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Molecular targets and mechanism of Longji Xiaozhong ointment in treating acute ankle sprain: A network pharmacology based analysis

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Acute ankle sprain is one of the most common lower limb injuries particularly in athletes and it accounts for 16-40% of all sports-related injuries. A significant proportion of patients experience persistent residual symptoms and recurrence. Traditional Chinese medicine (TCM) offers effective treatment in terms of pain relief, swelling reduction, functional improvement, and shorter disease duration. This study provides a comprehensive analysis of the active ingredients, molecular targets, signaling pathways, and Longji Xiaozhong ointment's clinical efficacy for treating acute ankle sprains. We followed network pharmacology approach to identify the molecular targets and mechanism of action of Longji Xiaozhong ointment in treating acute ankle sprain and to validate its clinical value using relevant clinical data. Initially, Longji Xiaozhong Ointment's active ingredients were screened using the TCMSP platform based on drug properties similar to DLP ≥0.18 and oral bioavailability OB ≥30%. The molecular targets were identified from the ETCM database, followed by constructing protein-protein interaction (PPI) network diagrams and GO and pathway enrichment analysis on key genes. Furthermore, the UniProt database was used to investigate the 3D structures of these key genes. Finally, the clinical data of 111 patients with acute ankle sprains were retrospectively analyzed and further explored by comparing the different curative effects of Longji Xiaozhong ointment with Celebrex and Shexiang Analgesic ointment to check the clinical value of Longji Xiaozhong ointment in treating acute ankle sprain. In the TCMSP database, we found that there were 42 active ingredients in Longji Xiaozhong ointment, including 13 kinds of *Strychnosnux-vomica* L., 7 kinds of *Ligusticum chuanxiong*, and 22 kinds of Safflowers (*Carthamus tinctorius*), and a total of 3206 target genes were obtained. After applying the OB ≥50% criteriaand DL ≥0.5, 19 key target genes were selected based on their correspondence to the active ingredients. Protein mutual aid network construction and module analysis yielded two high-scoring Clusters and identified seven key proteins, including SLC6A4, ADRB2, ADRA1B, CHRM1, F2, OPRM1, and OPRD1. Functional enrichment analysis (FEA) of candidate target genes of *Strychnosnux-vomica*, *Ligusticum chuanxiong*and Safflowers in Longji Xiaozhong ointment in ETCM database showed SUMOylation of intracellular receptors, blockade of NMDA receptors, and activation of GABA A receptors, lipid metabolism regulationby peroxisome proliferator-activated receptor alpha (PPARα). PPARA activates aspects of gene expression and transcriptional activation of mitochondrial biogenesis. On retrospective analysis, all patients were divided into Group A: Celebrex combined with brace immobilization group (36 cases); Group B: Shexiang Jietong ointment combined with brace immobilization group (37 cases); and Group C: Longji Xiaozhong ointment combined with brace immobilization group (38 cases). After treatment, the VAS score, swelling degree and scope score of Longji Xiaozhong ointment combined with brace immobilization group were significantly lower than those of the other two groups, and the effect was significantly better in terms of daily activities, sports, quality of life and occurrence of re-sprain than the other two groups. Our findings, supported by clinical data, demonstrate that Longji Xiaozhong ointment effectively alleviates swelling and pain, accelerates the repair of ankle ligaments, enhances ankle joint function, and improves ankle joint stability.

Keywords: *Carthamus tinctorius*, Gene expression map, *Ligusticum chuanxiong*, Nux vomica, Quaker buttons, Safflowers, Semen strychnos, Strychnine tree, *Strychnosnux-vomica*, Traditional Chinese medicine

Acute ankle sprain is a common lower limb injury, particularly in athletes which accounts for about 16-40% of all sports-related injuries[1]. Improper treatment can easily produce a variety of sequelae[2]. Ankle sprains not only have a huge impact on people's normal work and life, but also generate considerable health care costs and socioeconomic costs[3]. Although ankle joint fixation helps in repairing ankle joint injuries, it cannot speed up the repair process and there is no evidence that it restores the tension of the ankle joint[4,5]. The latest evidence-based medical evidence shows that oral non-steroidal anti-inflammatory drugs (NSAIDs) may reduce swelling

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and alleviate painfor acute ankle sprains, but it does not promote ligament repair, and there are side effects of gastrointestinal discomfort[6]. In contrast, traditional Chinese medicine (TCM) treatment for acute ankle sprain has been shown to be effective in pain relief, reducing swelling, improving function, and shortening the course of the disease[7,8]. Therefore, investigating TCM's molecular targets and related mechanisms in treating acute ankle sprains has clinical significance.

Network pharmacology is a relatively new field that combines various disciplines, including systems biology, proteomics, genomics, and pharmacology, to construct molecular networks of diseases and drugs. This approach utilizes high-throughput omics data analysis and network database retrieval to predict disease pathogenesis, identify drug targets, and elucidate the molecular mechanisms underlying disease treatment[9]. Network pharmacology has recently been used to investigate the mechanism of action of TCM in treating acute ankle sprain, which has become an area of active research.

Longji Xiaozhong ointment is a traditional preparation used for decades for treating ankle sprains in Shanghai Municipal Hospital of Traditional Chinese Medicine. Though it has a significant effect, it suffers from lack of clinical standardized research. Longji Xiaozhong ointment contains numerous active ingredients that can be screened using network pharmacology to identify the key compounds and predict their targets and associated pathways. Its analgesic effect can also promote rapid repair of ankle ligaments, improve the overall function of the ankle joint, and promote recovery of ankle joint stability. In this study, we aimed to identify the core components, targets and pathways of Longji Xiaozhong ointment and validate its clinical efficacy in treating acute ankle sprains using relevant clinical data.

