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ORIGINAL ARTICLE

Exploring the Potential Quality Marker of Traditional Tibetan Medicine *Erigeron multiradiatus* Based on Ultra-High Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry Analysis, Network Pharmacology, and Experimental Verification

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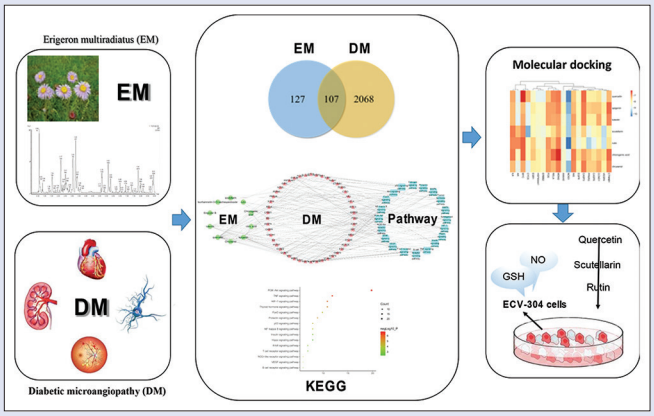
ABSTRACT

Background: *Erigeron multiradiatus* (EM), a well-known traditional Tibetan medicine, has been used for hundreds of years to treat various chronic metabolic diseases. However, previous studies on EM have primarily focused on its pharmacognosy, identification, and clinical efficacy, which lacks a systematic material basis research to identify therapeutic targets. **Objectives:** In this study, we investigated the potential quality marker (Q-marker) and the pharmacological mechanisms of EM during treatment of diabetic microangiopathy (DM) using both ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS) and network pharmacology. **Materials and Methods:** The chemical profile of EM was qualitatively identified for the first time by using UHPLC-QTOF-MS/MS. A network pharmacology methodology was then used to predict the major active components and key pharmacological pathways of EM in the treatment of DM. The compound-target network was constructed using Cytoscape software. Finally, virtual screening was conducted using molecular docking, and a cytopathological model was established by culturing vascular endothelial cells under *in vitro* conditions to verify the predicted bioactive markers. **Results:** A total of 26 compounds were identified and tentatively characterized in the chemical profile of EM based on the reference standards and mass spectral data. Network analysis was performed on 107 overlapping gene symbols, and seven bioactive constituents (e.g., quercetin, apigenin, luteolin, scutellarin, rutin, chlorogenic acid, and chrysoeriol) were identified as potentially bioactive markers. The results of molecular docking showed that quercetin, scutellarin, and rutin exhibited higher binding affinity to core targets than that of the remaining four compounds. The effects of quercetin, scutellarin, and rutin on vascular endothelial cells were investigated under *in vitro* conditions. According to the results, scutellarin and quercetin significantly inhibited the proliferation of vascular endothelial cells and decreased the levels of nitrous oxide, and reduced glutathione. **Conclusion:** This study showed that scutellarin and quercetin can be the Q-markers of EM due to their possible therapeutic efficacy in treating DM. It provided fundamental insight into chemical profiling, pharmacological mechanisms, and quality control of EM.

Key words: *Erigeron multiradiatus*, network pharmacology, quality marker, Tibetan medicines, ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

SUMMARY

- In this study, 26 compounds of EM were identified and tentatively characterized based on reference standards and mass spectral data. "Constituents-targets" net was constructed by network pharmacological methods. Network analysis was performed on 107 overlapping gene symbols, and 7 bioactive constituents were identified as potential quality markers. Quercetin, scutellarin, and rutin exhibited higher binding affinity to core targets. These three compounds were further investigated on vascular endothelial cells under *in vitro* conditions. This study declared the potential quality markers and mechanism of EM.



Abbreviations used: EM: *Erigeron multiradiatus*; Q-marker: Quality marker; DM: Diabetic microangiopathy; UHPLC-QTOF-MSMS: Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry; DMSO: Dimethylsulfoxide; MTT: 3-(4, 5-dimethylthia-zol-2-yl)-2,5-diphenyl tetrazolium bromide; IDF: International Diabetes Federation; DCCT: Diabetes Control and Complications Trial; BPI: The based peaks ions; OMIM: Online Mendelian Inheritance in Man; PPI: Protein-protein interactions; GO: Gene Ontology; KEGG: Kyoto encyclopedia of genes and genomes; BP: Biological process; MF: Molecular function; CC: And cell component; DMEM medium: Dulbecco's modified eagle medium; NO: Nitrous oxide; GSH: Reduced glutathione.

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INTRODUCTION

Diabetic microangiopathy (DM) is a common group of chronic complications of diabetes, which primarily include diabetic nephropathy, diabetic retinopathy, diabetic neuropathy, and diabetic cardiomyopathy.[1-3] Recently, the incidence of DM has gradually increased, becoming a significant threat to human health and mortality.[4] According to the survey data, the prevalence rate of diabetes in China increased from 9.7% in 2007 to an astonishing 11.6% in 2010.[5] According to the estimation of the International Diabetes Federation, the worldwide number of patients with diabetes will increase to 693 million by 2045.[6] The Diabetes Control and Complications Trial research conducted in the United States showed that the pathogenesis and severity of hyperglycemia were the critical factors that cause pathological changes in DM.[7] Although control of blood sugar levels could prevent and delay the development of DM, the frequency of diabetic nephropathy or diabetic retinopathy is still very high, resulting in a heavy financial burden for both the government and individuals.[8]

Traditional herbal medicines have long been used in the treatment of diabetes and the associated diseases.[9,10] In recent years, the application of traditional herbal medicines has shown stable curative effects with few adverse effects during the treatment of diabetes. Traditional herbal medicines can not only improve patients' physique but also reduce insulin resistance.[11,12] However, there is still very little understanding of the medical basis for its clinic applications.

Erigeron multiradiatus (EM) (Lindl.) Benth. is a biennial or perennial herb mainly found in the Qinhai-Tibet plateau of China at an altitude of 2600–4300 m. In traditional Tibetan medicine, EM has been used for hundreds of years to treat various chronic metabolic diseases.[13-15] Photochemistry research reported that flavonoids, phenolic acids, and sterols were isolated and identified in EM.[15] In our previous studies,[16] we have reported that extracts of EM have important pharmacological actions. The EM flavonoids significantly inhibit aldose reductase activity under *in vitro* and *in vivo* conditions, thereby improving hemodynamic behavior and protecting heart function in streptozotocin-induced diabetic rats. Our previous research also illustrated that EM has a protective effect on vascular inflammation induced by hyperglycemia both under *in vitro and in vivo* conditions.[17] It also regulates blood sugar, but the bioactive Q-markers, biochemical pathways, and mechanisms of action are still not clear.

Therefore, in this study, we analyzed the chemical profile of EM by using ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS). We used network pharmacology[18] to predict the key active components and the essential pathways modulated by EM during DM treatment. Finally, we performed molecular docking analysis and established a cytopathological model by culturing vascular endothelial cells *in vitro* to verify the activity of the predicted active components. This research represents the first comprehensively performed qualitative analysis of the significant constituents of EM. The potential Q-markers and therapeutic targets for the treatment of DM were investigated using network pharmacological analysis.

