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Untargeted metabolomics reveal the corrective effects of scorpion on epileptic mice

Lele Li[1], Shengyu Ge[1], Yang Wang[2]✉, Heyun Zhu[1]✉ & Bo Feng[1]✉

Scorpion is a commonly used drug in traditional Chinese medicine for treating epilepsy, although the exact mechanisms are not yet fully understood. This study aimed to compare the treatment effects of Scorpion water extract (SWE) and Scorpion ethanol extract (SEE) on mice with pentetrazole-induced epilepsy and investigate the possible mechanisms through metabolomics methods. A pentetrazole-induced epileptic mice model was used to assess the corrective effects of SWE and SEE. Untargeted metabolomics, utilizing UPLC-Q-TOF/MS, was employed to analyze the metabolic profiles of mice and identify metabolic changes following scorpion treatment. The results revealed that only SWE showed therapeutic effects in epileptic mice. Metabolomics analysis demonstrated significant alterations in metabolic signatures between the pentetrazole-induced epileptic mice and SWE groups. By utilizing orthogonal partial least squares discrimination analysis, 44 and 108 potential biomarkers in mouse serum were identified in positive and negative ion modes, respectively. Differential metabolites related to epilepsy were then used to pinpoint relevant pathways in epileptic mice, such as linoleic acid metabolism, biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, and ether lipid metabolism. In conclusion, this study highlights the corrective effects of Scorpion on epileptic mice and provides insight into the underlying metabolic pathways involved in its efficacy.

Keywords Scorpion, Untargeted metabolomics, UPLC-Q-TOF/MS, Epilepsy, Metabolic pathways, Metabolites

Epilepsy is a chronic neurological disorder characterized by a persistent tendency to experience seizures[1]. According to the World Health Organization (WHO), epilepsy contributes to about 1% of the global disease burden, ranking fourth among neuropsychiatric disorders after depression, alcoholism, and cerebrovascular disease, with a similar impact to breast and lung cancer[2]. It affects over 65 million individuals worldwide, with causes ranging from acute brain injuries and genetic mutations to metabolic disorders and autoimmune conditions[3,4]. The social, cognitive, and economic burdens of epilepsy are substantial, making the development of effective treatments a key focus in epilepsy research.

Traditional Chinese medicine (TCM) has shown promising efficacy in managing epilepsy with minimal side effects[5–7]. The dried body of *Buthus martensii* Karsch, known as Scorpion, is frequently used in China for epilepsy treatment[8–10]. Nevertheless, the precise mechanisms through which Scorpion alleviates epilepsy symptoms remain unclear. Metabolomics has emerged as a powerful tool for identifying disease-related biomarkers and potential drug targets across various stages of illnesses[11,12]. Metabolomics plays a crucial role in uncovering the corrective mechanisms of Chinese medicine, particularly in recent TCM research[13]. A widely adopted technique, UPLC-Q-TOF/MS[14], known for its high resolution and sensitivity, was employed in our study to investigate these mechanisms.

Specifically, we aimed to analyze how Scorpion treats epilepsy. We induced an epileptic mice model using pentetrazole. Using UPLC-Q-TOF/MS for untargeted metabolomics, we identified potential biomarkers and assessed the overall metabolic changes induced by Scorpion in epileptic mice. This research contributes to a deeper understanding of Scorpion's corrective mechanisms in epilepsy treatment.

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Materials and methods

Chemical reagents and materials

HPLC-grade acetonitrile, methanol, and formic acid were purchased from TEDIA (Fairfield, OH, USA). Ultrapure water was processed by the Milli-Q water purification system (Millipore, Bedford, USA). Ethanol was obtained from Beijing Shiji (Beijing, China). Pentetrazole (PTZ) was purchased from Aladdin (Shanghai, China). Scorpion was purchased from a local market in 2023.

Preparation of Scorpion extracts

Ten g of Scorpion was crushed and sieved through a No. 20 mesh. It was then added to 500 ml of water and boiled for 40 min. After filtering by vacuum suction filtration, the residue was subjected to the same process: adding another 500 ml of water and boiling it for another 40 min for extraction. The filtrate was combined and concentrated using rotary evaporator at 50 ℃ before being freeze-dried to obtain the scorpion water extract (SWE).

Ten g of Scorpion was crushed and sieved through a No.20 mesh. It was then added to 500 ml of 50% ethanol solution. Extraction was performed using a magnetic mixer under 50 ℃ and 1000 rpm for 12 h. After filtering by vacuum suction filtration, the residue was subjected to the same conditions for another extraction. The filtrates were combined and concentrated using rotary evaporator at 50 ℃ before being freeze-dried to obtain the scorpion ethanol extracts (SEE).

Animals and drug administration

Twenty-seven male Kunming mice weighing 20–22 g were supplied by the Experimental Animal Center of Jilin University. All animal experiments and husbandry have been carried out under the guidelines of the Animal Ethics Committee of Jilin Medical University (Approval Code: 20230126, Approval Data: Match 10th, 2023). All experimental methods were performed in accordance with the ARRIVE guidelines (https://arriveguidelines.org), guidelines of the Chinese Institution of Laboratory Animal Sciences (https://cnilas.org/en/).