Materials and Methods

Active ingredients and target genes screening

Use the TCMSP platform (http://lsp.nwu.edu.cn/tcmsp.php) to screen the active ingredients of Longji Xiaozhong ointment, including natural copper, earthworm, bletilla striata, nuxychia, xuejianchou, hibiscus leaf, wulingzhi, chuanxiong, Safflowers (*Carthamus tinctorius* L.), raw earth charcoal, nepeta charcoal, etc. Its drug properties are similar to DLP ≥0.18 and OB ≥30%. Molecular targets were screened from the ETCM database and target information was compared.

PPI of target genes

The PPI network of the target gene was analyzed using the online database STRING (https://string-db.org/), and the results were visualized and correlated using Cytoscape software. Key protein expression molecules were identified by applying the protein complex clustering algorithm through the Molecular Complex Detection (MCODE) plug-in.

GO and KEGG enrichment analysis

The candidate target genes of *Strychnosnux-vomica* L. (Strychnine tree or Nux vomica), *Ligusticum chuanxiong* Hort. and Safflowers were analyzed using the ETCM database (http://www.tcmip.cn/ETCM/index.php/Home/), which involved FEA and signaling pathway prediction. Next, a functional analysis of the three traditional Chinese medicines was performed using Metascape. The target gene was added to Metascape for this analysis.

Selection of clinical data

The clinical data of 120 patients with acute ankle sprain admitted to our hospital from October 2020 to September 2022 were selected. Diagnostic criteria: According to the diagnostic criteria for ankle sprains in the "Diagnostic and Curative Criteria for Diseases and Syndromes of Traditional Chinese Medicine"[10]; A clear clinical history of ankle trauma: damaged joint pain, swelling, subcutaneous ecchymosis, lameness, injured feet dare not use force while walking. The local tenderness is obvious, and the ankle joint is unstable. X-ray examination showed no signs of fracture and dislocation. Inclusion criteria: (i) Acute ankle sprain occurred within 72 h before enrollment, meeting the diagnostic criteria for grade I and grade II acute ankle sprain: if there is no loss of ankle joint function or ligament laxity (negative for anterior drawer test and talus tilt test), diagnosed as a grade I ankle sprain. Little or no bleeding, no point tenderness, total ankle range of motion decreased ≤5°, swelling ≤0.5 cm. Patient with partial loss of function, positive anterior drawer test (suggesting damage to the anterior talofibular ligament), negative talar tilt test (indicating no involvement of the calcaneofibular ligament), bleeding, spot tenderness, 5° <10° decrease in total ankle range of motion, 0.5 cm < swelling <2.0 cm, diagnosed as having a grade II acute ankle sprain[11]; and (ii) Aged 18-65 years old, male or female, who can accept the treatment in this project research. Exclusion criteria: (i) X-ray confirmed fracture or grade III ankle sprain (diagnostic criteria: almost complete loss of ankle joint function, positive

anterior drawer test and talus tilt test, bleeding, extreme point tenderness, and decreased overall ankle joint activity >10°, swelling >2.0 cm); (ii) Patients with fracture history of the same ankle joint in the previous year; (iii) Combined with serious diseases (such as liver, kidney, cancer and central nervous system diseases, dementia and hemophilia and other coagulation abnormalities); (iv) People with motor or sensory disturbances caused by nervous system disorders in the same leg; (v) Pregnant, pregnant and breastfeeding women; (vi) Use of NSAIDs and painkillers during the period from trauma to participation in clinical trials or analgesic steroids and other medications (excluding adherent inflammatory analgesics used on the screening day); (vii) allergic to test drug ingredients; (viii) infectious diseases, skin diseases, mental illness patients, who cannot cooperate with the treatment of this study; (ix) serious adverse reactions during treatment, intolerance to treatment or other complications; and (x) participation in other clinical research subjects.

Treatment methods of enrolled patients

Cases dropped out in each group. Among the finally enrolled patients, there were 36 cases of Celebrex combined with brace immobilization group (Group A); 37 cases of musk pain relief ointment combined with brace immobilization group (Group B); and 38 case of Longji Xiaozhong ointment combined with brace immobilization group (Group C).

Group A: Patients were given Celebrex orally, once a day, one tablet each time, and ankle joint brace was fixed at the same time, and the treatment continued for 3 weeks. Group B: Patients were given musk pain relief ointment (ingredients: aconite, clove, calamus, safflowers, xu changqing, rhubarb, nard pine, chuanxiong, artificial musk, borneol, camphor, peppermint oil, cinnamon aldehyde, specification: 7 cm*10 cm) for external application, and the operation method was as follows: wash the affected area, wipe dry, stick the plaster on the affected area, change it once a day, and fix the ankle joint brace at the same time, and treat continuously for 3 weeks. Group C patients were given Longji Xiaozhong ointment (ingredients: natural copper, earthworm, bletilla striata, maqianzi, Wumingyi, Xuejianshou, hibiscus leaves, Wulingzhi, Chuanxiong, Safflowers, Shengdi charcoal, Nepeta charcoal, size: 7 cm*10 cm) for external application following the operation method: wash the affected area, wipe dry, stick the plaster on the affected area, change it once a day, and fix it with an ankle brace at the same time, and treat it continuously for 3 weeks.

Curative effect observation and observation index of enrolled patients

Curative effect observation

The primary efficacy endpoint of this study was to assess pain relief using the visual analog scale (VAS). The secondary endpoints included the evaluation of oedema, the Foot and Ankle Outcome Scale (FAOS), and the European Five-Dimensional Five-Level Quality of Life Scale (EQ-5D-5L) scores. The VAS, oedema, FAOS, and EQ-5D-5L scores were recorded before and after the treatment to observe any changes in the patients.