RESULTS

Identification of chemical constituents in *Erigeron multiradiatus*

Currently, UHPLC-QTOF-MS methods are widely used in the chemical identification of herbal medicines. UHPLC efficiently separates components of herbal medicines, and QTOF-MS accurately identifies masses of precursor and fragment ions. High-resolution tandem MS/MS analysis supplies sufficient fragmentation ion information to characterize trace components in complex mixtures of natural products. Thus, QTOF-MS/MS has proven to be a powerful tool for efficiently identifying non-target compounds when there are no reference compounds in complex matrices. In this study, the chemical profiles of the secondary metabolites of EM were determined using UHPLC-QTOF-MS/MS analysis. Figure 1 shows the chromatogram of a representative image of the based peaks ions (BPI) from each species. A total of 37 compounds were extracted and separated by UHPLC [Table 1]; of them, 26 compounds were identified or tentatively characterized from the BPI chromatograms of the EM samples, including 16 phenolic acids and flavonoids and 10 diterpenoid/triterpenoid compounds. Five compounds were clearly identified by comparing the retention times and molecular ions with those of similar samples containing chlorogenic acid (compound 2); erigeside I (compound 4); scutellarin (compound 8); 3,5-dicaffeoylquinic acid (compound 12); and 3,4-dicaffeoylquinic acid (compound 15).

Collected component-targets and disease-targets and predicted potential targets

A total of 234 component-targets were collected and integrated from the databases, and 2175 disease-targets of DM were obtained from Online Mendelian Inheritance in Man (OMIM) and the GeneCards database.

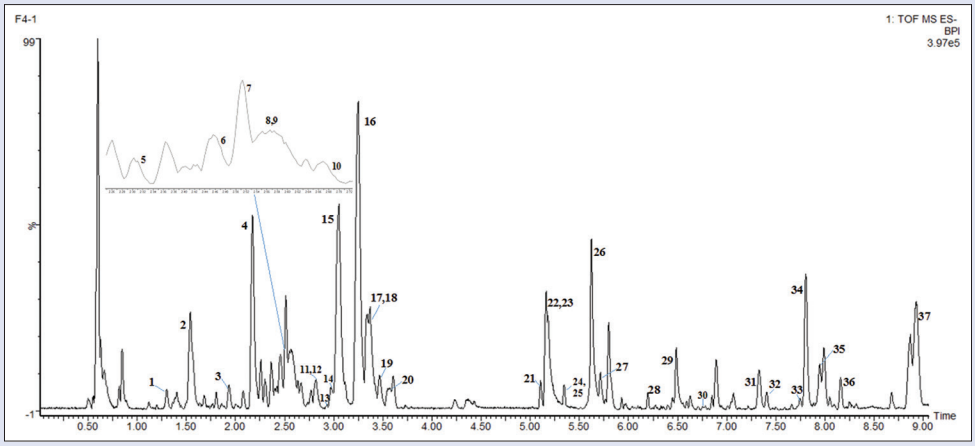

Figure 1: The base peak ion chromatograms of *Erigeron multiradiatus* by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry

Table 1: Components information of *Erigeron multiradiatus*

RT (min)	*m/z*(-)	Adductive ions	Error (ppm)	MS/MS	Formular	Identification
1.3	353.0882		2.5	191.0564, 179.0353, 173.0452, 135.0455	$C_{16}H_{18}O_9$	Cryptochlorogenic acid
1.54	353.0887	1061.2820 (3M-H)	4	191.0565, 179.0358, 173.0455, 135.0450	$C_{16}H_{18}O_9$	Chlorogenic acid
1.94	493.0629	987.1351 (2M-H)		317.0304, 287.0175, 166.9993, 139.0014	$C_{21}H_{18}O_{14}$	Myricetin 3-O-glucuronide
2.18	435.0934	871.1947 (2M-H)		323.0765, 221.0454, 179.0347, 161.0244, 135.0448	$C_{20}H_{20}O_{11}$	Erigeside I
2.3	609.1451	1219.296 (2M-H)		300.0270, 271.0244, 255.0294, 178.9984, 151.0035	$C_{27}H_{30}O_{16}$	Rutin
2.46	477.0663	955.1401 (2M-H)	−1.3	301.0354, 283.0229, 255.0291, 178.9988, 151.0037	$C_{21}H_{18}O_{13}$	Naringenin-7-O-β-glucuronide
2.51	477.066	955.1393 (2M-H)	−1.9	301.0413, 179.0013, 151.0059	$C_{21}H_{18}O_{13}$	Naringenin-3-O-β-glucuronide
2.53	461.0716	923.1514 (2M-H)	−0.9	300.0273, 285.0403, 271.0246, 255.02991, 243.0300, 151.0034, 135.0446	$C_{21}H_{18}O_{12}$	Scutellarin
2.57	463.0865	925.1634 (2M-H)	−2.6	300.0275, 286.0428, 271.0247, 255.0301, 243.0295, 151.0038, 135.0449	$C_{21}H_{20}O_{12}$	Isoquercitrin
2.66	461.0705	507.0763 (M + HCOOH) 969.1546 (2M-H), 1015.1609 (2M-H + HCOOH)	−2.4	446.0859, 331.0444, 316.0200, 301.0311, 285.0396, 269.0451, 255.0281, 151.0023	$C_{22}H_{20}O_{14}$	4'-O-Methylmyricetin-7-O-β-glucuronide
2.82	593.1495		−1.9	285.0390, 255.0295, 227.0347, 151.0053	$C_{27}H_{30}O_{15}$	Kaempferol-3-rutinoside
2.82	515.1182		−1.6	353.0865, 191.0559, 179.0348, 173.0454, 161.0243, 155.0347, 135.0450	$C_{25}H_{24}O_{12}$	Isochlorogenic acid A
2.93	623.1592			417.1557, 315.0503, 300.0260, 269.0426	$C_{28}H_{32}O_{16}$	Isorhamnetin-3-O-neohespeidoside
2.98	515.1190	1031.2417 (2M-H)	0	353.0865, 191.0562, 179.0350, 173.0455, 161.0229, 135.0452	$C_{25}H_{24}O_{12}$	1,5-O- Dicaffeoylquinic acid
3.04	515.1172	1031.2408 (2M-H)	−3.5	353.0868, 191.0547, 179.0350, 173.0455, 135.0452	$C_{25}H_{24}O_{12}$	Isochlorogenic acid A
3.24	543.1153	1087.2386 (2M-H)	2.6	381.0822, 363.0720, 319.0820, 221.0453, 203.0343, 179.0349, 161.0244, 135.0449	$C_{26}H_{24}O_{13}$	Erigoster B
3.34	445.0774	891.1633 (2M-H)	0.7	269.0451, 175.0238, 113.0240	$C_{21}H_{18}O_{11}$	Apigenin-7-O-β-D-glucuronopyranoside
3.35	515.1194	1031.2484 (2M-H)	0.8	353.0862, 191.0559, 179.0349, 173.0454, 135.0449	$C_{25}H_{24}O_{12}$	Dicaffeoylquinic acids
3.46	491.0829	983.1733 (2M-H)	0.6	447.1294, 315.0498, 300.0266, 269.0441, 175.0244, 161.0245	$C_{22}H_{20}O_{13}$	Carmine
3.57	475.0891	951.1861 (2M-H)	2.9	299.0549, 284.0317, 175.0232, 151.0035	$C_{22}H_{20}O_{12}$	Scutellarin methylester
5.11	585.1229		−2.6	423.0920, 301.0353, 285.0401, 179.0351, 161.0244	$C_{28}H_{26}O_{14}$	Caffeoylquinic acids
5.16	285.0398		−0.4	241.0501, 217.0502, 199.0396, 175.0402, 151.0037, 133.0294	$C_{15}H_{10}O_6$	Luteolin
5.18	301.0356		3.3	273.0404, 245.0446, 178.9984, 151.0037, 121.0291	$C_{15}H_{10}O_7$	Quercetin
5.46	1087.5015		−0.5	911.4655, 677.3472, 193.0351, 175.0248, 131.0344	$C_{45}H_{84}O_{29}$	Triterpenoid saponins
5.49	1073.5212		4	849.4597, 439.3217, 409.1338, 337.1133, 295.1025, 277.0926, 235.0824, 217.0714, 205.0706, 149.0452, 131.0347	$C_{52}H_{82}O_{23}$	Triterpenoid saponins
5.63	269.0457		2.6	225.0553, 201.0551, 181.0654, 159.0448, 151.0034, 117.0341	$C_{15}H_{10}O_5$	Apigenin
5.71	299.0564		2.7	284.0319, 256.0365, 227.0346, 151.0036	$C_{16}H_{12}O_6$	Chrysoeriol

Contd...

