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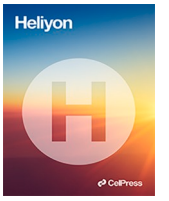

Research article

Mahuang Fuzi Xixin decoction: A potent analgesic for neuropathic pain targeting the NMDAR2B/CaMKIIα/ERK/CREB pathway

Yihui Chai [a,1], Siyu He [b,1], Dayi Liang [c], Chunsong Gu [d], Qian Gong [e], Ling Long [a], Peng Chen [a,**], Long Wang [b,*]

[a] Basic Medical School, Guizhou University of Traditional Chinese Medicine, Guiyang, 550000, China
[b] School of Pharmacy, Southwest Medical University, Luzhou, 646000, China
[c] Second Clinical Medical College, Heilongjiang University of Chinese Medicine, Haerbin, 150000, China
[d] Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine, Guiyang, 550000, China
[e] First Clinical Medical School, Guangzhou University of Chinese Medicine, Guangzhou, 510000, China

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ABSTRACT

Neuropathic pain (NeP) is a condition charactesized by nervous system injury or dysfunction that affects a significant portion of the population. Current treatments are ineffective, highlighting the need for novel therapeutic approaches. Mahuang Fuzi Xixin decoction (MFXD) has shown promise for treating pain conditions in clinical practice; however, its potential against NeP and the underlying mechanisms remain unclear. This study identified 35 compounds in MFXD using ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS). The analgesic effects of MFXD on chronic constriction injury (CCI) rats were evaluated through the detection of mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL). The analgesic effects of MFXD in rats with chronic constriction injury (CCI) were evaluated by measuring the mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL). Low-dose MFXD (L-MFXD) group (4.8 g/kg) and high-dose MFXD (H-MFXD) group (9.6 g/kg) exhibited significantly higher MWT and TWL values than the CCI group on days 11 and 15 post-CCI surgery, substantiating the remarkable analgesic efficacy of MFXD. Network pharmacology analysis identified 58 key targets enriched in pathways such as long-term potentiation (LTP) and glutamatergic synapse. The MCODE algorithm further identified core targets with significant enrichment in LTP. Molecular docking revealed that mesaconitine, rosmarinic acid, and delgrandine from MFXD exhibited high binding affinity with NMDAR2B (−11 kcal/mol), CaMKIIα (−14.3 kcal/mol), and ERK (−10.8 kcal/mol). Western blot and immunofluorescence confirmed that H-MFXD significantly suppressed the phosphorylation levels of NMDAR2B, CaMKIIα, ERK, and CREB in the spinal cord tissue of CCI rats. In conclusion, this study demonstrates that MFXD possesses potent analgesic effects on NeP by suppressing the NMDAR2B/CaMKIIα/ERK/CREB signalling pathway. This study unlocks a path toward potentially revolutionising NeP treatment with MFXD, encouraging further research and clinical development.

* Corresponding author.
** Corresponding author.
E-mail addresses: 740466982@qq.com (P. Chen), wanglongsdu1226@163.com (L. Wang).
[1] These authors contributed equally to this work.

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Abbreviations

NeP	Neuropathic pain
MFXD	Mahuang Fuzi Xixin decoction
UHPLC-HRMS	Ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry
CCI	Chronic constriction injury
ncRNAs	non-coding RNAs
LTP	Long-term potentiation
NMDARs	N-methyl-D-aspartate receptors
NMDA	N-methyl-D-aspartate
CaMKII	Calcium/calmodulin-dependent kinase II
MAPK	Mitogen-activated protein kinase
CREB	cAMP response element-binding protein
CNS	Central nervous system
GPCRs	G-protein-coupled receptors
TCM	Traditional Chinese Medicine
SJHXT	Shu-jing-huo-xue-tang
WTD	Wu-Tou decoction
SNL	Spinal cord ligation
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
SD	Sprague-Dawley
PGB	Pregabalin
L- MFXD	Low-dose Mahuang Fuzi Xixin decoction
M − H-MFXD	High-dose Mahuang Fuzi Xixin decoction
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	Protein-protein interaction
PDB	Protein Data Bank
MWT	Mechanical withdrawal threshold
TWL	Thermal withdrawal latency
NRPG	Nucleus reticularis paragigantocellularis
NRM	Nucleus raphe magnus
PAG	Periaqueductal gray
STZ	Streptozotocin
VGSC	Voltage-gated sodium channel
SNI	Spared nerve injury
GABAA	γ-aminobutyric acid A
PKC	Protein kinase C
MCODE	Motif Cluster Detection
KIF17	Kinesin family member 17
Eph	Erythropoietin-producing hepatoma
TRP	Transient receptor potential
DALDA	Dermorphin [D-Arg2, Lys4] (1–4) amide

1. Introduction

Pain is a deeply personal and distressing experience that is a complex blend of physical sensations and emotional turmoil, triggered by actual or perceived tissue damage [1]. Neuropathic pain (NeP) is a tormenting consequence of somatosensory damage, accompanied by pain hypersensitivity, pain abnormalities, and both persistent and spontaneous pain, often accompanied by non-painful oddities [1]. NeP is a widespread condition affecting a significant portion of the population, with estimates suggesting a prevalence as high as 7–8% in the general population and accounting for 15–25 % of chronic pain cases [2]. NeP is distinguished by recurrent flares, a chronic course, and commonly associated co-morbidities such as depression and anxiety. Its profound effects include disrupting patients' sleep, impeding social interactions, and affecting daily life, while simultaneously imposing a substantial economic burden on both the individual and society [1,2].

The pathogenesis of NeP is complex and encompasses a multitude of alterations within the nervous system, including ectopic excitability, peripheral and central sensitization, neuroinflammation, neuroplastic changes, pathological microglial activation, ion channel alterations, abnormal gene expression of mRNAs and non-coding RNAs (ncRNAs), and an imbalance between inhibitory and excitatory neurotransmitters [1,3]. Long-term potentiation (LTP) is a type of synaptic plasticity that is recognised as the cellular process responsible for learning and memory. This entails a sustained enhancement in the strength of synaptic transmission between

the two neurones. LTP is triggered by high-frequency stimulation of presynaptic neurones, resulting in an elevated release of neurotransmitters from the presynaptic neuron and an augmented response in the postsynaptic neurones [4]. LTP participates in NeP development and persistence. LTP is increases in the dorsal horn of the spinal cord, a key area of the brain involved in pain processing, following nerve injury. This increase in LTP is thought to contribute to the central sensitization and heightened sensitivity to pain that is characteristic of NeP [5–7]. LTP is induced by high-frequency stimulation of the presynaptic neurones, which triggers the activation of N-methyl-D-aspartate receptors (NMDARs) in the postsynaptic neurons. This activation allows calcium ions to enter the postsynaptic neuron, initiating a cascade of signalling pathways, including the calcium/calmodulin-dependent kinase II (CaMKII) pathway and mitogen-activated protein kinase (MAPK) pathways. CaMKII phosphorylates various proteins involved in synaptic plasticity, including AMPA receptors, which are the primary excitatory receptors in the postsynaptic membrane. The phosphorylation of AMPA receptors enhances their opening probability, leading to an increase in synaptic strength. ERK, a member of the MAPK pathway, contributes to LTP by phosphorylating cAMP response element-binding protein (CREB), which regulates the expression of genes essential for maintaining LTP, such as genes for AMPA receptors and other synaptic plasticity proteins [4–7].

Currently, the primary treatments for NeP in clinical practice include medication, physical therapy, pain psychology, and interventional therapy [1,3]. While these approaches offer diverse mechanisms of action, commonly used medications such as antidepressants and opioid analgesics primarily act on the central nervous system (CNS). These drugs often provide short-term relief by blocking nerve signals but are frequently associated with CNS-related adverse effects, including respiratory depression, substance use disorder, and opioid dependence [1,3]. Therefore, identifying novel drug targets and developing safer and more effective therapies is crucial. Recent studies have suggested promising avenues for the treatment of NeP. Long non-coding RNAs, such as Kcna2 antisense RNA, have emerged as potential therapeutic targets for NeP [8]. Targeting G-protein-coupled receptors (GPCRs) expressed on primary sensory neurones has emerged as a strategy for achieving pain relief without central side effects [9]. Studies have demonstrated that μ- and δ-opioid receptor heterodimers may be potential targets, with the agonist CYM51010 demonstrating potent analgesic effects in spinal cord ligation (SNL) mice [10]. NMDAR antagonists also hold promise for NeP treatment, with astaxanthin, a marine natural product, demonstrating efficacy as a potential NMDAR antagonist [11]. Nanomedicine offers additional avenues for NeP therapy [12]. Natural products, particularly those derived from plants, are attracting increasing interest because of their potential to manage chronic pain conditions, including NeP [13]. The exploration and development of novel therapeutic drugs from these natural sources represent a promising avenue for NeP treatment.