Observation indicators

Visual analogue score (VAS): The intensity of pain was measured using a 10 cm VAS, where patients marked the level of pain on a ruler. The score was recorded in a total range of 0-100 mm, where 0 indicated no pain and 10 cm indicated severe pain, corresponding to 100 points. Pain assessments were recorded before the treatment and after three weeks of treatment.

Oedema: The degree of oedema was measured using the 'figure-of-8' method at three time points viz. before treatment, 1 week after treatment, and 3 weeks after treatment. The measurement was taken in centimeters by placing tapes in a 'figure-of-8' pattern over the navicular tuberosity, distal medial malleolus, distal lateral tuberosity of the lateral malleolus, and base of the fifth metatarsal. The values obtained were compared with those of healthy ankles to assess the degree of edema[12].

FAOS: The FAOS is a self-administered assessment system specifically designed to evaluate symptoms and function of the foot and ankle after injury. The system comprises 42 items assessing five areas of recovery: pain (9 items), physical and recreational activities (5 items), activities of daily living (17 items), quality of life-related to feet and ankles (4 items), and other symptoms (7 items)[13]. Evaluations were performed at three different time points, which included baseline evaluation before treatment, evaluation 3 weeks after treatment, and evaluation 4 weeks after the entire treatment.

EQ-5D-5L scale: The EuroQoL Group developed the EQ-5D-5L scale, which has higher sensitivity and lower ceiling effects than the EQ-5D-3L scale[14,15]. The scale comprises two parts: the Descriptive System

and the VAS. The Descriptive System evaluates five dimensions of health, namely self-care, mobility, pain/discomfort, usual activities, and anxiety/depression, with five levels for each dimension, namely: no difficulty, a little difficulty, moderate severe difficulty, severe difficulty and extremely severe difficulty[16,17]. The intuitive scale is a vertical scale with a length of 20 cm. The value at the top is 100, representing "the most rational state of health in mind", and the value at the bottom is 0, representing "the worst state of health in mind". Evaluations were made at 4 time points before treatment, 3 weeks after treatment, 4 weeks and 26 weeks after treatment.

Statistical analysis

The statistical analysis of the bioinformatics section was performed by automatic calculation using the online databases mentioned above. Statistical analysis was performed using SPSS 22.0 software in the clinical data analysis. The baseline characteristics of the two groups were compared using the Wilcoxon rank sum test or an independent t-test for continuous variables presented as Mean±SD, and a chi-square test or Fisher's exact test for categorical variables presented as frequency and percentage. Pain VAS score and secondary outcomes (FAOS, edema, and EQ-5D-5L score) were assessed using RM ANCOVA. The dependent variable included values measured before, in the end, and 4 weeks after treatment. Changes in VAS scores from baseline to the end of treatment and 4 weeks after treatment were compared using paired t-test. Statistical significance was considered at $P < 0.05$.

Results

Active ingredients and target genes screening

By applying screening criteria of DLP ≥0.18 and OB ≥30% in the TCMSP database, we identified 13 active ingredients of Maqianzi, seven active ingredients of Chuanxiong, and 22 active ingredients of safflowers in Longji Xiaozhong ointment (Table 1). Furthermore, the active ingredients of Maqianzi corresponded to 384 target genes, those of Ligusticum Chuanxiong corresponded to 1356 target genes, and those of Safflowers corresponded to 1466 target genes.

We conducted additional screening of the target genes associated with the active ingredients according to OB ≥50% and DL ≥0.5, and finally obtained 19 key target genes, and determined the information of these target genes in the GeneCards database (Table 2).

Protein mutual aid network construction and module analysis of the above key target genes and core gene screening

The above 19 target genes were imported into Cytoscape software to build a PPI network diagram (Fig. 1A). Non-directional score calculations were performed on each node in the protein interaction network using the Network Analyzer tool in Cytoscape to obtain the value of the Degree of each node. The size of the node represented the value of the Degree. The Neighborhood Connectivity of each node was represented by the node's colour, from red to green, from high to low. The combined_score value of the edge was represented by the thickness of the edge. The layout of the Attribute Circle Layout was used to arrange all the protein nodes, and cluster association analysis was performed on these important protein molecules using the MCODE plug-in with the default parameters of Node Score Cutoff as 0.2, K-core as 2, and Max.Depth as 100. Two Clusters with higher scores were obtained (Fig. 1B), and 7 key proteins, including SLC6A4, ADRB2, ADRA1B, CHRM1, F2, OPRM1, and OPRD1, were finally obtained. The detailed information on these 7 key genes is shown in Suppl. Table S1 (*All supplementary data are available only online along with the respective paper at the journal website* (*http://ijeb.res.in*) *as well as NOPR repository at http://nopr.res.in*).

GO function and pathway enrichment of related targets

We firstly performed functional enrichment analysis on the candidate target genes of Maqianzi, Chuanxiong and Safflower in Longji Xiaozhong ointment in the ETCM database, and the GO analysis of Maqianzi showed that the gene functions involved in the target genes included cellular responses to jasmonic acid stimulation, cellular sodium ion homeostasis, cellular potassium ion homeostasis, establishment and maintenance of transmembrane electrochemical gradients, regulation of neuronal apoptosis, GABA signaling, chloride transmembrane transport and cellular responses to histamine, etc. [Fig. 2 A(i). See Suppl. Table S2 for details]. The results of the analysis of its action pathways include SUMOylation of intracellular receptors, activation of gene expression by RORA, phosphorylation of CREB through activation of CaMKII, blockade of NMDA receptors, binding and activation of glutamate, and activation of peroxisome proliferators regulation of lipid metabolism by receptor α (PPARα), PPARA-activated gene expression and transcriptional activation of mitochondrial biogenesis, etc. [Fig. A(ii). See Suppl. Table S3 for details].