Table 1: Contd...

RT (min)	*m/z*(-)	Adductive ions	Error (ppm)	MS/MS	Formular	Identification
6.19	307.1905		−1	235.1344, 209.1149, 185.1180, 137.0971, 125.0965, 121.0655	$C_{18}H_{28}O_4$	5-O-methyl embelin
6.48	293.1748		−1.7	236.1037, 221.1532, 205.1220, 192.1161, 177.0904	$C_{17}H_{26}O_4$	Dendocarbin J
6.79	487.3419		−0.8	469.3335, 360.9963, 235.0159	$C_{30}H_{48}O_5$	Triterpene acids
7.33	293.211		−2.4	275.2009, 235.1693, 223.1688, 211.1321, 195.1391, 183.1384, 171.1013, 155.1065, 121.1016	$C_{18}H_{30}O_3$	Long chain fatty acids
7.4	293.2111		−2	275.2005, 235.1693, 223.1333, 195.1391, 183.1384	$C_{18}H_{30}O_3$	Long chain fatty acids
7.74	295.2277		1.4	277.2165, 195.1387, 183.1022, 171.1026, 155.1078	$C_{18}H_{32}O_3$	Long chain fatty acids
7.8	295.2275		0.7	277.2165, 195.1387, 183.1022, 171.1026, 155.1078	$C_{18}H_{32}O_3$	Long chain fatty acids
7.96	497.3124		2	255.2326, 183.0121	$C_{27}H_{46}O_8$	Microlepin
8.16	293.2113		−1.4	249.2238, 197.1177, 185.1178, 149.0983, 141.1272, 125.0971	$C_{18}H_{30}O_3$	Long chain fatty acids
8.91	467.3008		−0.2	325.1827, 305.1714, 255.2315, 183.0116	$C_{26}H_{44}O_7$	2,16-Kauranediol 2-O-β-D-allopyranoside

RT: Retention time; MS: MS/MS represent fragment ion; HCOOH: formic acid

Component-targets and disease-targets were crossed using VENNY 2.1, and 107 overlapping targets were identified that could be used as potential targets for the anti-DM of EM [Figure 2a].

Network construction and analysis

An "ingredient-targets" network was constructed using Cytoscape software to elucidate the underlying mechanism of EM for the treatment of DM. As shown in Figure 2b, the green node represents the ingredients, the red nodes represent the DM targets, and the edges indicate those targets interacting with each other. The network shows viable protein target nodes (n = 126) connected by edges (n = 374) and an average node degree of 2.97. The network contained components with multiple targets, such as quercetin (degree = 58), apigenin (degree = 30), luteolin (degree = 27), scutellarin (degree = 17), rutin (degree = 13), chlorogenic acid (degree = 10), and chrysiol (degree = 6). Identifying these components with high degree numbers indicated that single constituents of EM could act on multiple targets.

Construction and analysis of protein–protein interaction network

In this study, protein–protein interaction (PPI) network was constructed using STRING, and the resulting PPI network diagrams were imported into the Cytoscape software for visualization [Figure 3a]. A total of 20 target genes with a high degree of connectivity (degree >30) were selected as the core genes for DM [Figure 3b], including *INS*, *IL6*, *EGFR*, *CASP3*, *MYC*, *VEGFA*, *CCND1*, and *ESR1* [Figure 3c].

Gene ontology term enrichment analysis and Kyoto encyclopedia of genes and genomes pathway

To investigate the molecular mechanism, gene ontology (GO) analysis and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment of the 107 candidate targets were conducted based on the DAVID database. The results of GO enrichment analysis were categorized using the biological process (BP), molecular function (MF), and cell component (CC) terms. The analysis yielded 336 GO entries ($P < 0.05$), including 234 BP entries, 42 CC entries, and 60 MF entries. The initial analysis yielded the top 10 enriched conditions in the BP, CC, and MF categories [Figure 4a]. The GO enrichment data correlate the clinical outcomes of EM treatment with regard to regulation of BPs such as apoptosis and gene expression, cellular components such as cytoplasm and extracellular space, and MFs such as enzyme and protein binding.

Subsequently, a total of 77 pathways were identified ($P < 0.05$). Figure 4b shows the 20 most noteworthy pathways, including the PI3K-Akt, TNF, and HIF-1 signaling pathways. To systematically and comprehensively explore the core mechanisms of therapeutic action of EM in treating DM, the "pathways–targets–ingredients" network was constructed using Cytoscape [Figure 4c]. According to the network analysis, the core ingredients of EM that treat DM include constituents such as quercetin, luteolin, scutellarin, rutin, and apigenin. These core ingredients primarily act on targets such as RELA, CCND1, RAF1, IL6, INS, CASP9, CASP3, EGFR, and VEGFR by regulating signaling pathways such as PI3K-Akt, TNF, and HIF-1.

Molecular docking

Molecular docking was performed using AutoDock Vina, considering the top 20 core target genes and 7 core active EM components. Figure 5a shows the docking results. The scale shows deeper blue which depicts lower free energy of association and a stronger binding affinity between the ligand and receptor. The results also show that multiple components have a biologically significant affinity with multi-targets based on the control compounds. The binding affinity for quercetin, scutellarin, and rutin identified them as better core targets than that of other compounds. The bioactivity of these three compounds will be further investigated under *in vitro* conditions in the next part of this study. Figure 5b shows some graphical representations of the molecular docking data. Quercetin can form multiple hydrogen bonds with NOS3 at ARG-A: 365, HIS-B: 461, PHE-B: 460, and SER-A: 102; scutellarin can form hydrogen bonds with VEGFA at LEU-A: 25, ASN-D: 55, ASP-D: 56, and CYS-D54D; and rutin can form hydrogen bonds with PPARG at GLY-G: 284, and SER-A: 342. Each of these