For millennia, Traditional Chinese Medicine (TCM) has served as a holistic approach to treating a wide range of health conditions, including NeP. TCM is gaining global recognition because of its complex composition, diverse targets, intricate mechanisms, high efficacy, and minimal toxicity. Variety of TCM modalities, including acupuncture, herbal medicine, moxibustion, and Tuina massage, can be used to treat NeP [14–16]. Herbal medicines play a pivotal role in TCM's treatment of NeP [14,15]. For instance, Shu-jing-huo-xue-tang (SJHXT), a formula composed of 17 crude drugs, suppresses NeP by acting on alpha-2 adrenoreceptors in chronic constriction injury (CCI) rats [14]. Wu-Tou decoction (WTD) alleviates NeP by inhibiting hippocampal microglial activation in SNL mice [15]. Mahuang Fuzi Xixin decoction (MFXD), a classical TCM formula, is comprised of Ephedrae Herba (Mahuang), Radix Aconiti Lateralis Preparata (Fuzi), and Asarum Heterotropoides (Xixin) [16]. Traditionally, MFXD has been used to treat conditions such as migraine, asthma, rheumatoid arthritis, major depressive disorder, allergic rhinitis, cold, and influenza in clinical practice [16–19]. However, despite its historical and clinical use, the potential therapeutic effects and mechanisms of action of MFXD in NeP remain unclear.

This study aimed to bridge this knowledge gap by investigating the analgesic properties and underlying mechanisms of MFXD in NeP. We first determined MFXD's composition of MFXD, followed by an evaluation of its analgesic effects in a rat model of CCI. Finally, network pharmacology and experimental verification were performed to elucidate the underlying mechanisms. Our findings highlight the potential of MFXD as a novel NeP therapeutic, potentially acting through inhibition of LTP via suppression of the NMDAR2B/CaMKIIα/ERK/CREB signalling pathway. This study not only established the analgesic efficacy of MFXD but also provided mechanistic insights into its development as a potential NeP treatment.

2. Materials and methods

2.1. Preparation of MFXD

MXFD herbal granules, consisting of Ephedrae Herba (batch NO. A301B051) and Radix Aconiti Lateralis Preparata (batch NO. A2071291), and Asarum heterotropoides (batch NO. A209A882) in a 2:1:3 ratio was procured from Guangdong Yifang Chinese Herbal Medicine Department.

2.2. Identification of the compounds in MFXD using UHPLC-HRMS

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of MFXD was performed using a UPLC-Orbitrap-HRMS platform (Thermo Fisher Scientific). The mobile phases consisted of 0.1 % (v/v) phosphoric acid (A) and acetonitrile (B). Samples were separated using a flow rate of 0.3 mL/min following the gradient: 0–1 min, 2 % A; 1–41 min, 2 %→100 % A; 41–50 min, 100 % A; 50–50.1 min, 100 %→2 % A; and finally, from minute 50.1 to minute 52, the composition returned to the initial condition with only solvent A at a concentration of 2 %. Mass spectra were obtained in both positive and negative electrospray ionisation modes using dd-MS2 mode within the mass range of 100–1500 *m/z*.

2.3. Animals

Thirty-six-week-old male Sprague–Dawley (SD) rats were obtained from the Guangdong Medical Laboratory Animal Center (Certificate NO. SCXK[Guangdong]2022-0002, Guangzhou, China). Rats were randomly divided into five groups (n = 6): sham, CCI, pregabalin (PGB), low-dose MFXD (L-MFXD), and high-dose MFXD (H-MFXD) group. Animal experiments adhered to the guidelines and regulations of the Animal Experimentation Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Licence No. GZTCMF1-2021100).

2.4. CCI surgery and MFXD treatment

CCI was performed according to a previously established protocol [20,21]. Briefly, the rats were acclimated to the surgical environment for one week and then anaesthetised with pentobarbital sodium. The left sciatic nerve was carefully exposed and subjected to moderate ligation using four ligatures (4-0 silk) with an average spacing of 1–2 mm. Surgical incisions were meticulously closed in a layered manner. In the sham group, only the sciatic nerve was exposed and not ligated. The PGB group received a PGB solution (15 mg/kg, batch NO. J20160021, Pfizer) via oral gavage, while the L-MFXD and H-MFXD groups were administered MFXD solution (4.8 g/kg and 9.6 g/kg, respectively) through oral gavage for 15 consecutive days following CCI surgery. Two key pain sensitivity indicators, mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were detected as previously reported [14,15]. For the MWT, individual rats were acclimated to a transparent plexiglass chamber with a metal mesh floor for 30 min. The ipsilateral plantar surface of the paw was stimulated five times at 5-min intervals using an electronic von Frey anesthesiometer (IITC Life Science Instruments, Woodland Hills, CA, USA). For the TWL test, a radiant heat source (Model 390; IITC Life Science Instruments, Woodland Hills, CA, USA) was directed toward the plantar surface of the ipsilateral hind paw. This stimulation was applied thrice at 10-min intervals. The nociceptive response, defined as the lifting or licking of the hind paw, was monitored and recorded.

2.5. Prediction of putative targets of MFXD

The targets of the MFXD compounds were extracted from the SwissTargetPrediction database (http://www.swisstargetprediction.ch/). Subsequently, all retrieved targets were queried against the UniProt database (https://www.uniprot.org/) to identify the annotated and reviewed gene symbols. Finally, duplicate and non-standardised targets were eliminated to establish the potential targets of MFXD.

2.6. Acquisition of therapeutic targets associated with NeP

Targets related to NeP were sourced from the GeneCards database (http://www.genecards.org/), retaining genes with a relevance score of $\geq$1. Subsequently, neuropathic pain-related targets were compiled by integrating data from multiple databases, including DisGeNET (https://www.disgenet.org/) and DrugBank (http://www.drugbank.ca/), among others. Finally, relevant targets of MFXD for treating NeP were identified by comparing the predicted targets of the active ingredients in MFXD with the retrieved NeP-related targets. Overlapping targets of MFXD and NeP have emerged as promising candidates for NeP treatment.

2.7. Network analysis

A Venn diagram was used to identify common targets between MFXD and NeP. To prioritise these common targets, they were subsequently subjected to network topological analysis using the STRING 11.0 database (https://string-db.org/), with a combined score exceeding 0.7 as the criterion for inclusion. To score key targets, three network topological parameters were calculated: degree, betweenness, and closeness. The definitions of these topological features are: “Degree” is the number of interactions a node has; “Node betweenness” is the count of shortest paths between node pairs that pass through the node; “Closeness” is the sum of the shortest distances from the node to all other nodes, indicating how quickly information spreads from it to the entire network. A node is more important in the network if its parameters are higher. Common targets surpassing the median in topological parameters were regarded as key targets. Cytoscape software (version 3.7.2) was used to visualise interaction networks.

2.8. Functional enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using Metascape (https://metascape.org/). The results of enrichment analyses of GO biological processes, cellular components, molecular functions, and KEGG signalling pathways were generated by uploading the key targets to Metascape, setting the parameter to “H species,” and selecting a significance threshold of $P \leq 0.05$ as the default criterion. The top-ranked results of GO and KEGG enrichment analyses were screened according to their physiological and pharmaceutical importance.

2.9. Modular analysis

Analysis of the protein–protein interaction (PPI) network for key targets of MFXD against NeP was conducted using the MetaScape

tool. Subsequently, the MCODE algorithm was employed to identify interconnected network components. ,Functional enrichment analysis was conducted for each MCODE component, and functional descriptions of the corresponding modules were established based on the top-ranked and best-scoring terms, as determined by their respective P values.

2.10. Molecular docking

Initially, three-dimensional structures of the targets were acquired from the Protein Data Bank (PDB) database (http://www.rcsb.org/) and UniProt (https://www.uniprot.org/) databases. Subsequently, two-dimensional structures of the MFXD compounds were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). These structures were then converted into three-dimensional formats using the Chem3D software. The AutoDock Vina docking model was employed to calculate the affinity between the receptor and ligand, where an affinity value of ≤ -5.0 kcal/mol indicated a strong interaction between receptor and ligand.