Table 1 — Active ingredients of Longji Xiaozhong ointment in ETCM database (OB ≥30.00% and DL ≥0.18)

Ingredients	Mol ID	Molecule name	OB (%)	DL
Strychni Semen	MOL001040	(2R)-5, 7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one	42.36	0.21
Strychni Semen	MOL001476	(S)-Stylopine	51.15	0.85
Strychni Semen	MOL003410	Ziziphin_qt	66.95	0.62
Strychni Semen	MOL003411	Icaride A	48.74	0.43
Strychni Semen	MOL003413	Isostrychnine N-oxide (I)	35.45	0.8
Strychni Semen	MOL003414	Isostrychnine N-oxide (II)	37.33	0.8
Strychni Semen	MOL003418	Lokundjoside_qt	32.82	0.76
Strychni Semen	MOL003432	vomicine	47.56	0.65
Strychni Semen	MOL003433	brucine-N-oxide	49.17	0.38
Strychni Semen	MOL003436	Isobrucine	33.58	0.8
Strychni Semen	MOL003440	Brucine N-oxide	52.63	0.38
Strychni Semen	MOL000449	Stigmasterol	43.83	0.76
Strychni Semen	MOL000492	(+)-catechin	54.83	0.24
Chuanxiong Rhizoma	MOL001494	Mandenol	42	0.19
Chuanxiong Rhizoma	MOL002135	Myricanone	40.6	0.51
Chuanxiong Rhizoma	MOL002140	Perlolyrine	65.95	0.27
Chuanxiong Rhizoma	MOL002151	senkyunone	47.66	0.24
Chuanxiong Rhizoma	MOL002157	wallichilide	42.31	0.71
Chuanxiong Rhizoma	MOL000359	sitosterol	36.91	0.75
Chuanxiong Rhizoma	MOL000433	FA	68.96	0.71
Carthami Flos	MOL001771	poriferast-5-en-3beta-ol	36.91	0.75
Carthami Flos	MOL002680	Flavoxanthin	60.41	0.56
Carthami Flos	MOL002694	4-[(E)-4-(3, 5-dimethoxy-4-oxo-1-cyclohexa-2, 5-dienylidene)but-2-enylidene]-2, 6-dimethoxycyclohexa-2, 5-dien-1-one	48.47	0.36
Carthami Flos	MOL002695	lignan	43.32	0.65
Carthami Flos	MOL002698	lupeol-palmitate	33.98	0.32
Carthami Flos	MOL002706	Phytoene	39.56	0.5
Carthami Flos	MOL002707	phytofluene	43.18	0.5
Carthami Flos	MOL002710	Pyrethrin II	48.36	0.35
Carthami Flos	MOL002712	6-Hydroxykaempferol	62.13	0.27
Carthami Flos	MOL002714	baicalein	33.52	0.21
Carthami Flos	MOL002717	qt_carthamone	51.03	0.2
Carthami Flos	MOL002719	6-Hydroxynaringenin	33.23	0.24
Carthami Flos	MOL002721	quercetagetin	45.01	0.31
Carthami Flos	MOL002757	7, 8-dimethyl-1H-pyrimido[5, 6-g]quinoxaline-2, 4-dione	45.75	0.19
Carthami Flos	MOL002773	beta-carotene	37.18	0.58
Carthami Flos	MOL002776	Baicalin	40.12	0.75
Carthami Flos	MOL000358	beta-sitosterol	36.91	0.75
Carthami Flos	MOL000422	kaempferol	41.88	0.24
Carthami Flos	MOL000449	Stigmasterol	43.83	0.76
Carthami Flos	MOL000006	luteolin	36.16	0.25
Carthami Flos	MOL000953	CLR	37.87	0.68
Carthami Flos	MOL000098	quercetin	46.43	0.28

Table 2 — Target genes associated with the active ingredients

Ingredients	Mol ID	Corresponding Target gene	Corresponding gene name in GeneCards Database
Strychni Semen	MOL001476	Prostaglandin G/H synthase 1	PTGS1
Strychni Semen	MOL001476	Muscarinic acetylcholine receptor M3	CHRM3
Strychni Semen	MOL001476	Muscarinic acetylcholine receptor M1	CHRM1
Strychni Semen	MOL001476	Sodium channel protein type 5 subunit alpha	SCN5A
Strychni Semen	MOL001476	Muscarinic acetylcholine receptor M5	CHRM5
Strychni Semen	MOL001476	Prostaglandin G/H synthase 2	PTGS2
Strychni Semen	MOL001476	5-hydroxytryptamine receptor 3A	HTR3A
Strychni Semen	MOL001476	Muscarinic acetylcholine receptor M4	CHRM4
Strychni Semen	MOL001476	Delta-type opioid receptor	OPRD1
Strychni Semen	MOL001476	5-hydroxytryptamine 2A receptor	HTR2A
Strychni Semen	MOL001476	Alpha-1B adrenergic receptor	ADRA1B

(*Contd*.)

Table 2 — Target genes associated with the active ingredients (*Contd*.)