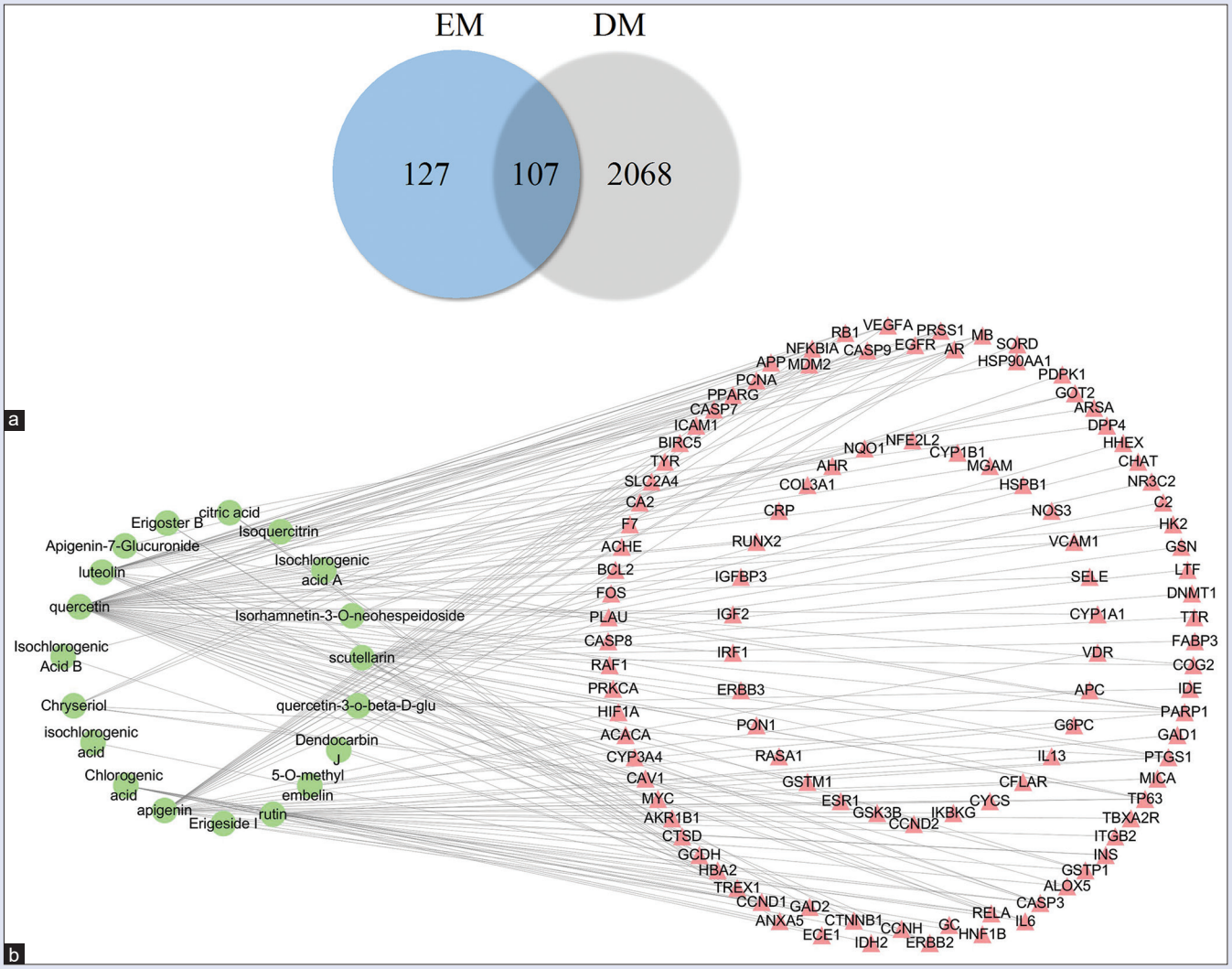

Figure 2: Network diagram of potential targets and ingredient-targets. (a) Venn diagram of potential targets of *Erigeron multiradiatus* and diabetic microangiopathy; (b) Network of ingredient-targets

ligands primarily bind with the active site via hydrogen bonding. Figure 5b shows the hydrogen bonding and bond length.

Experimental verification under *in vitro* conditions

Effects of quercetin, scutellarin, and rutin on vascular endothelial cell proliferation induced by treatment with high glucose levels

The value of optical density (OD) was less in the model control group (vascular endothelial cells grown in 30 mmol/L glucose for 48 h) and was statistically different from the normal control group (vascular endothelial cells grown in 5.5 mmol/L glucose for 48 h) ($P < 0.01$). The screening results showed that quercetin and scutellarin showed different levels of resistance to the proliferation of vascular endothelial cells grown in media containing high glucose, partly in a concentration-dependent manner. Rutin, however, did not show the same inhibitory response [Table 2].

Effects of quercetin, scutellarin, and rutin on the nitrous oxide and reduced glutathione levels of vascular endothelial cells induced with high glucose

The level of nitrous oxide (NO) in the media of vascular endothelial cells were significantly increased ($P < 0.01$) after treatment with 30 mmol/L glucose for 48 h. However, the level of reduced glutathione (GSH) was significantly decreased ($P < 0.01$). The screening results showed that both quercetin and scutellarin reduced the levels of NO and GSH in vascular endothelial cells stimulated by high glucose; however, rutin showed no significant effects [Table 3].

MATERIALS AND METHODS

Chemicals and herb materials

EM samples were collected during flowering from various Ganzi in the Sichuan Province. The botanical identification was authenticated using Chinese Monograph Flora by Professor Hao Zhang (School of Pharmacy, Sichuan University, China). Herbarium vouchers (No. EM190701) were prepared and deposited at the herbarium at the Institute of Qinghai-Tibetan Plateau, Southwest Minzu University. All samples were air-dried and ground with a commercial electric grinder.

Acetonitrile (LC-MS grade) was purchased from E. Merck (Darmstadt, Germany). Methanol, formic acid, and all other chemical reagents were of analytical grade (Chengdu Hengli Sci-Tech Co., Ltd., Chengdu, China). Ultra-pure water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Table 2: Effects of 3 herbal monomers on ECV-304 proliferation in high glucose medium ($\bar{X}$±s, *n*=3)

Groups	Concentration of glucose (mmol/L)	Concentration of monomers (μmol/L)	OD (570 nm)	Proliferaton (%)
Normal	5.5	-	0.671±0.07	-
Model	30	-	0.358±0.04**	-
	30	80	0.492±0.14$^{\#}$	37.43
Quercetin	30	8	0.457±0.12$^{\#}$	27.65
	30	0.8	0.386±0.08	-
	30	20	0.493±0.03$^{\#\#}$	37.71
Scutellarin	30	2	0.482±0.12$^{\#}$	34.64
	30	0.2	0.475±0.04$^{\#}$	32.68
	30	50	0.346±0.03	-
Rutin	30	5	0.351±0.05	-
	30	0.5	0.341±0.08	-

*P<0.05, **P<0.01: Compared with normal; $^{\#}P$<0.05, $^{\#\#}P$<0.01: Compared with model. OD: Optical density; ECV304: Human bladder epithelial cells