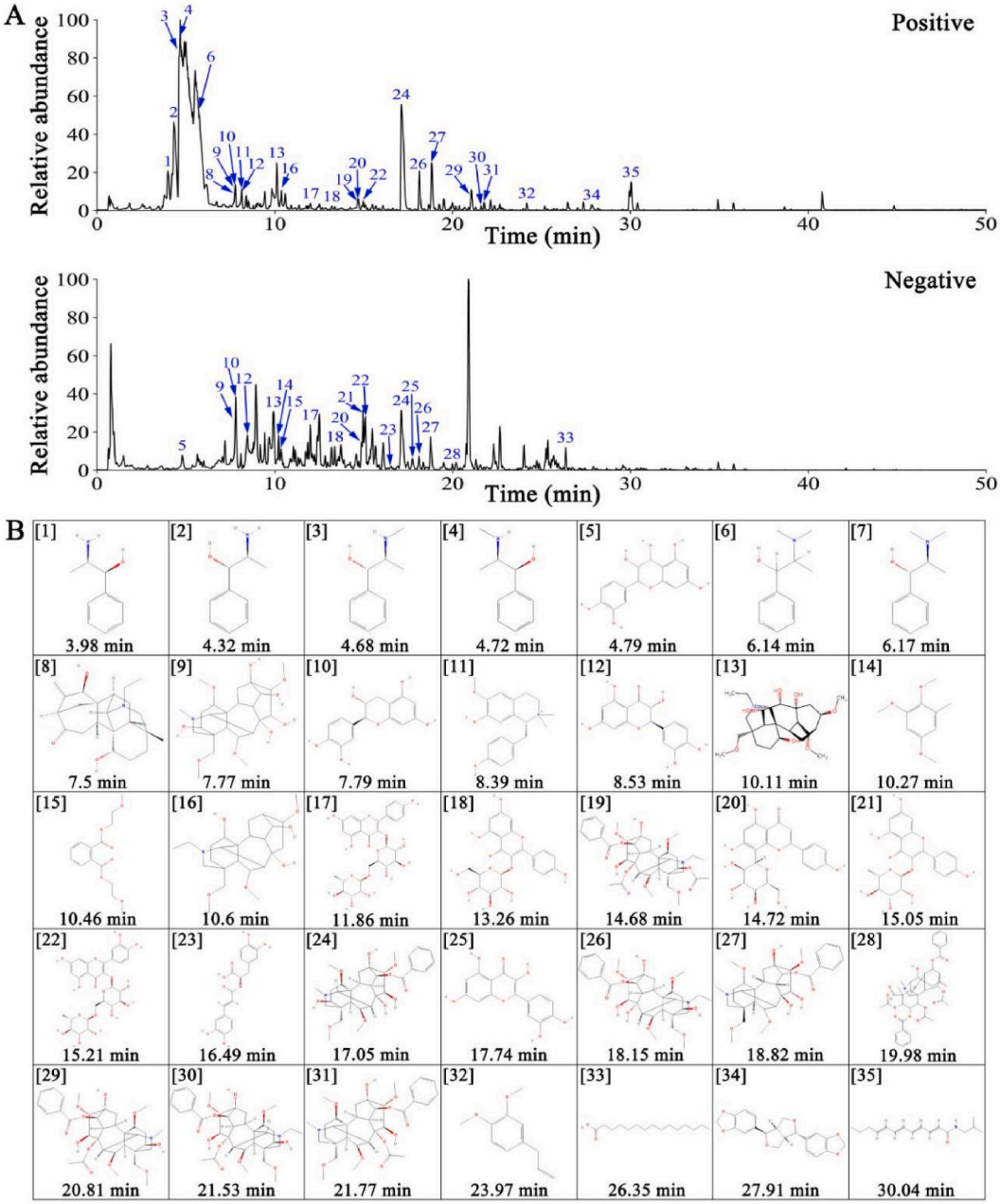

Fig. 1. Characterisation of MFXD components. (A) Total ion current chromatogram of MFXD in the positive and negative ionisation modes. (B) Chemical structures of identified compounds present in MFXD.

Lower affinity suggests more stable binding between the two. Finally, PyMOL software was used to visualise the results. The PDB ID of the target proteins were as follows: APP (1app), GRIN2A (5H8F), GRIN1 (5H8Q), ERK (4QUM), GRM5 (3LMK), GRIN2B (7UJR), CAMK2A (6X5G).

2.11. Western blot

Spinal cord tissue was minced and homogenised in a 2 mL EP tube containing steel beads and 200 μL of RIPA lysis buffer (Meilunbio, Dalian, China, MA0151) supplemented with 2 μL PMSF and 2 μL phosphatase inhibitors (Meilunbio, Dalian, China, MB12707) using a sonicator at maximum power for 10 s. The homogenate was incubated on ice for 30 min for complete lysis and the supernatant was collected. Forty micrograms of protein, a protein ladder for 20–120 kDa (GenScript, Piscataway, NJ, USA, M00521), or a protein ladder for 10–250 kDa (Mei5bio, Beijing, China, MF028-plus-01) were run on an SDS-PAGE gel and transferred to a PVDF membrane (Millipore, MA, USA, IPVH00010) using a semi-dry blotting apparatus. The membranes were blocked with 5 % milk in TBST buffer, incubated with the primary antibody diluted in TBST, and then incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (Boster, Wuhan, China, BA1054) or HRP-conjugated goat anti-mouse IgG (Boster, Wuhan, China, BA1051) diluted in TBST. After five washes with TBST for 5 min each, the PVDF membrane was incubated with ECL reagent (Affinity, USA, KF8003) for several minutes. The excess reagent was removed, and the membrane was covered with plastic wrap. An X-ray film (Beyotime, Shanghai, China, FF057) was then pressed onto the membrane and developed, fixed, and washed for final visualisation. The primary antibodies were as follows: p-NMDAR2B (Invitrogen, Carlsbad, CA, PA1-4633), NMDAR2B (Proteintech, Wuhan, China, 21920-1-AP), p-CaMKIIα (Abcam, Cambridge, UK, ab5683), CaMKIIα (Abmart, Shanghai, China, T56778S), p-ERK (Abcam, Cambridge, UK, ab201015), ERK (Proteintech, Wuhan, China, 11257-1-AP), p-CREB (Abcam, Cambridge, UK, ab32096), CREB (Proteintech, Wuhan, China, 122081-1-AP).

2.12. Immunofluorescence

Spinal cord tissues were dehydrated using a graded series of alcohols, cleared in xylene, and infiltrated with paraffin wax. Paraffin-embedded tissues were sectioned, de-paraffinised, and subjected to antigen retrieval using the appropriate repair solution. The tissue sections were encircled using an immunohistochemistry pen and incubated in diluted normal goat serum (GBCBIO Technologies,

Table 1
The compounds present in MFXD.

NO.	Identity	Mode	Molecular Formula	RT (min)	[M+H]+ (*m/z*)
1	L-Norephedrine	Pos	$C_9H_{13}NO$	3.98	134.0964
2	Norpseudoephedrine	Pos	$C_9H_{13}NO$	4.32	134.09636
3	Pseudoephedrine	Pos	$C_{10}H_{15}NO$	4.68	148.11188
4	Ephedrine	Pos	$C_{10}H_{15}NO$	4.72	166.12248
5	Resivit	Neg	$C_{15}H_{14}O_7$	4.79	305.06728
6	Methylephedrine	Pos	$C_{11}H_{17}NO$	6.14	180.13816
7	(+)-N-Methylpseudoephedrine	Pos	$C_{11}H_{17}NO$	6.17	162.127717
8	Songorine	Pos	$C_{22}H_{31}NO_3$	7.50	358.23733
9	Mesaconine	Neg	$C_{24}H_{39}NO_9$	7.77	530.261489
10	(+)-catechin	Pos	$C_{15}H_{14}O_6$	7.79	289.07214
11	Magnocurarine	Pos	$C_{19}H_{24}NO_3^+$	8.39	314.17486
12	Taxifolin	Pos	$C_{15}H_{12}O_7$	8.53	609.123946
13	6-Demethyldesoline	Neg	$C_{24}H_{39}NO_7$	10.11	498.27156
14	2,3,5-trimethoxytoluene	Neg	$C_{10}H_{14}O_3$	10.27	181.086251
15	DMEP	Neg	$C_{14}H_{18}O_6$	10.46	327.109116
16	Bullatine b	Pos	$C_{24}H_{39}NO_6$	10.60	438.284368
17	Nicotiflorin	Pos	$C_{27}H_{30}O_{15}$	11.86	595.165311
18	Astragalin	Pos	$C_{21}H_{20}O_{11}$	13.26	449.107545
19	3-acetylaconitine	Pos	$C_{31}H_{47}NO_{13}$	14.68	606.289833
20	Vitexin	Pos	$C_{21}H_{20}O_{10}$	14.72	433.112385
21	Kaempferol-3-rhamnoside	Neg	$C_{21}H_{20}O_{10}$	15.05	477.104115
22	Rutin	Pos	$C_{27}H_{30}O_{16}$	15.21	611.160179
23	Rosmarinic acid	Neg	$C_{18}H_{16}O_8$	16.49	359.078091
24	Benzoylmesaconine	Neg	$C_{31}H_{43}NO_{10}$	17.05	588.282693
25	Quercetin	Neg	$C_{15}H_{10}O_7$	17.74	301.035734
26	Benzoylaconine	Pos	$C_{32}H_{45}NO_{10}$	18.15	604.310505
27	Benzoylhypacoitine	Pos	$C_{31}H_{43}NO_9$	18.82	574.300199
28	Delgrandine	Neg	$C_{41}H_{43}NO_{12}$	19.98	786.276323
29	Mesaconitine	Pos	$C_{33}H_{45}NO_{11}$	20.81	632.306061
30	Aconitine	Pos	$C_{34}H_{47}NO_{11}$	21.53	646.321891
31	Hypaconitine	Pos	$C_{33}H_{45}NO_{10}$	21.77	616.310790
32	Methyleugenol	Pos	$C_{11}H_{14}O_2$	23.97	161.095943
33	Pentadecylic acid	Neg	$C_{15}H_{30}O_2$	26.35	287.223061
34	Asarinin	Pos	$C_{20}H_{18}O_6$	27.91	337.106756
35	Dodecatetraenamide, N-(2-methylpropyl)-	Pos	$C_{16}H_{25}NO$	30.04	248.200541

