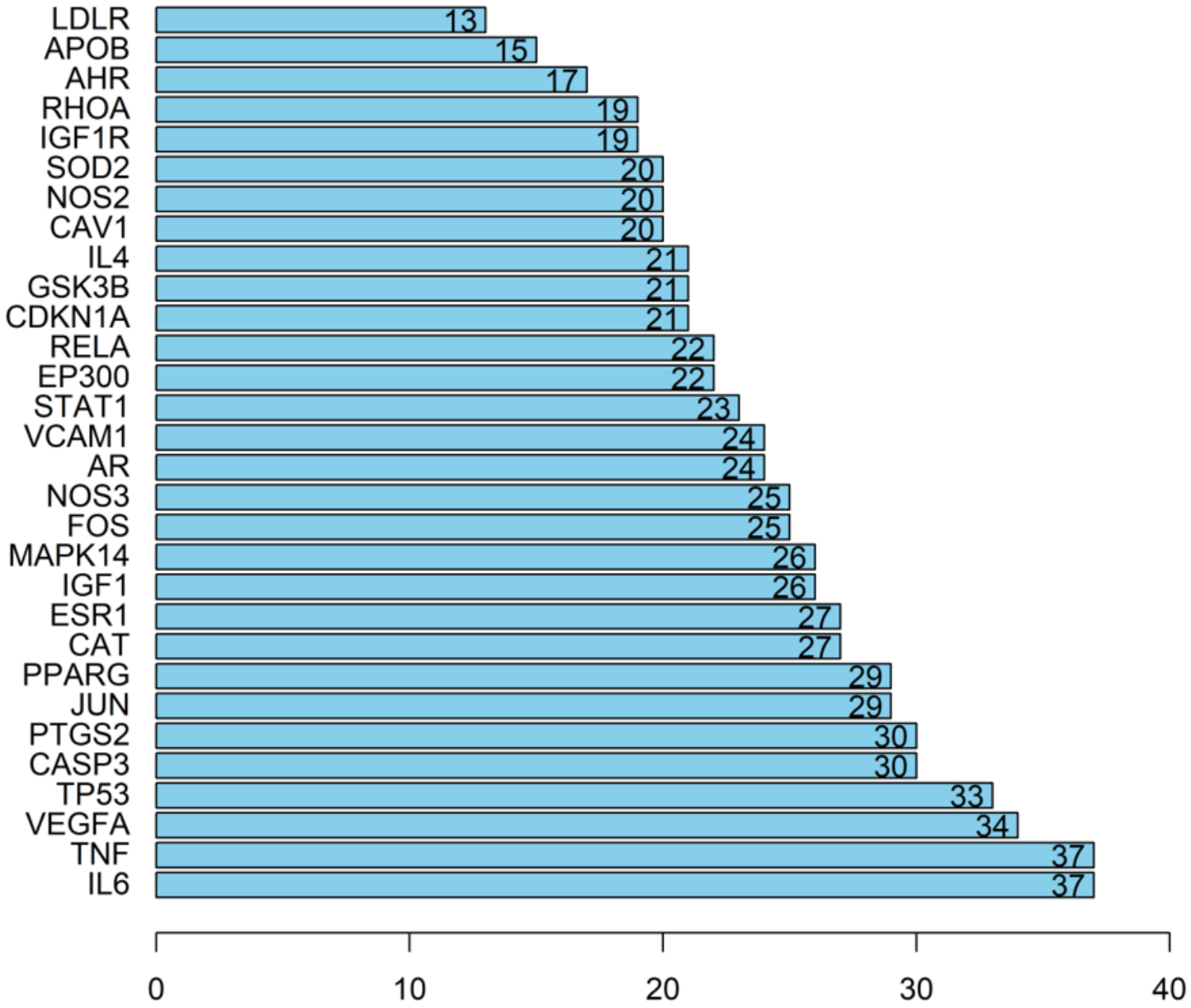

Figure 5

Histogram between genes or protein interaction related to GERA against RA. Results suggested critical genes related to GERA against RA were IL-6, TNF-α, VEGFA, TP53, CASP3, and PTGS2 (COX-2). The X-axis is the number of conjunctive nodes between genes or proteins. Y-axis is the gene symbol. GERA: Gastrodia Elata compared with Radix Aconitic Lateralis preparata. RA: rheumatoid arthritis.