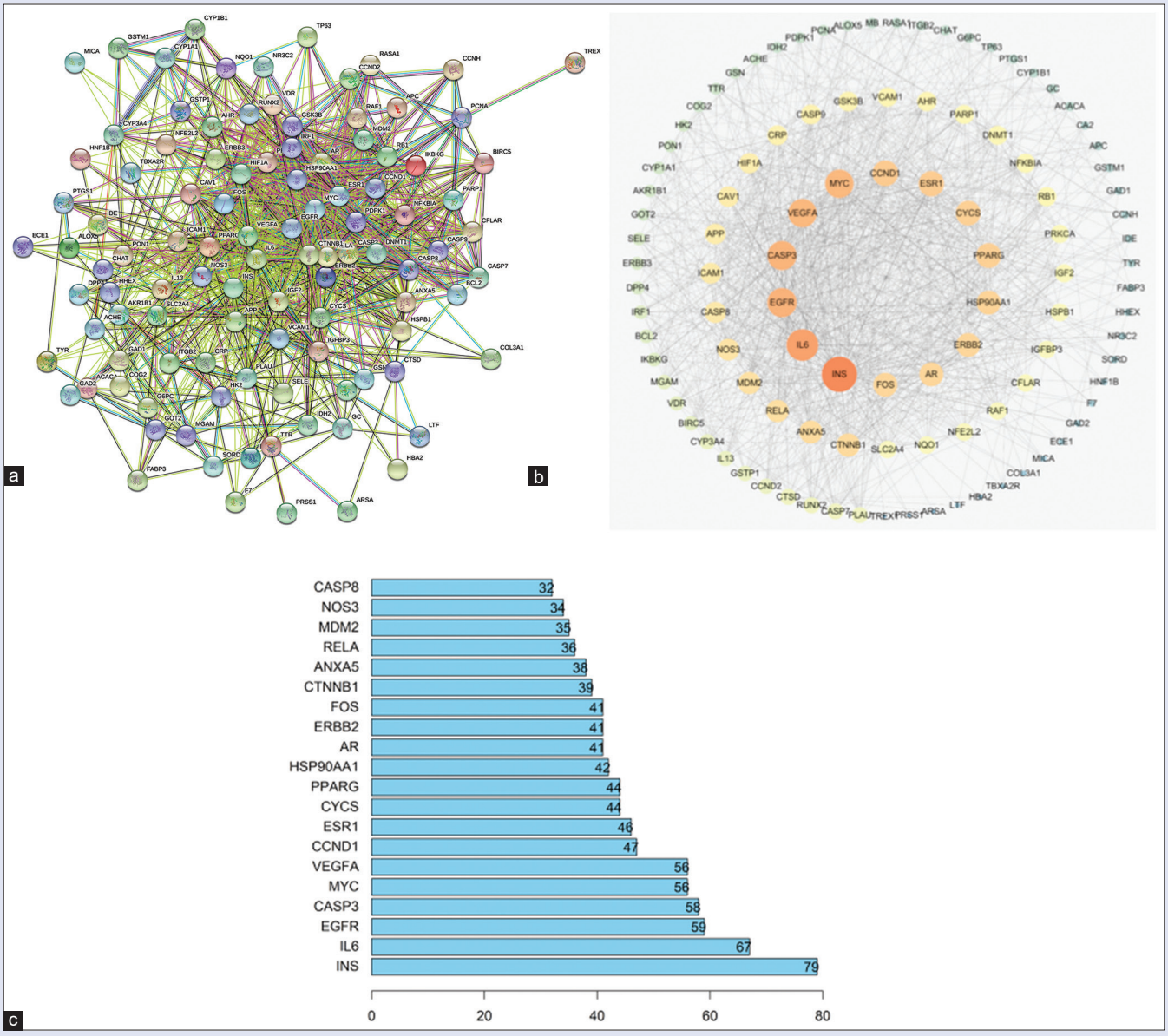

Figure 3: Protein interaction network and the key target of *Erigeron multiradiatus* (a) Protein-protein interactions network, (b) Protein interaction attribute by degree; (c) Top 20 key targets)

Table 3: Effect of 3 herbal monomers on the nitrous oxide, glutathione level in high glucose cultured ECV-304 cells ($\bar{X}$±s, *n*=3)

Groups	Concentration of glucose (mmol/L)	Concentration of monomers (μmol/L)	NO (μmol/L)	GSH (U/mg)
Normal	5.5	-	546.72±23.01	35.56±9.08
Model	30	-	696.21±31.69**	23.98±11.90**
	30	80	578.09±64.83#	26.90±17.87
Quercetin	30	8	564.98±39.46##	27.98±67.09
	30	0.8	600.64±12.86	24.14±4.90
	30	20	608.18±1.85	30.09±13.08##
Scutellarin	30	2	565.20±22.02#	31.67±4.83#
	30	0.2	590.46±39.93	32.87±9.05#
	30	50	621.98±53.5	25.98±12.00
Rutin	30	5	599.76±35.87	24.46±8.47
	30	0.5	607.84±5.09	22.90±5.78
	30	30	578.98±65.90##	25.98±4.86

**P<0.01: Compared with normal; #P<0.05, ##P<0.01: Compared with model. NO: Nitrous oxide; GSH: Glutathione; ECV-304: human bladder epithelial cells

Reference compounds, including chlorogenic acid (compound 2); erigeside I (compound 4); scutellarin (compound 8); 3, 5-dicaffeoylquinic acid (compound 12); and 3,4-dicaffeoylquinic acid (compound 15), were purchased from National Institutes for Food and Drug Control (Beijing, China). Their purity was more than 98% by HPLC. Cell culture materials were purchased from Gibco-BRL (MD, USA). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co.(St. Louis, MO, USA).

Qualitative analysis by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry.

Plant extraction and standard compound solutions

The samples were powdered and sieved through a 60-mesh sieve. Accurately weighed powder (about 0.2 g) was placed in a 25 mL volumetric flask with 80% methanol, used as an extraction solvent. The extract was sonicated for 30 min at room temperature in an ultrasonic machine (Kunshan, China). The final volume was then made up to 25 mL with 80% methanol. The extracts were centrifuged at 4000 rpm for 5 min. The supernatant was filtered through a 0.22 μm PTFE syringe filter and stored at 4°C before injecting into the UHPLC. Each sample was analyzed thrice.

For the preparation of stock solutions (1 mg/mL), 10 mg each of chlorogenic acid, erigeside I, scutellarin, 3,5-dicaffeoylquinic acid, and 3,4-dicaffeoylquinic acid were dissolved respectively with 80% methanol in a 10 mL volumetric flask. The stock solutions were stored at 4°C until further use.

Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry conditions

UHPLC-QTOF-MS/MS analysis was based on previous methods with some modifications.[19] The content of six reference compounds of all *Aster* samples was determined using a UHPLC-PDA. A Waters Acquity UHPLC TM system (Waters Corporation, Milford, MA, USA) equipped with a binary solvent delivery manager, auto-sampler manager, thermostatically-controlled column compartment, and PDA was used. The separation was performed in an Acquity HSS T_3 chromatography column (100 mm × 2.1 mm, 1.8 μm; Waters, USA) coupled with a C_{18} pre-column (2.1 mm × 5 mm, 1.8 μm, Van-Guard™ BEH, Waters, USA). Formic acid in water (0.1% v/v, solution A) and acetonitrile (solution B) were used as the mobile phase. The UHPLC-QTOF-MS/MS analysis was performed using the Waters ACQUITY UHPLC system coupled with a Q-TOF mass spectrometer (XEVO G2-S, Waters Corp., Manchester, UK). Chromatographic analysis was performed with a flow rate of 0.4 mL/min by using the ACQUITY HSS T3 column (100 mm × 2.1 mm, 1.8 μm). With 0.1% (v/v) formic acid (a) and acetonitrile (b) as a mobile phase, the peak shape and the separation efficiency were improved. The UHPLC gradient elution was optimized as follows: 10%–20% of solution B (0–0.8 min), 20%–28% of solution B (0.8–4.0 min), 28%–38% of solution B (4.0–4.5 min), 38%–60% of solution B (4.5–5.5 min), 60%–95% of solution B (5.5–10.0 min), 95%–95% of solution B (10.0–12.5 min), 95%–10% of solution B (12.5–13.0 min), and 10% of solution B (13.0–15.0 min).

MS parameters were set as follows: Negative polarity scanning mode, desolvation gas flow rate of 800 L/h, and temperature of 350°C. The cone gas was 20 L/h, and the source temperature was 120°C. The capillary and the cone voltage were 2700 and 40 V, respectively. During MS analysis, it is necessary to use LockSpray to ensure mass accuracy and reproducibility. Leucine enkephalin was used as the reference compound ([M – H]-=554.2615) and continually introduced together with the LC stream for accurate mass calibration. The LockSpray frequency was set to 10 s, and the scan time was 0.20 s. Centroided data were acquired for each sample from 100 to 1500 Da with a 0.20 s scan time and a 0.01 s interscan delay over a 15 min analysis time. Data were acquired and processed using the MassLynx 4.1 software (Waters Corp., Manchester, UK).