Guangdong, China). Sections were then incubated with a diluted primary antibody. Following three 3-min washes with PBST, the sections were incubated with diluted Cy3-labeled goat anti-rabbit IgG secondary antibody (Boster, Wuhan, China). Four 3-min washes with PBST were performed, followed by a 5-min incubation with DAPI. Finally, the sections were coverslipped with an anti-fluorescence quenching mounting medium (SouthernBiotech, Birmingham, AL, USA) and visualised under a fluorescence microscope. The primary antibodies were as follows: p-NMDAR2B (Invitrogen, PA1-4633), p-CaMKIIα (Abcam, ab5683), p-ERK (Abcam, ab201015), and p-CREB (Abcam, ab32096).

2.13. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) using IBM SPSS Statistics 26.0. All data are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization of compounds from MFXD

The chemical compounds in MFXD were identified using ultra-high performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS). Fig. 1A shows the total ion current chromatograms of the MFXD, providing a comprehensive view of the detected compounds. Detailed information on the identified ingredients of MFXD is presented in Table 1. Thirty-five compounds were preliminarily characterised and their respective structural formulas are shown in Fig. 1B and Table 1.

3.2. Analgesic effects of MFXD on CCI rats

An NeP rat model was constructed using CCI surgery to investigate the potential of MFXD in ameliorating NeP. Two key pain sensitivity indicators, the MWT and TWL, were assessed. Prior to CCI surgery, no significant differences were found in MWT and TWL values among the sham group, CCI, pregabalin (PGB), low-dose MFXD (L-MFXD), and high-dose MFXD (H-MFXD) groups (Fig. 2A and B). On days 3 and 7 post-CCI surgery, both the CCI group and the treatment groups (PGB, L-MFXD, and H-MFXD) exhibited a substantial reduction in MWT and TWL values compared to the sham group (Fig. 2A and B). Subsequently, MWT and TWL values gradually increased in the PGB, L-MFXD, and H-MFXD groups. In contrast, MWT and TWL values remained at a lower level in the sham group on days 11 and 15 (Fig. 2A and B). These findings underscore the significant analgesic efficacy of MFXD in CCI rats.

3.3. Target prediction of compounds of MFXD against NeP

Using a network pharmacology approach, we systematically analyzed the targets associated with both MFXD and NeP. A total of 835 targets for the 35 compounds in MFXD and 1321 therapeutic targets associated with NeP treatment were identified (Fig. 3A). This comprehensive analysis culminated in the identification of a subset of 226 common targets indicative of potential interactions between MFXD and NeP (Fig. 3A). To further elucidate the significance of these potential targets, all the 226 targets were subjected to network topological analysis, which led to the construction of an interaction network (Fig. 3B). Fifty-eight targets were identified as key targets (Fig. 3C).

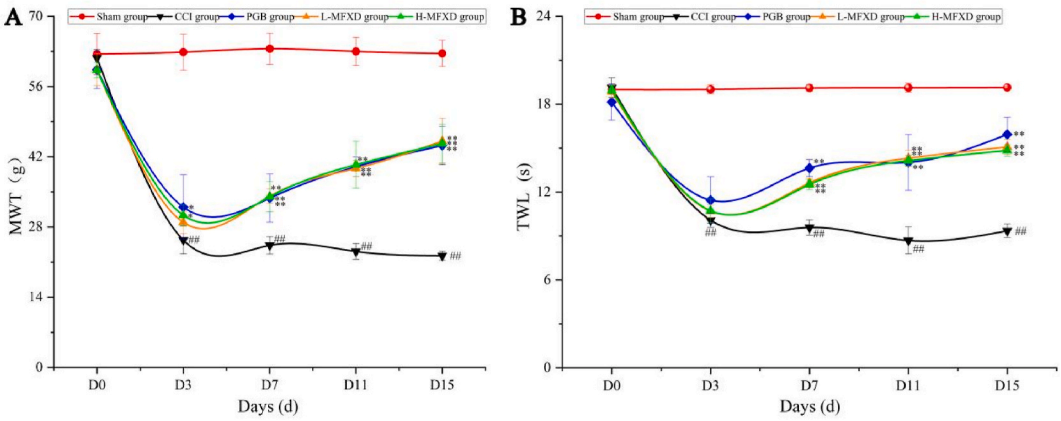

Fig. 2. Assessing the analgesic efficacy of MFXD in CCI rats. (A) The MWT values in the sham group, CCI group, PGB group, L-MFXD group and H-MFXD group in different test points. (B) The TWL values in each group. The data is presented as the mean ± SD and analyzed by one-way ANOVA with a post hoc Bonferroni test for multiple comparison (n = 3). $^{\#\#}p < 0.01$ compared to the sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the CCI group.

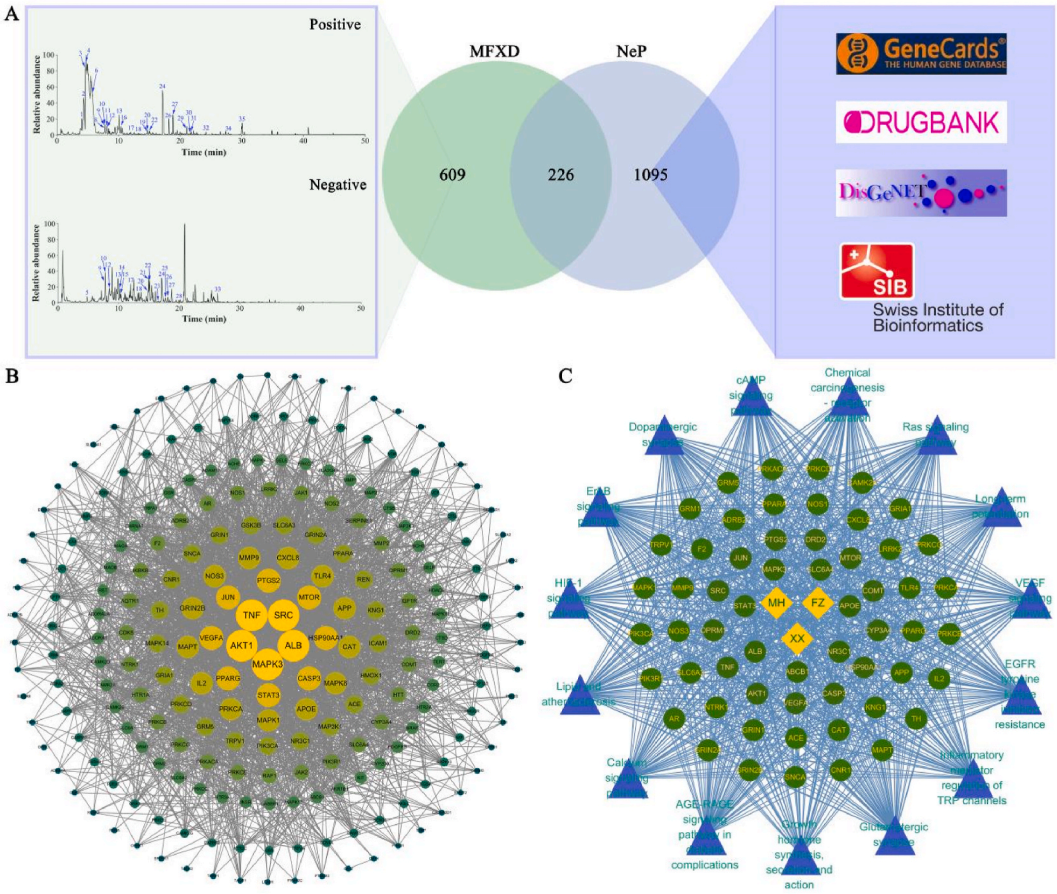

Fig. 3. Network pharmacological prediction of MFXD targets against NeP. (A) Overlap of MFXD and NeP targets identified using a Venn diagram. (B) PPI network of MFXD targets against NeP. (C) The MH/FZ/XX-key target-signalling pathway network.