Twenty-seven mice were divided into the model group (n = 10), SWE group (n = 8), and SEE group (n = 9). Every morning, mice in the SWE and SEE groups were orally administered the corresponding Scorpion extracts once at a dosage of 1g/kg[15,16]. The control group was given an equal amount of physiological saline. Administration was carried out continuously for 7 days. Thirty min after final administration on day 7, pentetrazole (80 mg/kg) was injected to induce acute epileptic seizures in mice. After injecting pentetrazole, the mice were immediately placed into transparent compartments marked with designated groups, and the camera was turned on to record the seizure activity of all mice within the compartments for 30 min. According to the Racine scale[17,18] (Grade 0, no convulsion observed,Grade I, head nodding, eye blinking, neck movement, salivation, or wet dog-like tremor,Grade II, rhythmic head nodding,Grade III, head clonus with forelimb tremor; Grade IV, Grade III with hindlimb tonic–clonic movements; Grade V, generalized tonic–clonic seizure with falling), we recorded the Grade V seizures of each mouse, calculated the seizure frequency, and analyzed the effect of the scorpion venom extract on mouse seizures. The seizure frequency data were further processed by Student's *t*-test (SPSS19.0, Chicago, IL, USA) to get p value. And $p < 0.05$ indicates significant differences between groups.

Sample collection and preparation

Whole blood samples were collected after recording seizures in mice. Blood was collected in an Eppendorf tube and then centrifuged at 2000×*g* for 10 min at 4 °C to obtain the serum. Methanol (400 µL) was added to the serum sample (100 µL) for protein precipitation. After vortex-mixing for 1 min and centrifuging at 12,000×*g* for 10 min, the supernatant was separated and lyophilized. Residues were re-dissolved in acetonitrile–water mixture (1:1, v/v) (100 µL) and centrifuged at 12,000×*g* for 15 min at 4 °C. The supernatant was subjected to UPLC-QTOF/MS analysis. A pooled "quality control" (QC) sample was prepared by mixing equal aliquots (10 µL) from all prepared serum samples. All prepared samples were stored at 4 °C.

UPLC-Q-TOF/MS analysis

Chromatographic separation was performed using a Shimadzu LC20AD ProminenceTM UPLC system equipped with a Thermo Fisher Golden C18 column (2.1 × 50 mm, 1.9 µm) maintained at a temperature of 35°C. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 0.1% formic acid in ACN. The gradient for mobile phase B was programmed as follows: 5% (0 to 1 min), 5–35% (1 to 14 min), 35–95% (14 to 20 min), 95% (20 to 22 min), and 95–5% (22 to 22.1 min). The system was then equilibrated at 5% for 2.9 min. The injection volume was set to 5.0 µL, and a 0.3 mL/min flow rate was employed.

Q-TOF/MS analysis was conducted on a Triple-TOF 5600 + MS (SCIEX, Concord, Canada) with an ESI source. The MS parameters were as follows: positive or negative ion mode, source temperature: 500 ℃, ion spray voltage: + 5500 or − 4500 V, nebulizer gas (N2): 55 psi, heater gas (N2): 60 psi; curtain gas (N2) of 35 psi, and declustering potential: 100 V. Full-scan MS data were acquired from m/z 100 to 1000 in TOF/MS mode with collision energy 5 eV. The MS/MS data were acquired in IDA mode using the following settings: collision energy of 35 eV, rolling collision energy with a spread of 15 eV, the mass range of precursors were from m/z 100 to 1000, and the mass range of MS/MS spectra were from m/z 100 to 1000.

Data analysis and identification of potential biomarkers

After UPLC-Q-TOF/MS analysis, the raw data obtained from the model group, SWE group, and QC samples were imported into MS-DIAL software 4.10 (http://prime.psc.riken.jp/) to perform data preprocessing, including peak identification, peak matching, and peak alignment of retention time and mass. The peak alignment was performed using a reference file (QC data). The retention time tolerance was set at 0.1 min, and the MS1 tolerance was set at 0.01 Da. The data collection parameters were as follows: an MS1 tolerance of 0.01 Da and an

MS2 tolerance of 0.02 Da. The peak detection parameters were a minimum peak height of 3000 amplitude and a mass slice width of 0.1 Da.

The resultant dataset, containing m/z value, retention time, the normalized intensity, and the sample code, was used to perform the multivariate statistical analysis. Then, the dataset was saved as .csv files and imported into SIMCA software 13.0 (Umetrics, Umea, Sweden) to conduct the orthogonal partial least squares-discriminant analysis (OPLS-DA). In the OPLS-DA model, ions with variable importance in projection (VIP) 1 values larger than 1 were highlighted and were further filtered by Student's t-test (SPSS19.0, Chicago, IL, USA). The metabolites with $p < 0.05$ were considered significant and were selected as potential biomarkers. After screening for the accurate molecular weight and fragment information of the candidate variable fragments from MS-DIAL software 4.10, biomarkers were identified. MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) was used to identify metabolic pathways.