Ingredients	Mol ID	Corresponding Target gene	Corresponding gene name in GeneCards Database
Strychni Semen	MOL001476	Beta-2 adrenergic receptor	ADRB2
Strychni Semen	MOL001476	Alpha-1D adrenergic receptor	ADRA1D
Strychni Semen	MOL001476	Sodium-dependent serotonin transporter	SLC6A4
Strychni Semen	MOL001476	Mu-type opioid receptor	OPRM1
Strychni Semen	MOL001476	Calmodulin	CALM1
Chuanxiong Rhizoma	MOL000433	Cell division protein kinase 2	CDK2
Chuanxiong Rhizoma	MOL000433	Thrombin	F2
Chuanxiong Rhizoma	MOL000433	Glycogen synthase kinase-3 beta	GSK3B

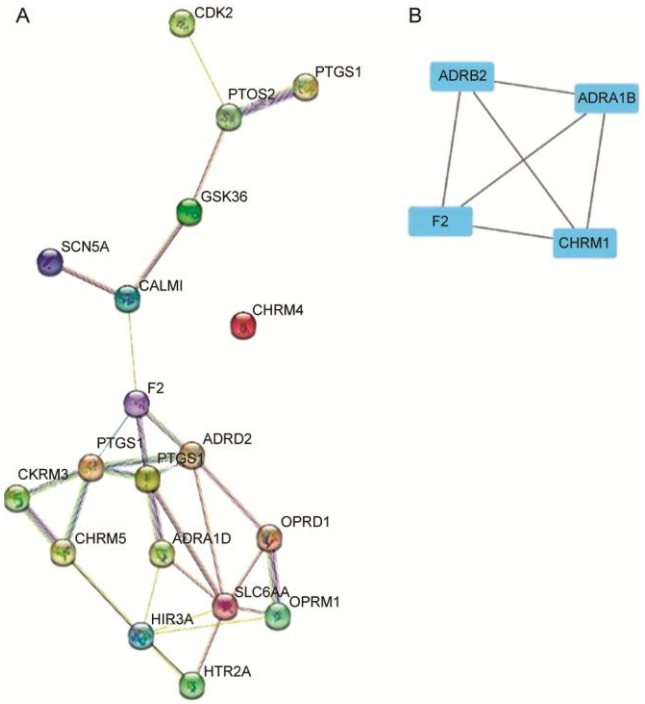

Fig. 1 — Protein mutual aid network construction and module analysis of key target genes and core gene screening. (A) Protein interaction network diagram; and (B) Cluster with higher score.

Further, we performed functional enrichment analysis on the candidate target genes of Chuanxiong in Longji Xiaozhong Ointment. The GO analysis of Chuanxiong showed that the gene functions involved in the target genes include membrane depolarization in the process of cardiomyocyte action potential, urea Cycling, ATP hydrolysis coupled to anion transmembrane transport, L-glutamate transport, negative regulation of macrophage-derived foam cell differentiation, drug metabolism processes, GABA signaling, glucocorticoid response, and calcium transmembrane Transshipment, etc. [Fig. 2 B(i). See Suppl. Table S4 for details]. The analysis results of its action pathway include GABA A receptor activation, neurotransmitter receptors and postsynaptic signal transmission, sumoylation of intracellular receptors, biosynthesis of maresin-like SPMs, interaction between L1 and ankyrin and NMDA receptors. Body blocking, glutamate binding and activation, etc. [Fig. 2 B(ii). See Suppl. Table S5 for details].

Finally, we performed functional enrichment analysis on the candidate target genes of safflower in Longji Xiaozhong ointment. The GO analysis of safflower shows that the gene functions involved in the target genes include cobalamin metabolism, mitochondrial electron transport, cell Pigment c to oxygen, neuronal action potential, arginine catabolism process, steroid hormone-mediated signaling pathway, drug metabolism process and precursor metabolite production and energy, etc. Fig. 2 C(i). See Suppl. Table S6 for details). The analysis results of its action pathway include cobalamin (Cbl, vitamin B12) transport and metabolism, GABA A receptor activation, intracellular receptor SUMOylation, neurotransmitter receptor and post-synaptic signal transmission, L1 and ankyrin The interaction of citric acid cycle (TCA cycle) and PPARA activates gene expression etc. (Fig. 2 C(ii). See Suppl. Table S7 for details).

Structure (3D) of key proteins in the above gene network in UniProt database

We obtained 7 key proteins including SLC6A4, ADRB2, ADRA1B, CHRM1, F2, OPRM1 and OPRD1 in the above research. Then we further explored the 3D structures of these key proteins in the UniProt database, as shown in Fig. 3.

VAS scores of three groups of patients before and after treatment

The final study was divided into Group A: Celebrex combined with brace immobilization group (n=36); Group B: ShexiangJietong ointment combined with brace immobilization group (n=37); and Group C: Longji Xiaozhong ointment combined with brace immobilization group (n=38). There was no significant difference in the visual analogue score (VAS) scores of the three groups before treatment (P >0.05). After 3 weeks of treatment, the VAS scores