3.4. Analysis of key targets of MFXD against NeP

Gene annotation and functional enrichment analyses were conducted to identify the putative functions of 58 key targets. GO biological processes revealed three primary aspects relevant to NeP treatment: regulation of neuronal signal transmission, regulation of neuronal death, and other related processes (Fig. 4A). GO cellular components included membrane raft, membrane microdomain, dendritic spine, and neuron spine (Fig. 4B). GO molecular functions were predominantly associated with protein kinase activity, glutamate receptor activity, and neurotransmitter receptor activity (Fig. 4C). Furthermore, KEGG signalling pathway enrichment analysis revealed that the key targets of MFXD treatment for NeP were primarily involved in cAMP signaling pathway, calcium signaling pathway, long-term potentiation, dopaminergic synapse, inflammatory mediator regulation of TRP channels, and glutamatergic synapse (Figs. 3C and 4D).

3.5. PPI network and MCODE enrichment analysis of core targets of MFXD against NeP

The MCODE algorithm identified highly correlated core targets within the PPI network, resulting in five significant clusters (Fig. 5A). Functional enrichment analyses revealed diverse functions across the different clusters. Notably, cluster 2 exhibited enrichment specifically related to neuronal synapse and glutamate receptor function (Fig. 5B). The top three biological processes included glutamate receptor signaling pathway regulation, neuronal synaptic plasticity regulation, and trans-synaptic signaling regulation (Fig. 5B). The top three cellular components were the postsynaptic density, dendritic spines and neuron spines (Fig. 5B). Additionally, the molecular functions were mainly associated with glutamate receptor activity, calcium-dependent protein serine/threonine kinase activity, and neurotransmitter receptors (Fig. 5B). Moreover, KEGG enrichment analysis showed that core targets of MFXD treatment for NeP exhibited the most significant enrichment in LTP (Fig. 5C). These findings suggest that the crucial mechanisms underlying MFXD treatment of NeP may involve modulation of LTP.

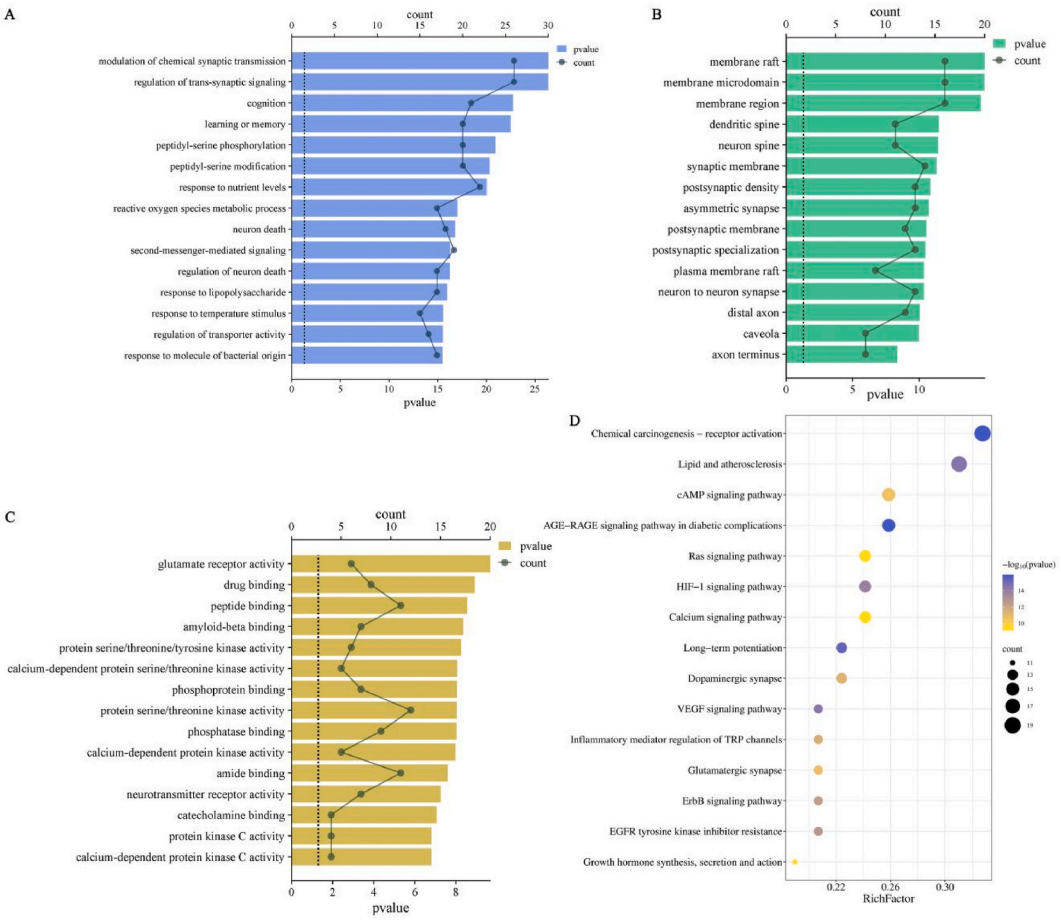

Fig. 4. GO and KEGG enrichment analysis of key targets of MFXD against NeP. GO enrichment analysis of biological processes (A), cellular components (B) and molecular functions (C). (D) KEGG enrichment analysis.

3.6. Relationship between MFXD compounds and targets

Molecular docking confirmed the binding affinity of the identified components of MFXD to the core targets. The top three pairs exhibiting strong binding abilities: APP-mesaconitine (−10.2 kcal/mol), rosmarinic acid (−10.7 kcal/mol), and delgrandine (−9.5 kcal/mol); CAMK2A-mesaconitine (−12.9 kcal/mol), rosmarinic acid (−14.3 kcal/mol), and delgrandine (−12 kcal/mol); ERK-mesaconitine (−8.7 kcal/mol), rosmarinic acid (−9.1 kcal/mol), and delgrandine (−10.8 kcal/mol); GRIN1-mesaconitine (−11.4 kcal/mol), rosmarinic Acid (−10.9 kcal/mol), and delgrandine (−12 kcal/mol); GRIN2A-mesaconitine (−11.4 kcal/mol), rosmarinic acid (−10.9 kcal/mol), and delgrandine (−12 kcal/mol); GRIN2B-mesaconitine (−11 kcal/mol), rosmarinic acid (−12.8 kcal/mol), and delgrandine (−10.9 kcal/mol); GRM5-mesaconitine (−10.6 kcal/mol), rosmarinic acid (−11.6 kcal/mol), and delgrandine (−10.4 kcal/mol) (Fig. 6A). The typical binding pairs are shown in Fig. 6B. Collectively, these findings demonstrate that mesaconitine, rosmarinic acid, and delgrandine exhibit robust binding to core targets, suggesting their dominant roles in MFXD activity.

3.7. MFXD inhibit LTP by suppressing the NMDAR2B/CaMKIIα/ERK/CREB signalling pathway

Based on the outcomes of functional enrichment analysis, MCODE enrichment analysis, and molecular docking, LTP stood out as the most pivotal signalling pathway regulated by MFXD, contributing to its analgesic effects in CCI rats. To investigate this further, the expressions of key proteins associated with LTP was assessed. Western blot results showed a distinct increase in the phosphorylation levels of NMDAR2B, CaMKIIα, ERK, and CREB in the CCI group compared with the sham group (Fig. 7; Supplementary Fig. 1). Conversely, the phosphorylation levels were lower in the H-MFXD group than that in the CCI group (Fig. 7; Supplementary Fig. 1). Furthermore, immunofluorescence results corroborated these findings, illustrating distinctly higher phosphorylation levels of NMDAR2B, CaMKIIα, ERK, and CREB in the CCI group than in the sham group (Fig. 8). In contrast, the H-MFXD group exhibited markedly lower phosphorylation levels than did the CCI group (Fig. 8). Taken together, these results suggest that LTP is activated following CCI surgery, whereas MFXD exerts its analgesic effects by inhibiting LTP through the suppression of the NMDAR2B/CaMKIIα/ERK/CREB signalling pathway in the spinal cord of CCI rats (Fig. 9).