Results

Effects of Scorpion on epileptic mice

As shown in Fig. 1, the number of seizures in the model, SWE, and SEE groups were 2.63 ± 1.32 (n = 8), 1.00 ± 1.19 (n = 10), and 2.22 ± 1.23 (n = 9), respectively. Compared with the model group, the number of seizures in the SWE group was significantly lower. Moreover, there was no significant difference in the number of seizures between the model and SEE groups. The results indicated that only SWE showed good effects on epileptic mice, and the extraction method played an important role in the pharmacological function of Scorpion. Therefore, the serum samples from the SWE group were used in the following metabolomics study.

Metabolomic analysis

To systematically elucidate the mechanisms by which SWE ameliorate epilepsy, serum samples were analyzed using UPLC-Q-TOF/MS in both positive and negative ion modes. Representative total ion chromatograms (TIC) of serum samples from both the model and SWE groups were obtained under optimal conditions (Fig. 2). After data preprocessing, 880 and 655 peaks were detected in positive and negative ion modes, respectively.

Before statistical analysis, method validation was performed to evaluate the repeatability and stability of this established method. A QC sample was analyzed three times at the beginning of an analytical run and then once every five samples to provide robust quality assurance for each peak. The repeatability of the established methods was evaluated with the relative standard deviation (RSD) of the peaks in 7 QC samples. More than 80% of ions were detected in a QC sample with an RSD of less than 20% for positive and negative ion modes, indicating the excellent repeatability and stability of the present methods (Fig. S1). There were 1 positive ion peak and 8 negative ion peaks which has high RSD (more than 30%) between QC samples. And the peaks with high RSD had been excluded before OPLS-DA.

To evaluate the systemic changes of the metabolome in epileptic mice treated with SWEs, OPLS-DA was used to explore the tendency of metabolic profiles between the model and SWE groups. A positive ion OPLS-DA model was established with R^2 (Y) = 0.9581 and Q^2(cum) = 0.8670, suggesting that the established model had prominent fitness and predictability. Similarly, the negative ion OPLS-DA model had prominent fitness and predictability (R2 = 0.9354 and Q2 = 0.9127). Permutation test (n = 200) was further performed to validate the model. No overfitting was found because the permutated R2 and Q2 values on the left are lower than the original point on the right (Fig. S2). As shown in Fig. 3A,B, all samples were within the Hotelling T2 (0.95) ellipse, and the OPLS-DA sore plot suggested that clear separations from the model and SWE groups were observed in both positive and negative ion modes. The excellent separation between the SWE group and model groups in the OPLS-DA score plots indicated that the SWE group had completely different metabolic profiles compared with the model group.

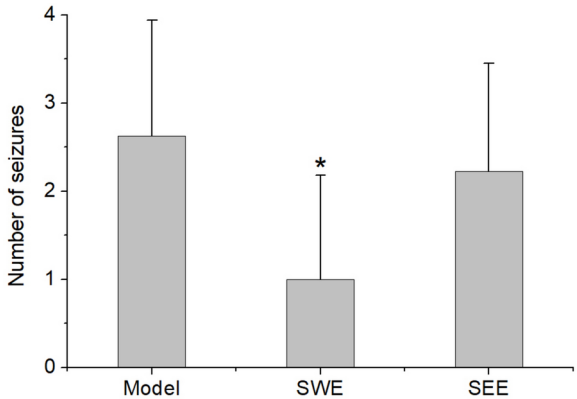

Fig. 1. Number of seizures in the model, SWE, and SEE groups. *Significant differences exist compared to the model group, $p < 0.05$.