Identification of chemical constituents of *Erigeron multiradiatus*

UHPLC-QTOF-MS/MS detected chemical profiling for the extracts of EM. The detected peaks were identified and temporarily characterized by comparison with reference compounds and previous studies regarding their retention times, UV spectra, molecular ions, fragment ions, and MS/MS fragmentation patterns.[19] The reference compounds were used for preliminary identification and elemental composition confirmation, which showed an *m/z* error of ≤3.0 ppm in the detected molecular ions. The mass spectra of molecular ions were investigated by correlation with the Chemspider, MassBank, and Metlin online databases for compounds matching previous documents for *Erigeron* species or their

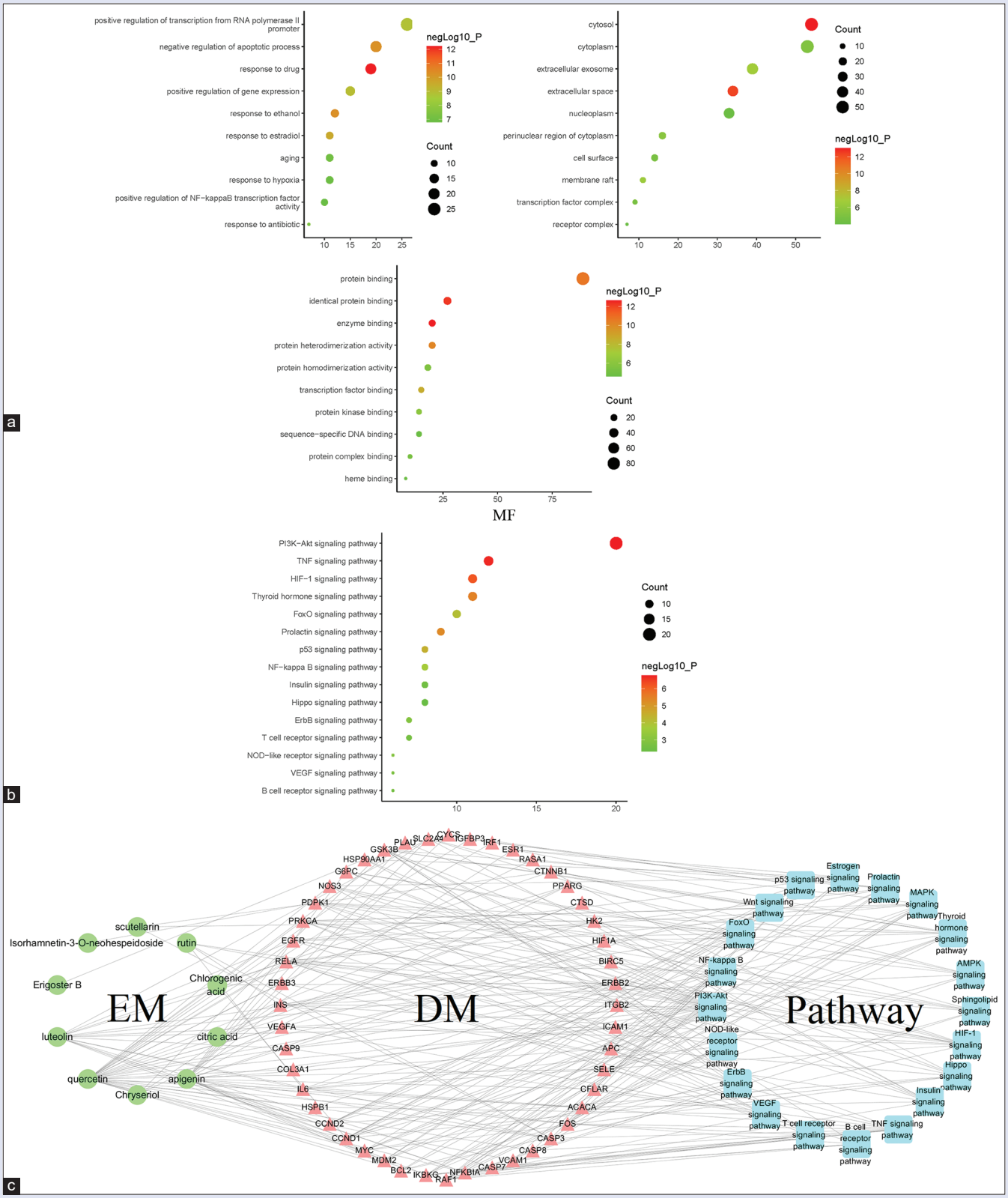

Figure 4: Gene ontology and Kyoto encyclopedia of genes and genomes enrichment analysis. (a) Gene ontology function enrichment analysis; (b) Kyoto encyclopedia of genes and genomes pathway enrichment analysis; (c) Ingredients-target-pathway network

relative species. The compound data were assembled using a protocol with automatic peak detection, peak filtration, artificial compound identification, compound alignment, and cross-species metabolite profile analysis.