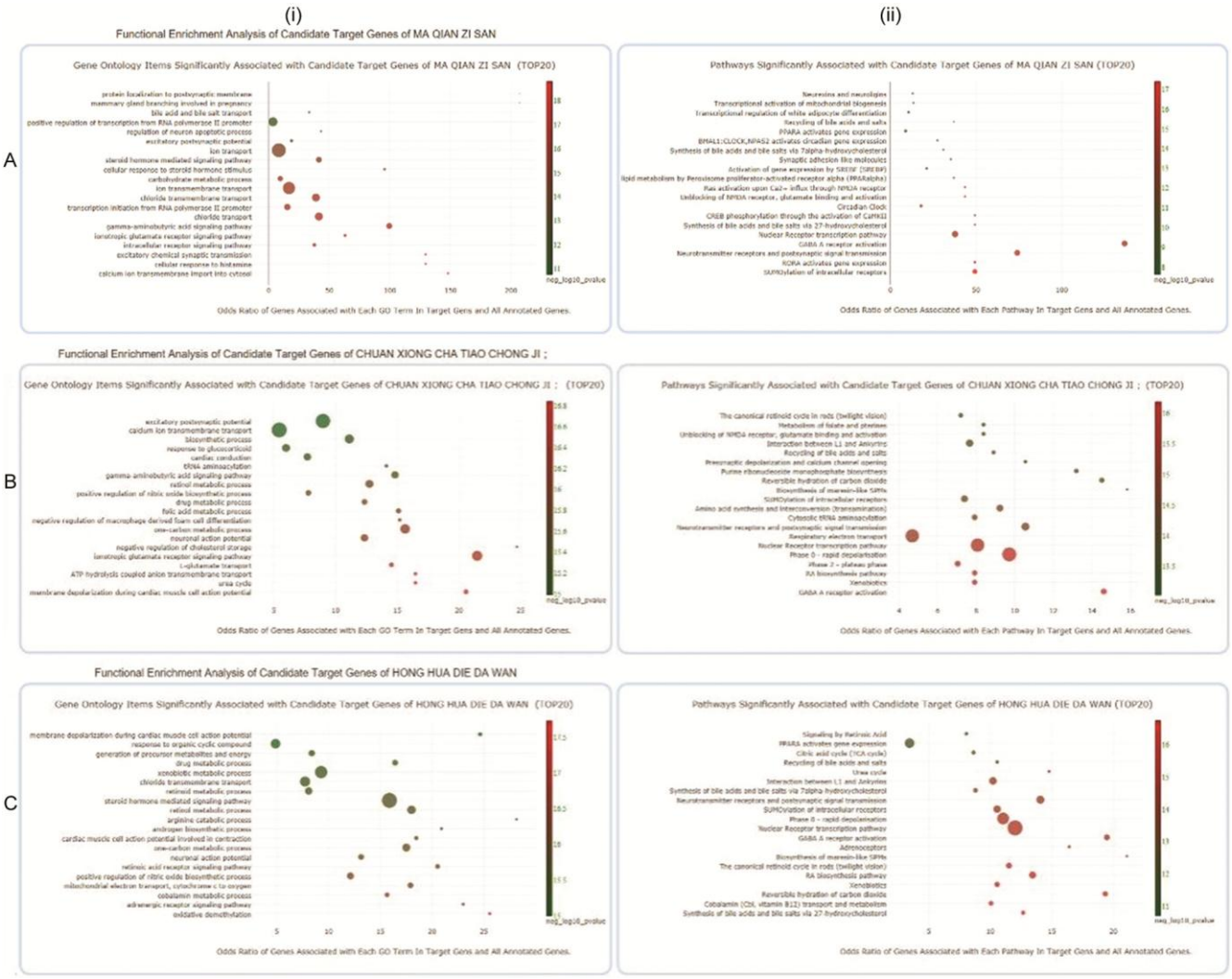

Fig. 2 — GO functions of candidate target genes of (A) Strychnosnux-vomica; (B) Ligusticum chuanxiong; and (C) safflower in Longji Xiaozhong ointment and pathway enrichment of related targets. (i) GO analysis of the target genes; and (ii) Pathway analysis of the target genes.

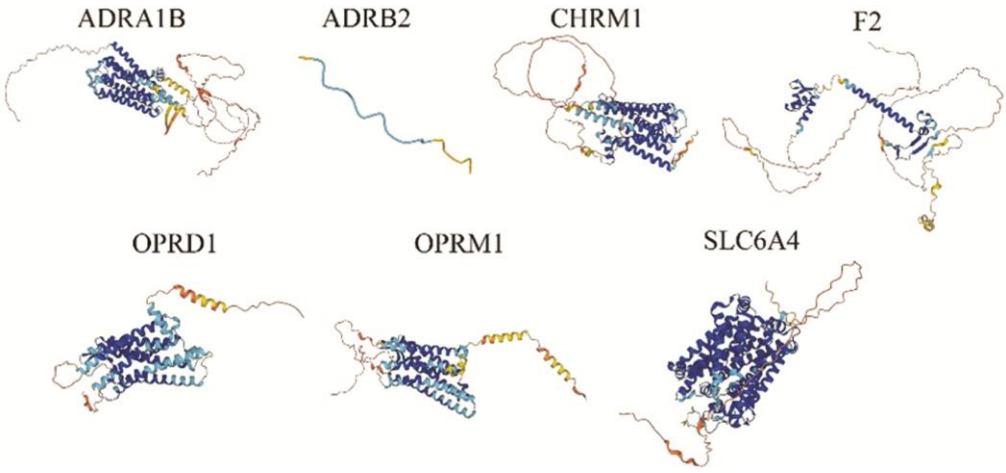

Fig. 3 — The 3D structure information of 7 key protein genes in the UniProt database.

of the Longji Xiaozhong ointment combined with brace immobilization group were significantly lower than those of the other two groups, and the differences were statistically significant (P <0.01) (Table 3).

Oedema before and after treatment in three groups of patients

There was no significant difference in the degree of swelling and range scores before treatment among the three groups (P >0.05). After 1 week of treatment and 3 weeks after treatment, the degree of swelling in the Longji Xiaozhong ointment combined with brace immobilization group was significantly better than that before treatment. And lower than the scores of the other two groups after treatment, the difference was statistically significant (P <0.05) (Table 4).

FAOS Functional Score in the three groups of patients

The FAOS scores of all the patient groups before treatment showed no significant difference. However, after 3 weeks of treatment and 4 weeks after the treatment, the FAOS scores of all three groups of patients increased significantly compared to the scores before treatment (all P *<0.05*). Furthermore, the FAOS score of the combined brace immobilization group treated with Longji Xiaozhong ointment was significantly higher than the scores of the other two groups (P *<0.05*) (Table 5).

Different EQ-5D-5L scores of the three groups of patients after treatment

When evaluating the EQ-5D-5L scores of the three groups of patients, we found that the overall condition of the patients in the Longji Xiaozhong ointment combined with brace immobilization group was better than that of the other two groups, especially in the following five aspects of "serious problems" and the number of patients with "extremely serious problems" was significantly less than that of the other two groups (Table 6).