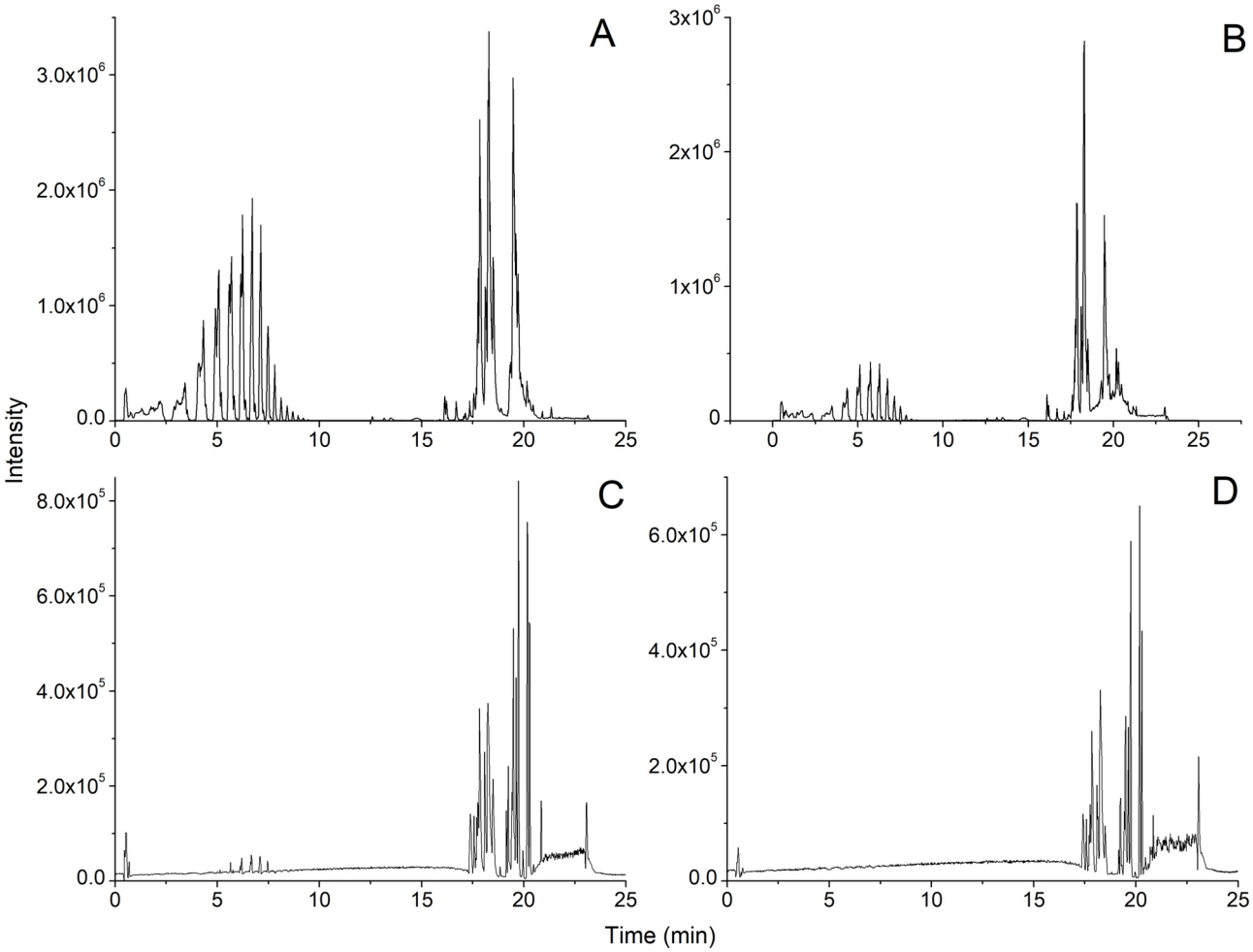

Fig. 2. Representative total ion chromatograms (TIC) of the (**A**) model and (**B**) SWE groups based on positive ion mode. Representative TIC of the (**C**) model and (**D**) SWE groups based on negative ion mode.

Identification of potential endogenous biomarkers

To identify key metabolites distinguishing between the model and SWE groups, OPLS-DA was employed to generate S-plots (Fig. 3C,D). These plots visualize covariance and correlation relationships from the model, along with Variable Importance in Projection (VIP) values. This approach reduces false positives in metabolite selection. Initially, features with VIP greater than 1 were selected as significant variables, further screened using Student's t-test to identify metabolites significantly differing between groups. Finally, total 154 ions (46 from positive and 108 from negative) with $p < 0.05$ were retained as potential biomarkers, highlighted in red on the S-plot (Fig. 3C,D).

Next, the selected biomarkers were identified based on the MS and MS/MS information recorded in the MS-DIAL metabolomics database (https://systemsomicslab.github.io/compms/msdial/main.html#MSP). The ion features with statistical significance ($p < 0.05$ and VIP > 1.0) were identified and are summarized in Table 1. Specifically, compared to the model group, the SWE-treated mice exhibited altered levels in a total of 19 metabolites, all of which were found to be decreased (Fig. 4).

Analysis of metabolic pathway of potential biomarkers

Topology and pathway enrichment analyses were conducted to assess the role of metabolites in biological reactions based on their positions within related pathways and to identify key metabolomic pathways. All identified potential biomarkers were analyzed using MetaboAnalyst for metabolic pathway analysis, presented through an interactive visualization system (Fig. 5A,B). The results of metabolic pathway enrichment and topological analysis indicated that treatment of PTZ-induced epileptic mice primarily affected metabolic pathways such as linoleic acid metabolism, biosynthesis of unsaturated fatty acids, glycerophospholipid metabolism, and ether lipid metabolism. These pathways are interconnected, as illustrated in Fig. 6 and Table S1. The corrective effects of SWE on epileptic mice were exerted by reducing the levels of ten metabolites, including stearic acid, palmitic acid, docosahexaenoic acid, eicosapentaenoic acid, gamma-linolenic acid, arachidonic acid, linolenic acid, lysophosphatidylcholine 18:1, 1-stearoyl-sn-glycero-3-phosphocholine, and phosphatidylcholine lyso 17:0.