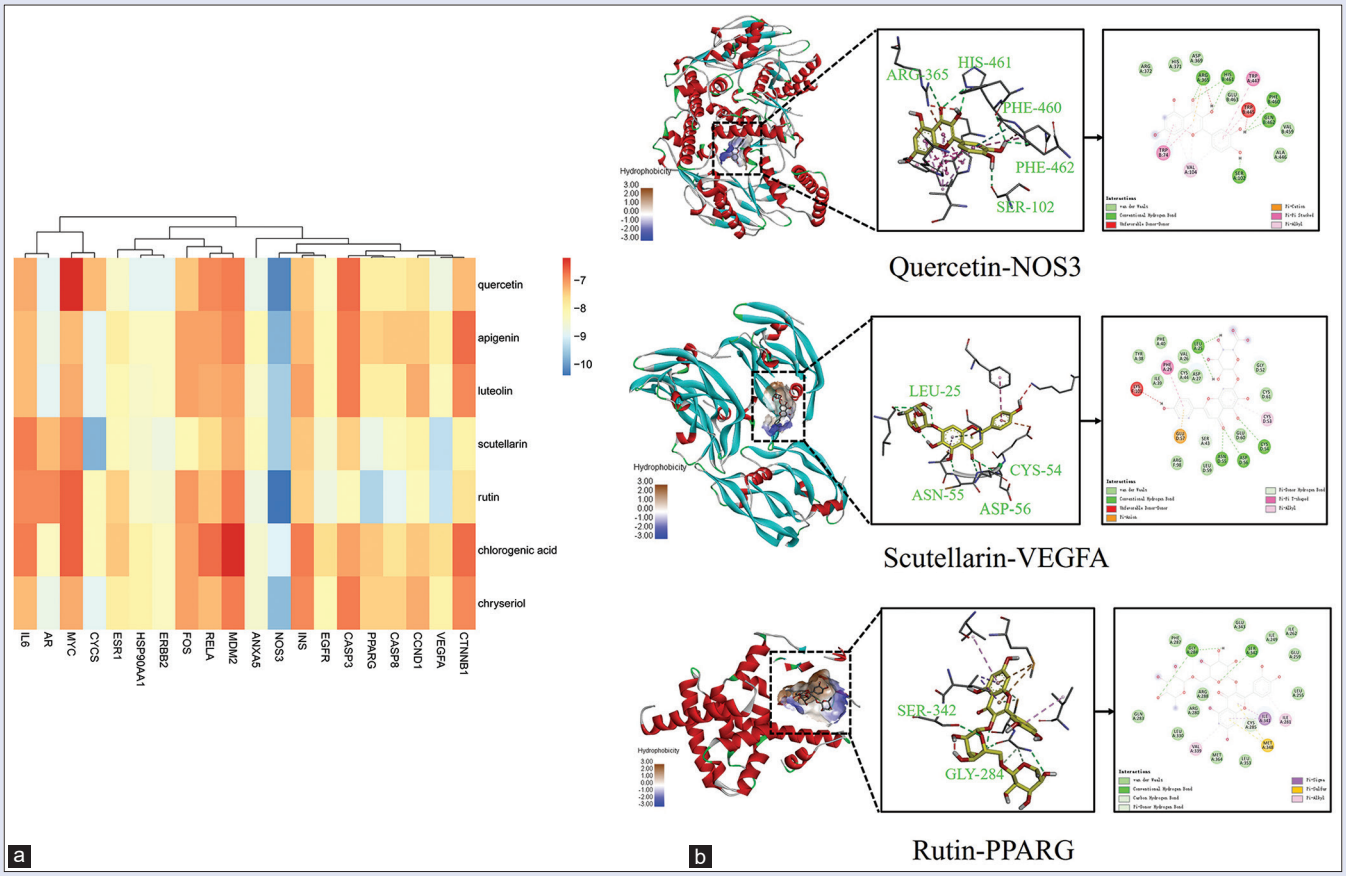

Figure 5: Molecular docking results. (a) Molecular docking heatmap; (b) Molecular docking mode of partial active ingredients

Component-targets and disease-targets collection

Collection of targets related to the identified compounds

From the constituents identified by UHPLC-QTOF-MS/MS, each active ingredient's relevant targets were identified from the following databases. (1) TCMSP (http://lsp.nwu.edu.cn/tcmsp.php),[20] (2) Pharmmapper (http://www.lilab-ecust.cn/pharmmapper/),[21] and (3) Swiss Target Prediction (http://www.swisstargetprediction.ch/).[22] Targets were converted into gene symbols using Uniprot (http://www.uniprot.org/), and gene symbols were combined.

Gene dataset collection of diabetic microangiopathy

In this study, "diabetic microangiopathy" was used as a keyword to search for relevant DM targets in the OMIM database (http://www.ncbi.nlm.nih.gov/omim) and GeneCards database (https://www.genecards.org/).

Construction of "Ingredients-Targets-Disease" network and protein–protein interaction network

Venny 2.1 (BioinfoGP, CNB-CSIC, http://www.liuxiaoyuyuan.cn/) was used to cross EM-related targets with DM-related targets and obtain the overlapping specifically expressed genes, which were then used as potential target-proteins for the treatment of DM.[23] The active ingredients and potential target data were imported into Cytoscape3.7.1 (University of California and Institute for Systems Biology, etc.; http://www.cytoscape.org/download.php),[24] to build an "ingredients-targets-disease" network.

In the network, the node indicates ingredients, targets, or diseases, while the edge indicates the interactions between these nodes. The degree, one fundamental topological parameter, is the number of edges associated with each node. It is evaluated by the plugin network analyzer of cytoscape, representing each node's importance in the networks.

The analysis pf PPI of the 107 target genes was undertaken using STRING11.0 (https://STRINGdb. org/), and the screening condition "Homo sapiens" was used. The results were imported into Cytoscape to analyze the protein interactions. The leading 20 target genes with higher levels of correlation were considered the core genes for DM.

Gene ontology and Kyoto encyclopedia of genes and genomes pathway enrichment analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) identifies the most significantly enriched biological annotations for genes or proteins.[25] The DAVID database was used to analyze the GO function and KEGG pathway enrichment ($P < 0.05$) of the PPI network proteins. The associated pathways and GO functions based on their enrichment were found and appropriately described. The output data of pathways, targets, and ingredients were imported into Cytoscape to construct a "pathways-targets-ingredients" network.

Molecular docking

The structural data of core target proteins were downloaded from the RSCB protein data bank for molecular docking. AutoDock Tools1.5.6 software (Molecular Graphics Laboratory, The Scripps Research Institute) was used to delete hydrogen in receptor protein–complex structures, separate the ligand and receptor moieties, add nonpolar hydrogens, calculate Gasteiger charges, and save the resulting

structures as pdbqt files.[26] The 3D structure file of core components with the largest degree parameter values was downloaded from PubChem, and then the spatial structure was verified in Pymol. The 3D structure was then loaded into the Autodock tool, atomic charges were added, atomic types were included, all flexible keys were enabled to rotate by default, and the data file was saved in pdbqt format as the docking ligand. The core target–proteins were considered receptors and the small molecular components as ligands. The active sites for molecular docking were determined according to the coordinates of ligands in the target protein–complexes. The Gridbox coordinates and size were set according to the active pockets of each receptor protein. AutoDock Vina was used for molecular docking, and the docking results enabled analysis and characterization of the core active components. THE co-crystallized compound or a standard drug of each core target-proteins was used as controls to provide the reference docking score.

Experimental verification

Cell culture

ECV304 cells, a spontaneously transformed line derived from the human umbilical vein endothelial cell, were purchased from the China Center for Type Culture Collection. The cells were maintained in an environment containing CO_2 at 37°C and grown on culture plates with 10% fetal bovine serum-supplemented Dulbecco's modified Eagle's medium. The MTT assay for cell survival assessment was performed as described by Mosmann,[27] and the cells with >95% viability were used for subsequent procedures.

Effects of quercetin, scutellarin, and rutin on the proliferation of vascular endothelial cells induced by hyperglycemia

The cells were inoculated at a density of 2×10^4 cells per well and allowed to adhere for 24 h. The cells were then starved in a serum-free medium for 24 h. The cells were divided into blank, high glucose, and high glucose + different concentrations of each test compound groups. Quercetin was tested at a concentration of 0.8 μmol/L, 8 μmol/L, and 80 μmol/L; scutellarin was tested at a concentration of 0.2 μmol/L, 2 μmol/L, and 20 μmol/L; and rutin was tested at a concentration of 0.3 μmol/L, 3 μmol/L, and 30 μmol/L. OD was read at 570 nm. MTT assay is a simple and accurate method to detect the survival and growth of cells. MTT participates in the energy metabolism of mitochondria of living cells, which is only decomposed by mitochondrial adenosine triphosphate of living cells, while dead cells without mitochondrial metabolism cannot metabolize MTT into blue formazan crystals. Within a certain range of cell numbers, the amount of MTT crystal is directly proportional to the number of cells. Therefore, the MTT assay was used to assess the proliferation of vascular endothelial cells in each sample group. Following steps were performed for MTT assay: (1) first, the cell supernatant was removed, and added 20 μL of MTT solution (5 mg/mL) was added to each well, incubated at 37°C for 4 h in a 5% CO_2 incubator. (2) After discarding the MTT solution, 150 μL of DMSO was added to each well and the plates were shaken and mixed well to dissolved the crystal formed. (3) Finally, the OD was quantitatively measured via enzyme-linked immunosorbent assay at 570 nm wavelength, and the OD reflected the cell proliferation level.