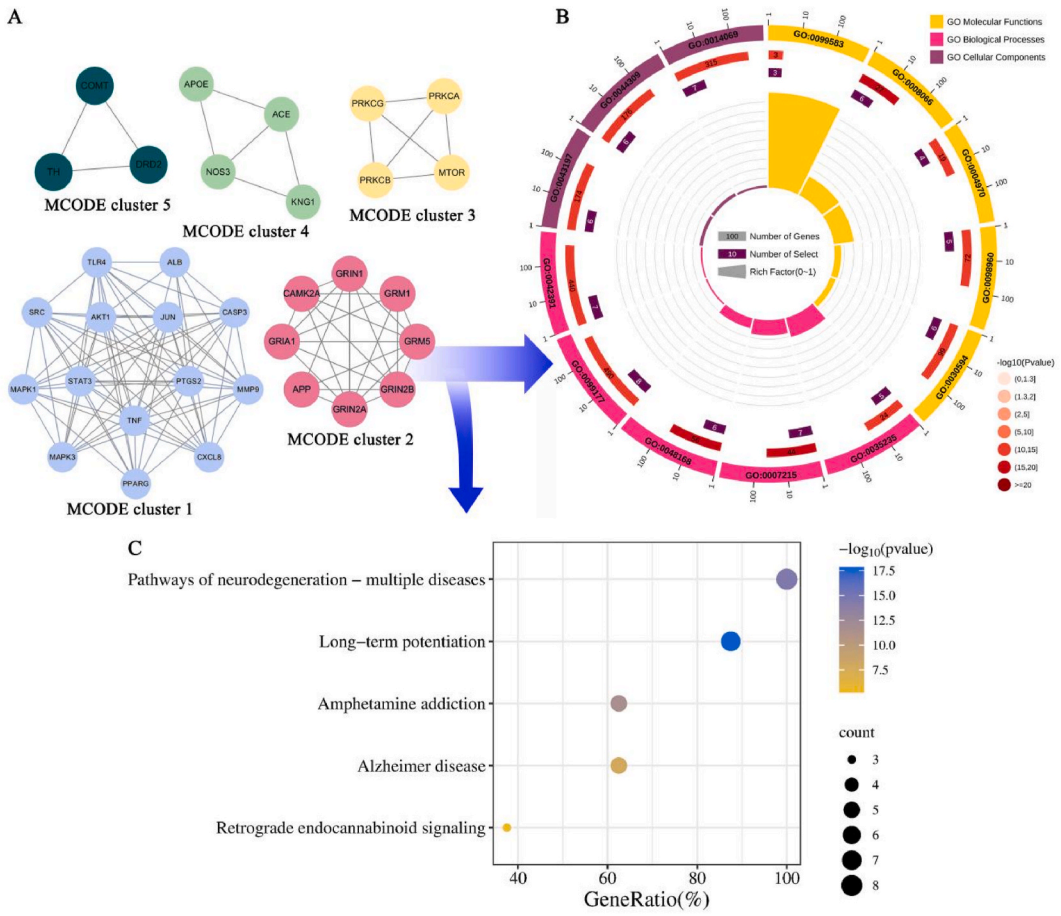

Fig. 5. Prediction of MFXD core targets against NeP. (A) MCODE clustering of core targets. (B) GO enrichment analysis of MCODE cluster 2. (C) KEGG enrichment analysis of MCODE cluster 2.

4. Discussion

NeP, the most prevalent, intractable, and challenging form of chronic pain, arises from a diverse range of causes such as spinal cord injury, diabetic neuropathy, herpes zoster virus infection, stroke and post-surgical complications. Despite advancements in treatment, the existing options for NeP remain limited and are often accompanied by a multitude of adverse effects. Consequently, a significant proportion of patients continue to experience the debilitating consequences of the condition [1–3]. Hence, there is a crucial need to explore novel and effective treatments to alleviate NeP. TCM has a long history of clinical application in pain management, offering valuable contributions and innovative insights into NeP treatment. The multifaceted components, targets, and mechanisms of TCM align well with the diverse pathogenic mechanisms underlying NeP, suggesting its potential to effectively treat this condition through multiple pathways [14–16].

MFXD is a classic TCM formula that renowned for its efficacy in treating various types of pain in the clinic [16–19]. Multiple studies have investigated the efficacy and safety of MFXD in patient cohorts diagnosed with bradyarrhythmia, allergic rhinitis, or mild bronchial asthma. These studies collectively demonstrate promising clinical effects and a favorable safety profile for MFXD in these patient populations [17,22,23]. MFXD comprises three herbs: Ephedrae Herba (*Mahuang*), Radix Aconiti Lateralis Preparata (*Fuzi*), and Asarum Heterotropoides (*Xixin*) [16,17]. Ephedrae Herba (*Mahuang*) was derived from the dried stems of Ephedra sinica Stapf. The primary active compounds, ephedrine and pseudoephedrine, are known for their anti-inflammatory properties, which are believed to contribute to the herb's analgesic effect [24]. Radix Aconiti Lateralis Preparata, commonly known as *Fuzi*, is a TCM derived from the processed lateral root of Aconitum Carmichaelii Debx. *Fuzi* holds a prominent position in Chinese medicine and is recognised for its effectiveness in alleviating a spectrum of neurological disorders. Due to its analgesic properties, *Fuzi* is believed to offer therapeutic benefits in conditions such as depression, epilepsy, and dementia [25]. The dried roots and rhizomes of a plant called *Asarum heterotropoides* Fr. Schmidt var. *mandshuricum* (Maxim.) Kitag, also known as *Xixin* in Chinese or Asarum Heterotropoides, is a highly valued TCM belonging to the genus *Asarum* (Aristolochiaceae). It is used to treat pain and inflammation in various Asian countries [26].

The chemical composition of MFXD was initially determined using UHPLC-HRMS and 35 compounds were identified. Among these, ephedrine alkaloids, a group of naturally occurring stimulants found in plants, particularly in the Ephedra genus, are prominent. Six

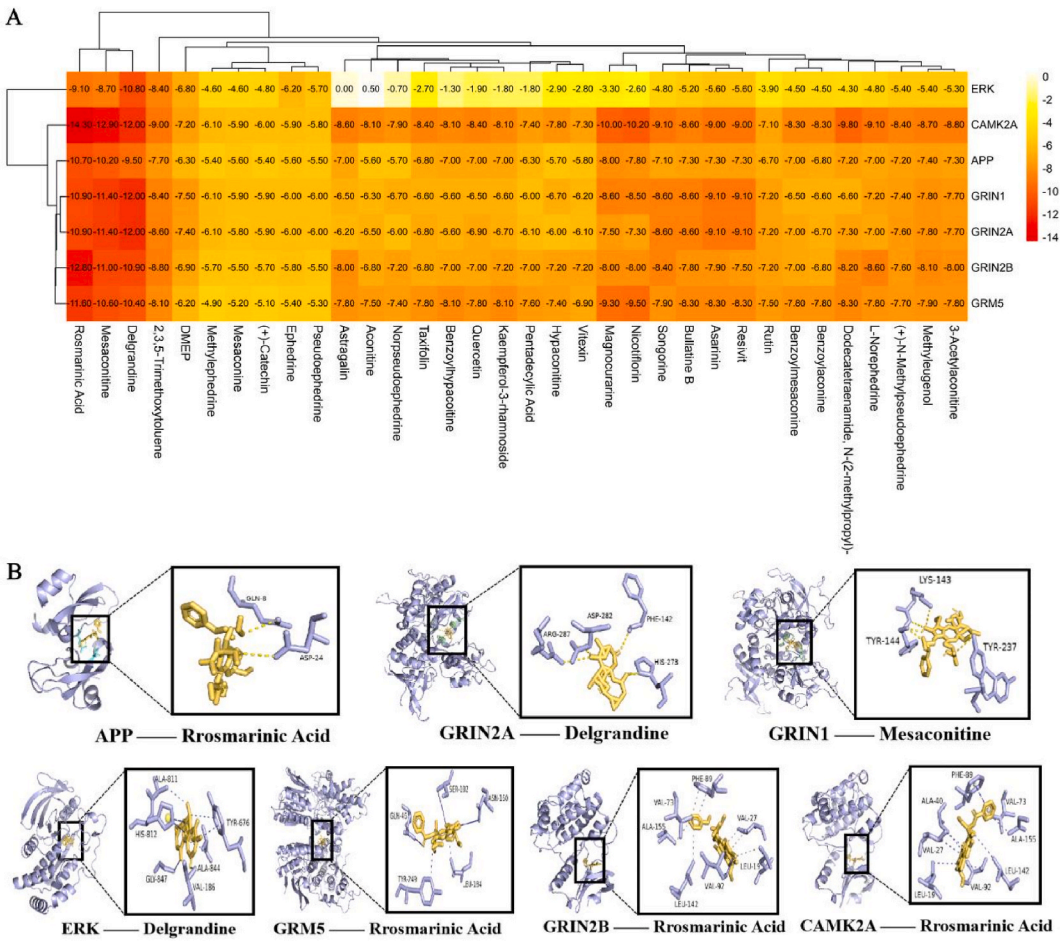

Fig. 6. Molecular docking analysis of MFXD compounds with core targets. (A) Heatmap depicting docking scores for MFXD compounds with core targets. (B) Representative docking complexes illustrating the interactions between specific MFXD compounds and core targets.