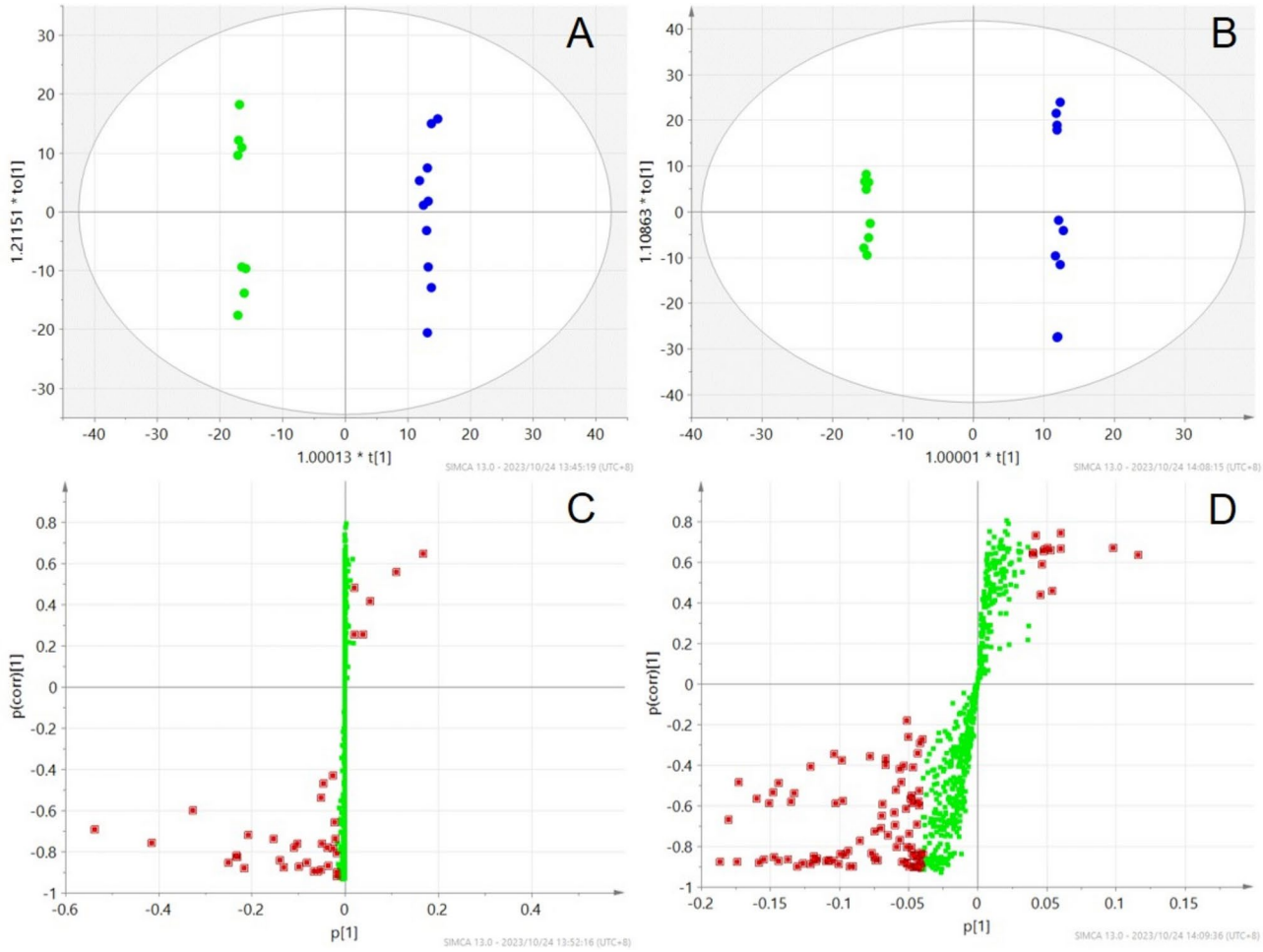

Fig. 3. Orthogonal partial least squares discrimination analysis (OPLS-DA) model score plot of samples based on (**A**) positive ion and (**B**) negative ion modes, respectively. The corresponding loading plot is based on (**C**) positive ion and (**D**) negative ion modes, respectively.

Discussion

Scorpion is a type of animal-derived traditional Chinese medicine, commonly used in clinical practice as a decoction and sometimes prepared as a tincture. According to the results of this study, SWE have a certain corrective effect on epileptic mice, while SEE show no significant corrective effect. When clinically using Scorpion to treat epilepsy, more consideration may be given to its use as a decoction.