Effects of quercetin, scutellarin, and rutin on the nitrous oxide and glutathione of vascular endothelial cells induced with high glucose

The level of NO was determined using the nitrate reductase method.[28] GSH levels were measured using a Fluorescence spectrophotometric assay.[29] The OPT fluorescent agent is combined explicitly with GSH at pH 8.0 to form the fluorescent GSH-OPT. The fluorescence value of the latter compound has a linear relationship with GSH concentrations (16-100 μmol/mL), and the fluorescence spectrophotometer detects fluorescence overexcitation wavelengths of 488-505 nm and emission wavelengths of 515-575 nm. The cell treatment is the same as before. After the cells are broken, the supernatant is taken and diluted, and the OPT solution is added, mixed, and allowed to stand at room temperature for 30 min before testing.

Statistical analysis

Statistical analysis of each set of data was undertaken using a one-way analysis of variance (ANOVA), and Duncan's multiple range test using SPSS11.5 (SPSS Inc, USA), and P values <0.05 were considered statistically significant.

DISCUSSION

The Tibetan natural product EM has been used for many years to treat various diseases. EM and scutellarin showed significant therapeutic effects when administered for diabetes and diabetic-derived vascular disorders in our previous study.[30] However, as herbal medicines have multi-ingredient and multi-target nature, the pharmacological effects of specific underlying mechanisms are neither understood nor characterized. The network pharmacology strategy provides an innovative method for investigating the mechanisms between multi-components and multiple targets. UHPLC-QTOF-MS provides precise molecular and fragment ions of all the individual chemical constituents in the EM sample. The use of network pharmacology in combination with UHPLC-QTOF-MS for the first time enabled an investigation of potential quality markers and underlying biological mechanisms of EM when being used in the treatment of DM.

In this study, 26 main constituents were identified from EM using UHPLC-QTOF-MS. Correlation of potential targets for these 26 ingredients identified 107 common targets through integration with possible genes of DM, which may be EM targets when treating DM. INS, IL6, EGFR, CASP3, MYC, VEGFA, and other target proteins are potential core targets of DM by compounds within EM from the PPI network analysis. The "ingredients-targets" network also confirmed that EM elicits an anti-DM outcome through a multi-targets action. The GO enrichment results suggest that EM treats DM via regulation of BPs such as apoptosis and gene expression, CCs such as cytoplasm and extracellular space, and MFs such as enzyme binding and protein binding. The KEGG pathway enrichment results indicated that EM treats DM by regulating, for example, the PI3K-Akt signaling pathway, TNF signaling pathway, and HIF-1 signaling pathway. The molecular docking results and the "ingredients-targets" network infer that quercetin, scutellarin, and rutin potentially have biologically relevant binding affinities with several core targets. Therefore, quercetin, scutellarin, and rutin were selected as developmental candidates for more thorough experimental verification.

Hyperglycemia is a common feature of all types of diabetes mellitus and can cause changes in vascular endothelial metabolic pathways, structure, and function, resulting in a significant decrease or even disappearance of vascular endothelial cells.[31] Therefore, high glucose concentrations were used as a pathogenic factor on vascular endothelial cells (ECV-304 cell line) cultured *in vitro* to simulate endothelial dysfunction in the diabetic state. The influence of quercetin, scutellarin, and rutin on cell proliferation, NO release, and GSH levels induced by high glucose were investigated. Both quercetin and scutellarin have different resistance responses to high glucose levels inhibiting the proliferation of vascular endothelial cells. Moreover, quercetin and scutellarin have varying degrees of reducing NO and GSH levels in vascular endothelial cells stimulated by high glucose levels. These results infer that quercetin and scutellarin play an essential role in EM clinical outcomes when used to

treat DM. Furthermore, previous studies suggested that scutellarin shows a protective anti-inflammatory effect on vascular inflammation induced by hyperglycemia under *in vitro* and *in vivo* conditions.[17] Finally, due to the high content of scutellarin in EM, it should serve as a Q-marker of EM.

Oxidative stress has been shown to compromise the two major failing mechanisms during diabetes: Insulin secretion and insulin action.[32] Previous studies have demonstrated that insulin resistance is inextricably linked to oxidative stress.[33] The PPI analysis predicted that INS is a crucial protein target of EM when used to treat DM. EM may play a role in treating DM by acting on INS to regulate oxidative stress. Moreover, the pathological products of the oxidative stress response can act on retinal cells, which may cause abnormal expression of caspase-3 protein in retinal cells and induce apoptosis, mediating programmed cell death of retinal cells.[34] Our results indicate that the clinical outcome of DM after treatment with EM is mediated via CASP3 through the oxidative pathway. In addition, our results confirm that quercetin and scutellarin are effective against hyperglycemia-induced oxidative stress in endothelial cells, indicating that EM has an antioxidant defense mechanism. The KEGG analysis identified PI3K/AKT, TNF, and HIF-1, as well as other signaling pathways that have been reported to be related to oxidative stress and diabetes.[35-37] EM may play an antioxidative defense mechanism by regulating these signaling pathways, and subsequent experiments will verify this hypothesis. In addition, research has shown that the increase in the level of intracellular glucose in diabetes leads to the overproduction of adhesion molecules, further causing leukocyte-endothelium adhesion, which is considered one of the earliest vascular inflammatory events.[17]

PPI network analysis suggested that interleukin-6 (IL-6) is also a vital EM target when treating DM. IL-6 is associated with many intraocular inflammatory diseases such as macular edema and diabetic retinopathy via acting as a pro-inflammatory cytokine.[38] Previous studies have reported that increased levels of IL-6 generally exist in patients with diabetic retinopathy, potentially correlating to the severity of diabetic retinopathy.[39] Previous studies have suggested that scutellarin has a specific protective effect against vascular inflammation induced by hyperglycemia under *in vitro* and *in vivo* conditions. Furthermore, a previous study has reported that scutellarin treats DM through regulation of the NF-κB signal pathway.[13] In this study, KEGG pathway analysis infers that EM regulates the NF-κB signal pathway, confirming previous predictions. Finally, essential pathogenesis stages of DM include insulin resistance, hyperglycemia, and diabetes. A previous study has reported that EM has aldose reductase inhibitory activity, thereby improving hemodynamic behavior and protecting heart function.[13] Therefore, EM not only for treating DM but should also be considered as an antidiabetic medicine. Overall, the results of this study suggest that EM acts via multiple diabetes-related targets (e.g., INS and IL-6) and regulates multiple diabetes-related signaling pathways (e. g., PI3K-Akt, AMPK, and Wnt) to treat DM thereby improving the symptoms of diabetes, which is worthy of further substantial investigations.

CONCLUSION

In this study, we performed combined chemical ingredient analysis, target predictions, network analysis, and experimental validations to determine the potential active constituents and underlying biological mechanisms for EM's clinical outcomes when used to treat DM. Seven active ingredients were screened by molecular docking. And quercetin, scutellarin, and rutin could more easily bind to the core targets and were further investigated on vascular endothelial cells. The results confirmed that scutellarin and quercetin played an important role in treating DM and can be considered the Q-markers of EM. For the first time, this study provides scientific empirical evidence for the mechanisms of the clinical outcomes of the Tibetan medicine, EM. However, the collected disease targets and drug targets were based on databases and previous literatures, which may be incomplete or incorrect. Therefore, further target verification and animal experimental verification are necessary in the future.

Author's contributions

Zhifeng Zhang designed the experiments; Amu Hamamozhi, Xueyan Su, and Jing Li performed the experiments and wrote the paper; Wenfu Cai, Riza Zhao and Jing Li built and analyzed the database of chemical components of EM; Jing Li performed the network pharmacology analysis; All authors participated in the preparation of the manuscript and approved the final manuscript.

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Conflicts of interest

There are no conflicts of interest.

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