Table 3 — Visual analogue score (VAS) scores of patients in the three groups before and after treatment

Group	Before treatment	3 weeks after treatment	t	p
A (n=36)	39.40±14.56	16.84±6.30*	8.532	<0.0001
B (n=37)	40.20±14.27	15.16±5.13*	10.04	<0.0001
C (n=38)	41.60±12.84	12.26±4.37	13.33	<0.0001

[Group A, Celebrex combined with brace immobilization group; ShexiangJietong ointment combined with brace immobilization group; and Group C, Longji Xiaozhong ointment combined with brace immobilization group. *P<0.05 *vs.* C group]

Table 4 — Comparison of degree of oedema before and after treatment in the three groups

Group	Before treatment	After treatment	
		1 week	3 weeks
A (n=36)	0.90±0.32	0.45±0.22$^{\#,*}$	0.29±0.17$^{\#,\Delta,*}$
B (n=37)	0.90±0.55	0.42±0.24$^{\#,*}$	0.26±0.20$^{\#,\Delta,*}$
C (n=38)	0.93±0.48	0.31±0.23$^{\#}$	0.12±0.06$^{\#,\Delta}$

[Group A, Celebrex combined with brace immobilization group; Group B, ShexiangJietong ointment combined with brace immobilization group; and Group C, Longji Xiaozhong ointment combined with brace immobilization group. $^{\#}P$<0.05 *vs.* before treatment; $^{\Delta}P$<0.05 *vs.* after 3 weeks of treatment; *P<0.05 *vs.* C group]

Table 5 — Comparison of each item of the Foot and Ankle Outcome Scale (FAOS) functional score among the three groups of patients

Group	Before treatment	Treated	
		3 wk after starting	4 wk after finishing
A (n=36)	312.10±73.04	356.30±80.62$^{\#,*}$	445.16±89.72$^{\#,\Delta,*}$
B (n=37)	332.25±79.31	375.33±97.64$^{\#,*}$	460.22±64.22$^{\#,\Delta,*}$
C (n=38)	317.29±80.83	415.20±76.10$^{\#}$	496.39±78.27$^{\#,\Delta}$

[Group A, Celebrex combined with brace immobilization group; Group B, musk pain relief ointment combined with brace immobilization group; and Group C, Longji Xiaozhong ointment combined with brace immobilization group. $^{\#}P$<0.05 *vs.* before treatment; $^{\Delta}P$<0.05 *vs.* after 3 weeks of treatment; *P<0.05 *vs.* C group]

Discussion

Acute ankle sprain is a common disease in orthopedic outpatient and emergency departments. According to a survey that has been used for many years, one in every ten thousand people suffers from acute ankle sprain every day[18]. Acute ankle sprains are more common in people who participate in sports and recreational physical activities. Ankle sprains are also highly prevalent in the general population, as can be seen from hospital emergency department data[19,20]. Up to 70% of the general population report suffering an ankle injury during their lifetime[21]. Since the foot is the only structure of the human body that touches the ground, its function is mainly to maintain the body upright, walk and exercise, and the ankle joint is the first relatively flexible main joint connected to the foot, so an injury to the ankle joint will directly affect human basic activity functions.

Ankle sprains not only have a huge impact on people's normal work and life, but also generate considerable health care costs and socioeconomic costs. The recurrence rate of ankle sprains is very high, and several studies have shown that the most common risk factor for ankle sprains is a history of at least one ankle sprain. After an ankle sprain, there are many long-term sequelae such as pain, persistent swelling, ankle instability, ankle

Table 6 — Different EQ-5D-5L scores of the three groups of patients after treatment

Group	Level	Movement ability (n)	Self care (n)	Daily activities (n)	Pain/ discomfort (n)	Anxiety/ depression (n)
A (n=36)	No problem	18	15	13	23	13
	Light problem	15	14	13	8	16
	Moderate problem	1	6	7	4	5
	Serious problems	2	1	2	1	1
	Extremely serious problems	0	0	1	0	1
B (n=37)	No problem	18	18	21	19	15
	Light problem	16	14	14	17	14
	Moderate problem	2	5	1	1	6
	Serious problems	1	0	1	0	1
	Extremely serious problems	0	0	0	0	1
CA (n=38)	No problem	22	24	25	27	19
	Light problem	15	12	10	10	14
	Moderate problem	1	2	3	1	5
	Serious problems	0	0	0	0	0
	Extremely serious problems	0	0	0	0	0

[Group A, Celebrex combined with brace immobilization group; Group B, Muskpainrelif ointment combined with brace immobilization group; Group C, Longji Xiaozhong ointment combined with brace immobilization group]

"weakness", recurrent injury, decreased ankle function, and loss of control[22,23]. "Out of control" refers to the sudden involuntary turning of the ankle joint to one side during dynamic functional activities, which is similar to the movement of sprained feet, but quickly returns
to the normal position of the ankle joint out of control, usually when going up and down stairs, on uneven ground It is easy to appear during walking, running, jumping and other activities. Anandacoomarasamy*et al*.[24] found that 74% of patients with ankle sprains had at least one sequelae. Braun[25] found that within 6-18 months after ankle sprain, 72.6% of ankle sprain patients reported residual symptoms; 40.4% reported at least one moderate or severe residual symptom; 40.4% of the people complained of joint instability; 40.3% of the people still had pain in the ankle joint when walking. Verhagen *et al*.[26] found that 30% of patients with ankle sprains still had residual symptoms 9 months after the injury, and this proportion reached 39% after 6.5 years. It can be seen that the residual symptoms will persist after ankle sprains and may slowly deteriorate over time. Recurrent ankle sprains are closely related to the sequelae of ankle sprains. Therefore, the delay in treatingacute ankle sprains or the lack of timely treatment or improper treatment methods may lead to a certain degree of delayed healing of the condition, and many types of complications are prone to occur, such as instability of the ankle joint, weight bearing or pain after exercise, dysfunction, traumatic arthritis, etc., seriously affect the daily life and normal work of patients[27]. This has also prompted the prevention, treatment and rehabilitation of ankle sprains to become a research hotspot in the field of clinical and biomechanics. We need to further optimize the current treatment of ankle sprains and explore a more effective treatment plan.