Epilepsy is a chronic condition characterized by recurrent seizures resulting from abnormal neuronal activity in the brain. This abnormal activity can be caused by neuronal overexcitation or dysfunctional coordination of neuronal firing. The etiology of epilepsy involves various factors, including genetic predisposition, brain injury, metabolic abnormalities, and others[19–21]. Recent studies reveal that epilepsy might be induced by a metabolic etiology and epileptic seizures also contribute to changes of small endogenous metabolites[22], Ingrid E Scheffer et al., 2017). A report about children with drug-refractory epilepsy indicated that the top two enriched metabolic pathways involved in the drug-refractory epilepsy condition were the biosynthesis of unsaturated fatty acids and linoleic acid metabolism, and a decrease in fatty acids and an increase in triglycerides were associated with the response to anti-seizure medications therapy[23]. Similarly, our study showed that the Scorpion's ability to treat epileptic mice was also related to these two metabolic pathways by decreasing the levels of stearic acid, palmitic acid, docosahexaenoic acid, eicosapentaenoic acid, gamma-linolenic acid, arachidonic acid, linolenic acid. The biomarkers stearic acid, palmitic acid, docosahexaenoic acid, and eicosapentaenoic acid have been identified in cerebrospinal fluid, as previously reported[24,25]. The observed fluctuation in the levels of these four metabolites within both serum and cerebrospinal fluid post-SWE treatment in epileptic mice indicates potential correlations. However, a deeper understanding of the true connections between these serum and cerebrospinal fluid variations and the specific changes in these four metabolites within the cerebrospinal fluid remains a subject for further investigation.

Additionally, the study failed to pinpoint the distinct ways SWE and SEE regulate the metabolism of epileptic mice, nor did it identify the differing components of the two extracts. The differences in the chemical composition of SWE and SEE, and how their related active compounds impact the metabolism of epileptic mice, will be explored in our future research.

No	Metabolites	Ion Mode	RT(min)	m/z	Molecular formula	Adduct ions	Mass error (ppm)
1	1-Stearoyl-sn-glycero-3-phosphocholine	Positive	23.051	524.3703	$C_{26}H_{54}NO_7P$	$[M+H]^+$	− 1.53
2	Lysophosphatidylcholine 18:2	Positive	17.857	520.339	$C_{26}H_{50}NO_7P$	$[M+H]^+$	− 1.54
3	(2R,2'R,4a'S,6'S,7'R,8a'S)-4,6',7'-Trihydroxy-2',5',5',8a'-tetramethyl-3',4',4a',5',6',7,7',8,8',8a'-decahydro-2'H-spiro[furo[2,3-e]isoindole-2,1'-naphthalen]-6(3H)-one	Positive	5.162	402.2301	$C_{23}H_{31}NO_5$	$[M+H]^+$	6.46
4	2-decyl-3-hydroxypentanedioic acid	Positive	7.226	311.1856	$C_{15}H_{28}O_5$	$[M+Na]^+$	8.68
5	Aspartate conjugated cholic acid	Positive	5.946	524.3167	$C_{28}H_{45}NO_8$	$[M+H]^+$	− 9.73
6	Methionine conjugated cholic acid	Positive	17.918	540.3309	$C_{29}H_{49}NO_6S$	$[M+H]^+$	− 8.14
7	2',3'-Dideoxythymidine	Positive	5.654	227.1048	$C_{10}H_{14}N_2O_4$	$[M+Na]^+$	9.68
8	Methionine conjugated chenodeoxycholic acid	Positive	17.441	524.3428	$C_{29}H_{49}NO_5S$	$[M+H]^+$	4.58
9	Lysophosphatidylcholine 18:1	Positive	18.506	522.3555	$C_{26}H_{52}NO_7P$	$[M+H]^+$	0.19
		Negative	18.50	566.3475	$C_{26}H_{52}NO_7P$	$[M+HCOO]^-$	2.12
10	Lysophosphatidylcholine 16:0	Negative	18.271	540.3295	$C_{24}H_{50}NO_7P$	$[M+HCOO]^-$	− 2.22
11	Linoleic acid	Negative	19.742	279.2316	$C_{18}H_{32}O_2$	$[M-H]^-$	− 5.01
12	Phosphatidylcholine lyso 17:0	Negative	19.436	568.363	$C_{25}H_{52}NO_7P$	$[M+CH3COO]^-$	1.76
13	Palmitic acid	Negative	20.181	255.2331	$C_{16}H_{32}O_2$	$[M-H]^-$	0.39
14	Gamma-Linolenic acid	Negative	19.242	277.2187	$C_{18}H_{30}O_2$	$[M-H]^-$	5.05
15	Docosahexanoic acid	Negative	19.486	327.2309	$C_{22}H_{32}O_2$	$[M-H]^-$	− 6.42
16	Phosphatidylcholine lyso 16:0	Negative	18.836	554.3477	$C_{24}H_{50}NO_7P$	$[M+CH3COO]^-$	2.53
17	Arachidonic acid	Negative	19.625	303.2332	$C_{20}H_{32}O_2$	$[M-H]^-$	0.66
18	Stearic acid	Negative	20.861	283.2648	$C_{18}H_{36}O_2$	$[M-H]^-$	1.77
19	Eicosapentaenoic acid	Negative	19.156	301.217	$C_{20}H_{30}O_2$	$[M-H]^-$	1.00

Table 1. Serum biomarkers for Scorpion treatment of epileptic mice.