ephedrine alkaloids were identified, namely ephedrine, pseudoephedrine, norpseudoephedrine, l-norepinephrine, methylephedrine, and (+)-N-methylpseudoephedrine. Studies have shown that ephedrine and ephedrine can inhibit formalin-induced pain in mice [27]. In clinical settings, combination therapy with acetylsalicylic acid and pseudoephedrine can significantly ameliorate throat pain in adult patients with confirmed acute pharyngitis and rhinosinusitis associated with upper respiratory tract infection [28]. Additionally, oral pseudoephedrine has demonstrated efficacy in relieving ear-drum pain during aviation flights [29]. Nine aconitine, mesaconitine, hypaconitine, mesaconine, songorine, 3-acetylaconitine, benzoylmesaconine, benzoylaconine, and benzoylhypacoitine were identified as aconitum alkaloids, a group of diterpenoid alkaloids found in Aconiti Lateralis Preparata. In particular, mesaconitine has been shown to possess antinociceptive effects in different nociceptive test models of rats and mice [30]. Aconitine and mesaconitine reduce in formalin-induced nociceptive behaviour in mice [31]. Another study reported that songorine, mesaconitine, and hypaconitine exhibited antinociceptive effects in a mouse model of acetic cramps [32]. Moreover, when mesaconitine was microinjected into the nucleus reticularis paragigantocellularis (NRPG), nucleus raphe magnus (NRM), and periaqueductal gray (PAG) of rats, it produced a dose-dependent antinociceptive effect on the brain stem. In contrast, benzoylmesaconine, when injected into NRM or PAG, demonstrated antinociceptive action; however, this effect was not observed in NRPG [33]. Catechins, polyphenolic compounds found in various fruits, vegetables, Chinese herbal medicines, and beverages, have also been identified in MFXD. They have undergone exploration for antioxidant and neuroprotective properties of these compounds have also been explored. Catechin exhibits analgesic effects on the CCI-induced NeP rat model by blocking NF-κβ activation, leading to a decrease in NF-κB-regulated inflammatory cytokines [34]. Taxifolin is a naturally occurring flavonoid with antioxidant, anti-microbial and anti-inflammatory properties. It also alleviates alloxan-induced hyperglycamiarelated neuropathy and NeP in rats [35]. Bullatine B, also known as neoline, is a diterpenoid alkaloid characterised by a complex structure and a diverse range of biological activities. It has been observed to significantly alleviate NeP in rat models induced by either paclitaxel or partial ligation of the sciatic nerve [36]. Bullatine B demonstrates the ability to relieve mechanical hyperalgesia in a streptozotocin (STZ)-induced diabetic mouse model. This effect was attributed to the inhibition of the Nav1.7 voltage-gated sodium channel (VGSC), a pivotal element in the transmission of pain signals [37]. Astragalin, a prevalent natural flavonoid in TCM, possesses a spectrum of pharmacological properties, including analgesic effects. Astragalin mitigates NeP in

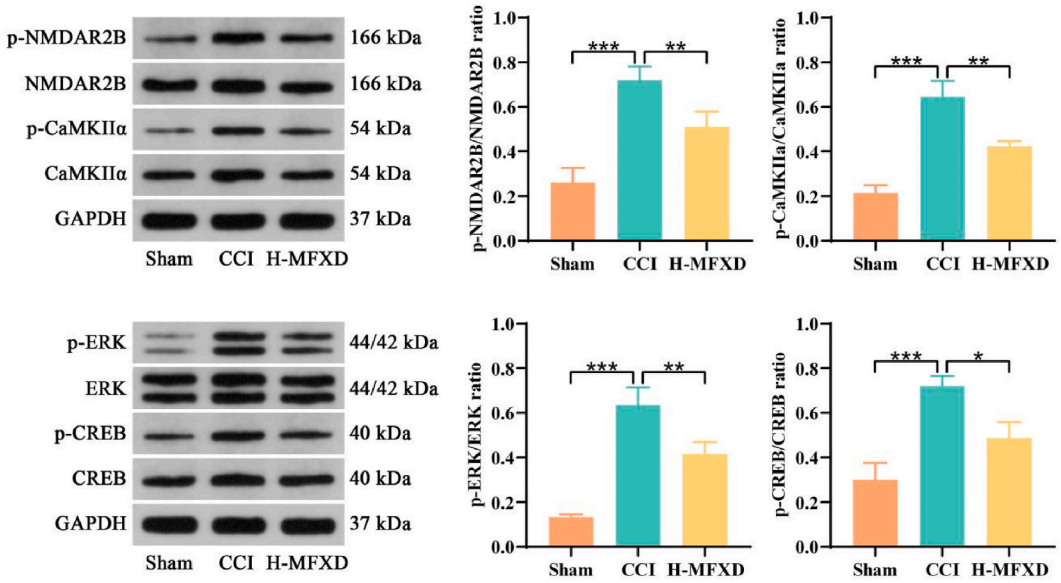

Fig. 7. Western blot analysis of the phosphorylation of NMDAR2B, CaMKIIα, ERK, and CREB in the spinal cord of the sham, CCI and H-MFXD groups. Data are presented as mean ± SD and analyzed by one-way ANOVA with a post hoc Bonferroni test for multiple comparison (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 compared to the CCI group.

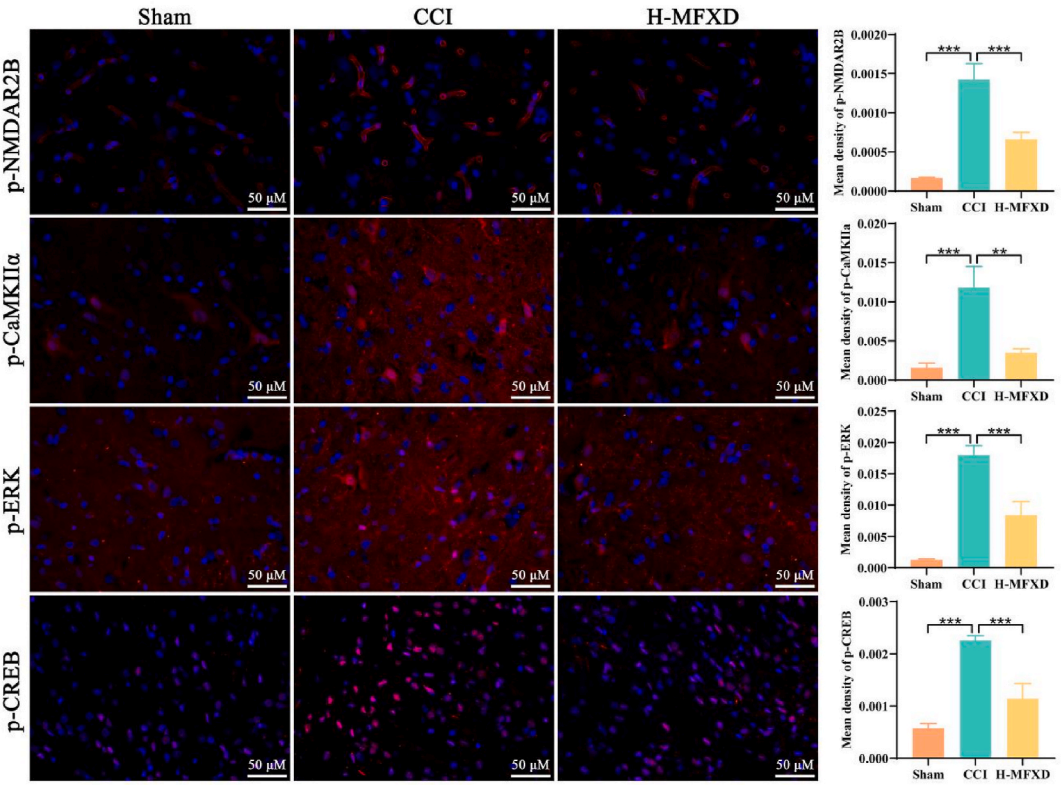

Fig. 8. Immunofluorescence analysis of the phosphorylation levels of NMDAR2B, CaMKIIα, ERK, and CREB in the spinal cord of the sham, CCI and H-MFXD groups. Data is presented as the mean ± SD and analyzed by one-way ANOVA with a post hoc Bonferroni test for multiple comparison (n = 3). **p < 0.01, ***p < 0.001 compared to the CCI group.