Western medicine currently has no uniform standard for treatingacute ankle sprains. Commonly used methods include wearing bandages, ankle braces, plaster fixation, and surgery. Many current studies have shown that conservative treatment is the mainstream treatment method[28]. Although immobilization therapy can provide a healing environment for ankle joint injuries, it does not accelerate the repair of ankle joint injuries, and there is no clear evidence that it can restore ankle joint tension. The latest evidence-based medical evidence shows that oral NSAIDs can play an analgesic and detumescent role in acute ankle sprains[29]. But it does not have the effect of promoting ligament repair, and there are side effects of gastrointestinal discomfort. And TCM treatment of acute ankle sprain has good accumulative effects in various aspects such as pain relief, detumescence, improvement of function, and shortening the course of disease. At the same time, Chinese medicine treatment has the potential to promote the rapid repair of ankle ligaments and improve the overall function of the ankle joint.

Acute ankle sprain belongs to the category of "soft tissue injury" in traditional Chinese medicine. A muscle soft tissue injury in damage to the blood

vessels. Blood overflows the veins and stasis under the skin, so swelling is seen; blood stasis and Qi stagnation cause pain, so pain is felt. Therefore the general TCM treatment principle of this disease is to remove blood stasis and promote Qi circulation, reduce swelling and relieve pain. Longji Xiaozhong ointment was developed on this basis. It is composed of natural copper, earthworm, bletilla striata, nuxychia, wumingyi, xuejianshou, hibiscus leaf, wulingzhi, chuanxiong, Safflowers, shengdichar, nepeta and other drugs. This ointment is currently the hospital preparation in our hospital. It has been clinically used for decades to treat various bone and trauma diseases, and the effect is very fast. It is especially suitable for the treatment of soft tissue injuries and fractures. The compatibility rules of Longji Xiaozhong ointment are as follows: firstly, use natural copper and Wumingyi to dissipate blood stasis to relieve pain; secondly, use products that promote blood circulation and remove blood stasis, such as Wulingzhi, Xuejianshou, Safflowers, Chuanxiong, etc. to pass through the meridians; the third is to use anti-swelling products such as Baiji, Nuxyche, and Folium *Hibisci Mutabilis*, etc., which must be compatible with each other to promote blood circulation and remove blood stasis, reduce swelling and relieve pain. All the medicine were made into ointment to enhance the drug's transdermal effect. Modern pharmacological studies have shown that natural copper and anonymity in prescriptions are mineral drugs, and there are many studies on regulating bone metabolism and promoting fracture healing, and have the functions of promoting bone healing and antimicrobial effects. Some studies have also found that adding Wumingyi as an auxiliary material can enhance the curative effect of promoting blood circulation and relieving pain[30]. Clinical experiments have shown that externally applied Chinese medicine has a positive effect on the permeability of microvessels[31]. Years of clinical practice have proved that Longji Xiaozhong ointment can significantly relieve swelling and pain caused by acute ankle sprains, and can promote the rapid healing of ankle sprains.

Therefore, we used network pharmacology analysis to further analyze the active ingredients, targets and possible related mechanisms in Longji Xiaozhong ointment, and finally verify its use value from our clinical data.

In the TCMSP database, according to the screening conditions of DLP ≥0.18 and OB ≥30%, we found that in Longji Xiaozhong ointment, there are only nuxychonzi, Chuanqiong and safflowers have corresponding active ingredients, and there are 13 kinds of nuxychonzi in it active ingredients. Chuanxiong has 7 active ingredients, safflowers has 22 active ingredients. When exploring the target genes of its action, we found that there are 384 target genes corresponding to the active ingredients of *Strychnosnux vomica*, 1356 target genes corresponding to the active ingredients of *Ligusticum chuanxiong*, and 1466 target genes corresponding to the active ingredients of Safflowers. In order to further screen the key target genes, we improved the screening conditions on this basis, and finally obtained 19 key target genes. The protein mutual aid network construction and module analysis were carried out on these genes, and finally 7 key proteins including SLC6A4, ADRB2, ADRA1B, CHRM1, F2, OPRM1 and OPRD1 were obtained. Then, GO analysis and pathway enrichment analysis were performed on the target genes of the above three drug ingredients in the ETCM database, and the results showed that these target genes may participate in inflammatory pathways and stabilize the internal environment to play a role. These research results provide a good reference for our future basic experiments, and are also consistent with the anti-inflammatory and analgesic effects of the drug in clinical use, and also reflect the value of our research from the side.

In order to further explore the clinical application value of Longji Xiaozhong ointment, we designed a efficacy comparison with Celebrex and Musk Pain Relief Creamon using retrospective clinical data of 111 patients with acute ankle sprains admitted to our hospital from October 2020 to September 2022. The results showed that the combined treatment of Longji Xiaozhong ointment with brace immobilization had a significantly lower VAS score than the other two treatment groups and significantly improved daily activities, sports, and quality of life.

Conclusion

The above study, although with some limitations, has demonstrated Longji Xiaozhong ointment's potential in treating acute ankle sprains from various levels and perspectives. These findings have important implications for future multi-center, large-scale randomized controlled trials, clinical applications, and new insights into the potential of

TCM for treating acute ankle sprains. Future studies can be conducted to perform related fundamental cytological experiments based on the identified primary active ingredients, targets, and signaling pathways, which could provide more precise insight into treating acute ankle sprains with traditional Chinese medicine.

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Conflict of Interest

Authors declare no competing interests.

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