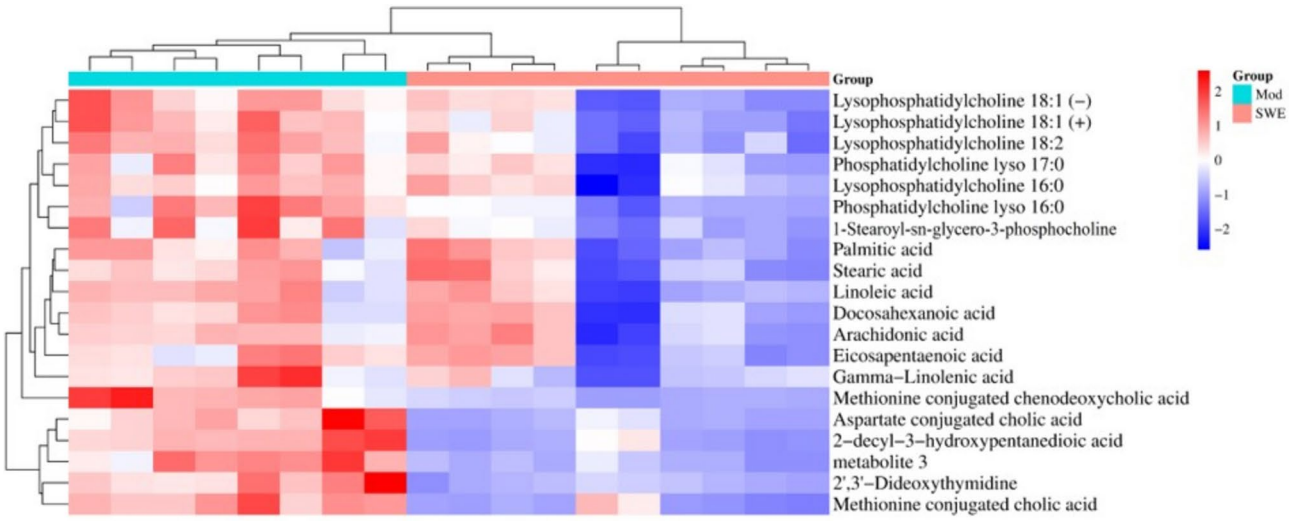

Fig. 4. Hierarchical clustering heat map of the differential metabolites with the degree of change marked in red (up-regulation) and blue (down-regulation).

Conclusion

The study focused on the effects of Scorpion on epileptic mice, with results demonstrating significant corrective effects specifically from SWE treatment. An untargeted metabolomic analysis using UPLC-Q-TOF/MS was then implemented to investigate the serum metabolome profiles in epileptic and SWE-treated mice systematically. This approach aimed to uncover the corrective effects of SWE on metabolic imbalances associated with epilepsy. The application of OPLS-DA further elucidated the action mechanisms of SWE, leading to the identification of 19 potential biomarkers in serum samples linked to metabolic pathways such as linoleic acid metabolism. In conclusion, our research offers a comprehensive analysis of SWE's corrective effects in epilepsy, providing insights into metabolic abnormalities and revealing the corrective mechanisms of animal-derived Traditional Chinese Medicine at a holistic level.