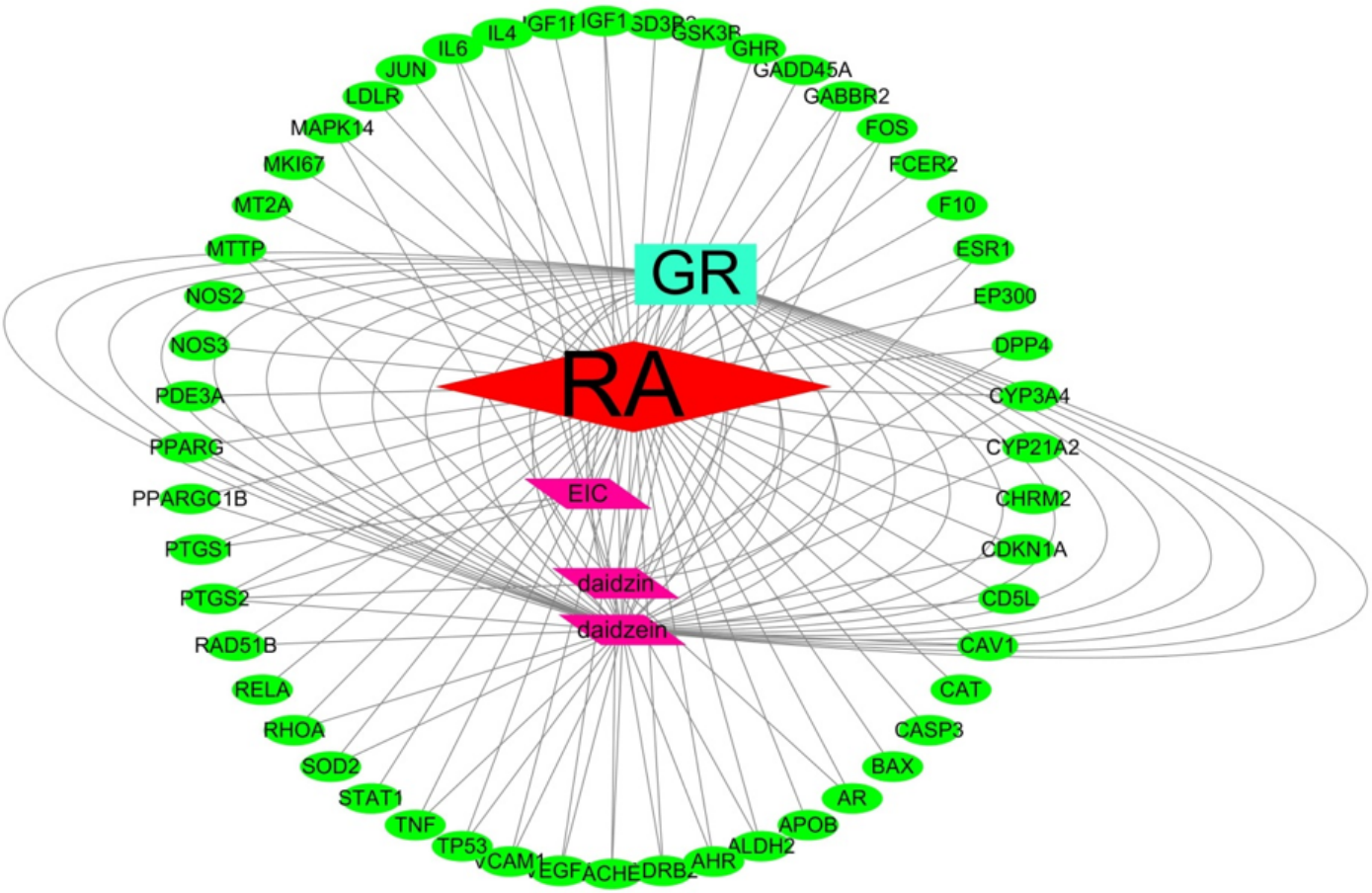

Figure 6

The interactive network graph of GR-active compounds-potential targets-RA. Green represent the targets of GR active compounds against RA. Red represent the RA disease. Purple represent key differential expressed metabolites between three GR formulae (1:1, 3:2, and 2:3). Lightblue represent GR, a couplet medicinals. GR: Gastrodia elata and Radix aconitic lateralis preparata. EIC: linoleic acid. RA: rheumatoid arthritis.