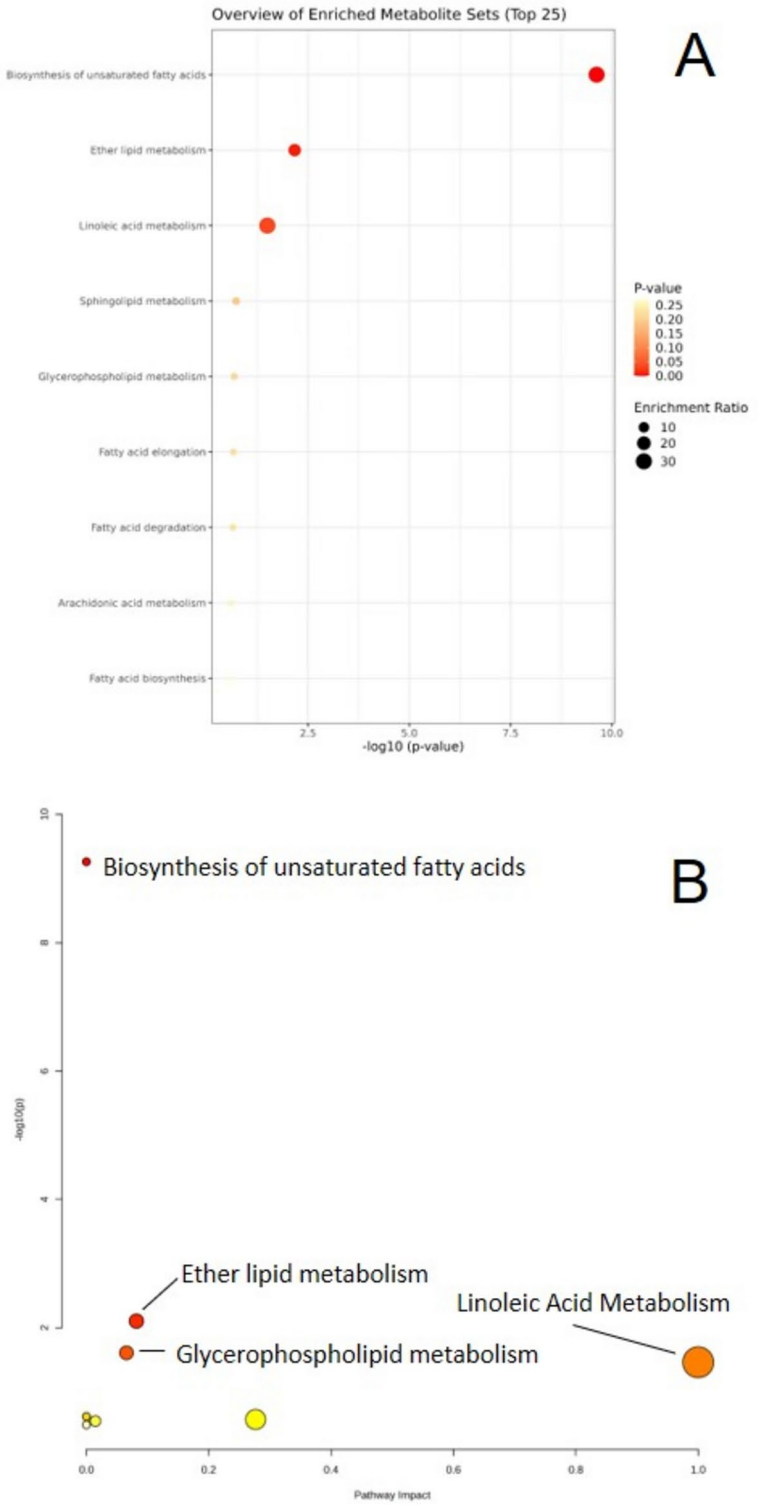

Fig. 5. Pathway analysis of significantly altered metabolites for epilepsy. (**A**) Visual analysis of enrichment pathway of altered metabolites. (**B**) Pathway analysis of typical metabolites in response to epilepsy.

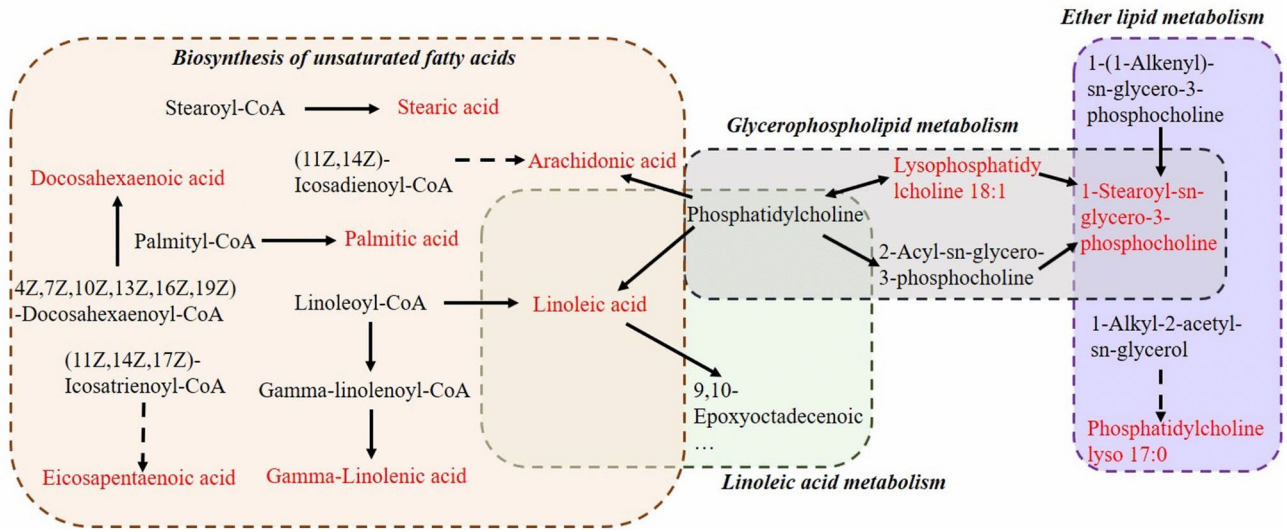

Fig. 6. The network is based on the potential biomarkers changing for Scorpion modulation according to the KEGG PATHWAY database.

Data availability

The data supporting the findings of this study are available within the article. Additional datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

L.Z. data collection and analysis; G.W. conception and design; W.S. material preparation; X.M. review the manuscript.

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Competing interests

The authors declare no competing interests.

Ethical approval

The Animal Ethics Committee of Jilin Medical University (Approval Code: 20230126, Approval Data: Match 10th, 2023) reviewed and approved this animal study. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org) and guidelines of the Chinese Institution of Laboratory Animal Sciences (https://cnilas.org/en/).

Additional information

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