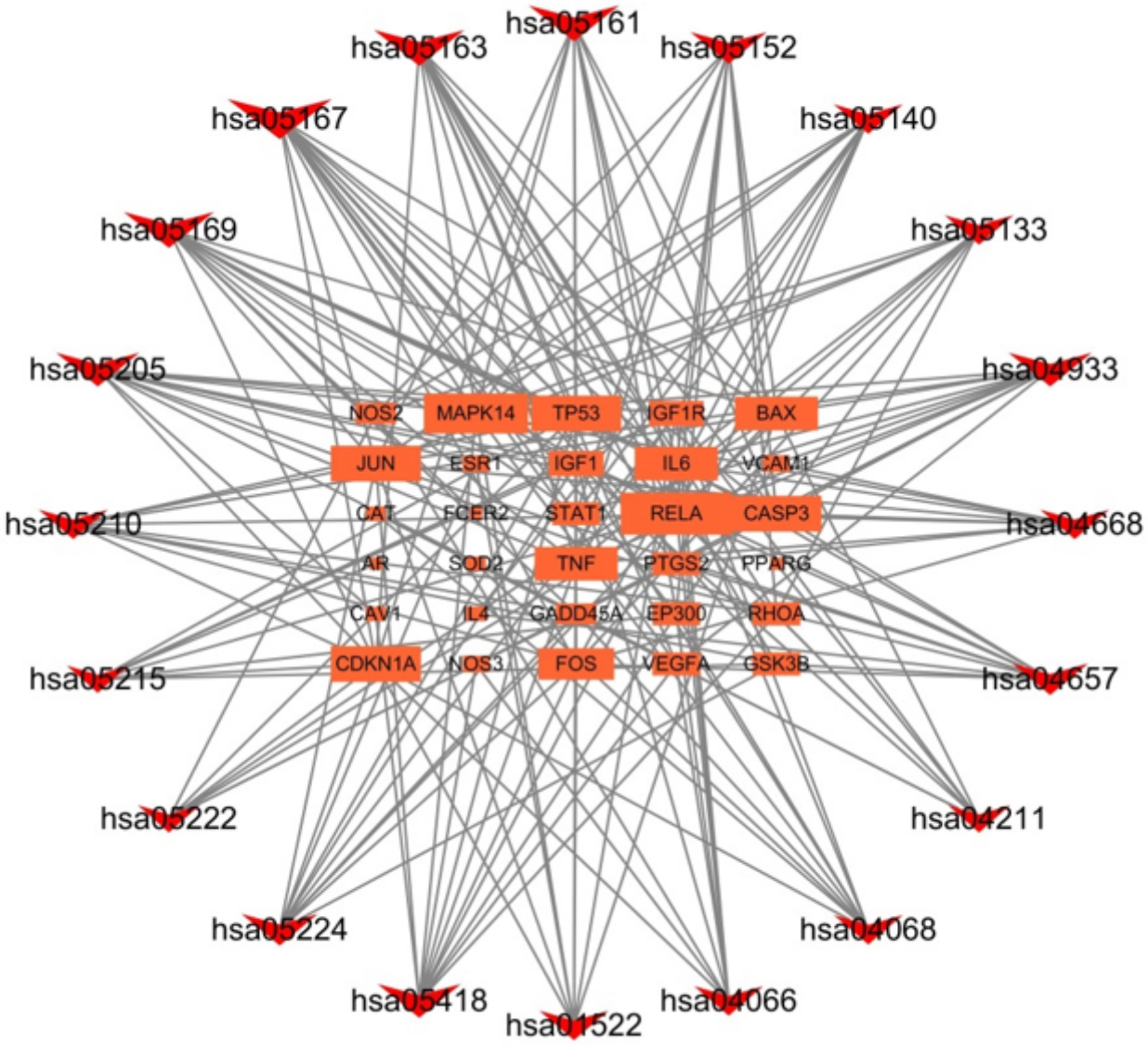

Figure 7

The interactive network of target-pathway. Red represent for pathway ID. Brown represent for gene.