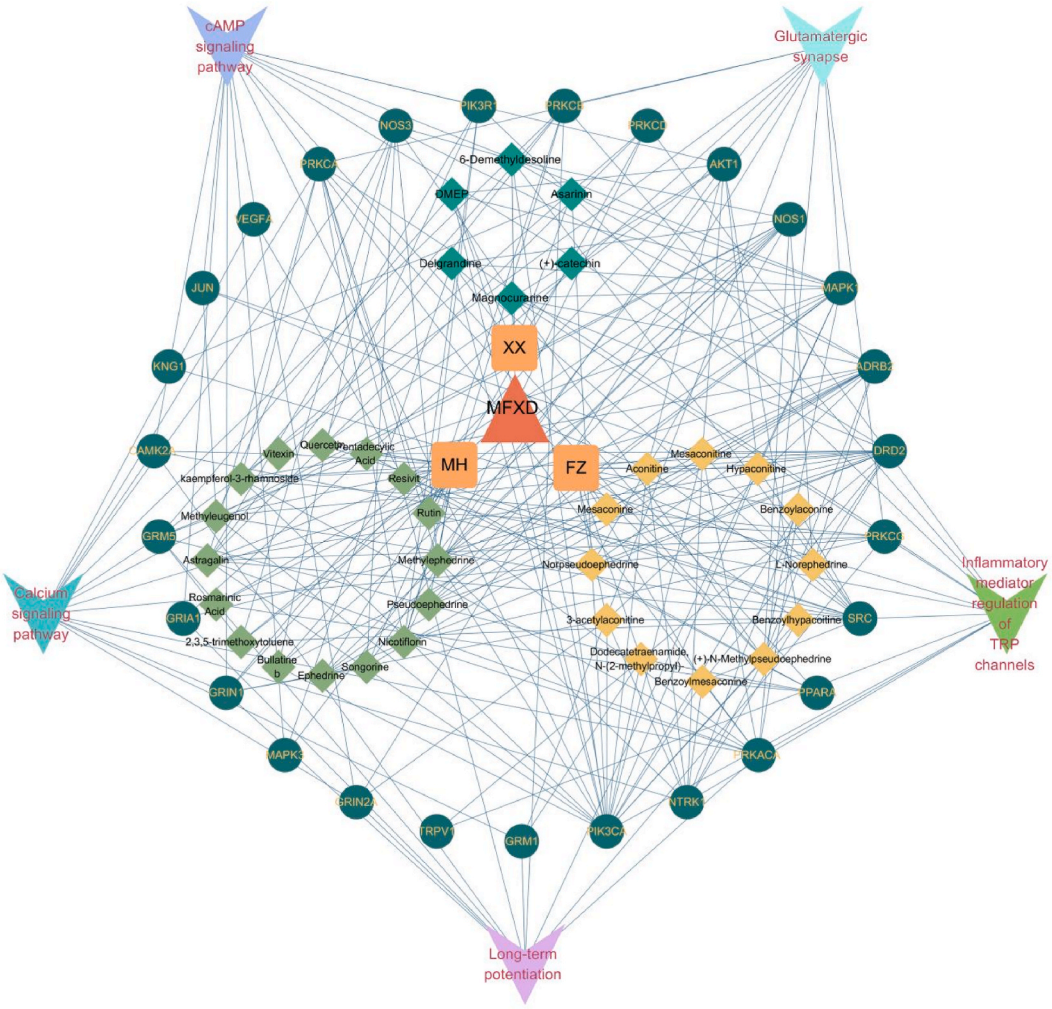

Fig. 9. MFXD mechanism in the treatment of NeP.

a rat model of CCI. This effect is achieved through inhibition of the P2X4/ERK-mediated pathway and attenuation of satellite glial cell activation in the dorsal root ganglia [38]. Vitexin is a flavonoid with antinociceptive and antioxidant activities. This demonstrates the ability to alleviate pain-related behaviours induced by different stimuli. In inflammatory pain models, the analgesic effect is attributed to the inhibition of TRPV1 activity, reduction in oxidative stress, suppression of hyperalgesic cytokine production, and elevation of anti-hyperalgesic cytokine levels [39]. Furthermore, vitexin shows analgesic effects in both acute and chronic pain, mediated by the TRPV4 pathway [40]. Rutin is a citrus flavonoid glycoside with anti-inflammatory, analgesic, and antioxidant properties that occurs naturally in various plants. This water-soluble is converted into quercetin upon entry into the bloodstream. Rutin has been shown to significantly inhibit oxaliplatin-induced chronic painful peripheral neuropathy in mice by improving oxidative stress-induced damage to the dorsal horn neurones [41]. Rosmarinic acid, a polyphenolic compound, possesses multiple pharmacological activities and potential health benefits. In a rat model of NeP induced by CCI, rosmarinic acid exhibited antinociceptive properties through anti-apoptotic and anti-inflammatory effects [42]. Moreover, in a Spared Nerve Injury (SNI) mice model, rosmarinic acid was effective in reducing hyperalgesia and NeP-associated symptoms. This improvement was linked to the amelioration of microglial senescence in both the hippocampus and spinal cord of SNI-treated mice [43]. Quercetin, a highly abundant dietary flavonoid derived from numerous fruits and vegetables, exhibits anti-inflammatory and analgesic effects. In a mouse model of intense acute swimming-induced muscle pain, quercetin demonstrates the ability to alleviate mechanical muscle hyperalgesia [44]. Furthermore, quercetin exhibits diverse analgesic effects against chronic pain in various rodent models [45]. Methyleugenol, an alkenylbenzene compound derived from several essential oils found in plants such as *Asiasari radix*, has been identified as an agonist of γ-aminobutyric acid A (GABAA) receptors. This compound has demonstrated efficacy in inhibiting formalin-induced pain in mice by suppressing NMDA receptor-mediated hyperalgesia [46,47].

Network pharmacology integrates computational biology, network analysis, and experimental pharmacology to study the complex interactions between drugs and their targets within a biological network [48]. Using network pharmacology, 226 common and 58 key

targets of the 35 components of MFXD against NeP were identified. Through functional enrichment analysis, key targets were significantly enriched in the cAMP signaling pathway, calcium signaling pathway, LTP, inflammatory mediator regulation of TRP channels, and glutamatergic synapse, which are all closely related to NeP.

LTP is a fundamental cellular mechanism underlying learning and memory. It manifests as a sustained increase in the synaptic strength and connections between neurones [4]. LTP initiation involves high-frequency stimulation of presynaptic neurones and is believed to be orchestrated by various signalling pathways, among which the N-methyl-D-aspartate (NMDA) receptor signalling pathway holds particular significance. NMDA receptors located on postsynaptic neurones are activated in response to glutamate binding, allowing calcium ions to enter the postsynaptic neurones. This influx triggers the activation of key signalling molecules, including CaMKII and protein kinase C (PKC). These molecules, in turn, facilitate the insertion of additional AMPA receptors into the postsynaptic membrane, thereby enhancing synaptic strength [5–7]. The MAPK signalling pathway is another player in LTP. MAPKs, a family of enzymes activated by diverse stimuli, including calcium ions, phosphorylate various proteins, including transcription factors. In the context of LTP, MAPKs activate transcription factors that drive the increased the expression of genes essential for synaptic plasticity [5–7]. In the context of NeP development, has emerged as a contributing factor that increases the sensitivity of pain neurones to sensory inputs. LTP can strengthen synapses among nociceptors and neurones that detect pain and facilitate the transmission of pain signals to the brain. Additionally, LTP may induce the formation of new synapses between nociceptors and other spinal neurones, establish new pain pathways and intensifying pain signals. Moreover, LTP induces alterations in the spinal cord neuronal properties, making them more excitable and more likely to fire in response to pain signals, thereby amplifying pain sensitivity [4–7]. Considering its potential therapeutic implications, LTP has emerged as a promising target for NeP treatment. Inhibiting LTP holds promise for mitigating pain sensitivity in individuals grappling with NeP. NMDA blockade can impede LTP, thereby curtailing the transmission of pain signals. NMDA receptor antagonists such as ketamine, dextromethorphan, and memantine have demonstrated efficacy in clinical trials, offering a promising avenue for NeP treatment [4].

Glutamatergic synapses are chemical synapses where the neurotransmitter involved is glutamate. Glutamate serves as an excitatory neurotransmitter responsible for the transmission of pain signals. In NeP, there is an increase in glutamate release and a decrease in glutamate uptake, leading to excess of glutamate at the synapse. This excess glutamate can activate NMDA receptors, which are a type of glutamate receptor that is involved in pain signalling. Activation of NMDARs causes changes in neurones, such as increased excitability and plasticity, which can contribute to the development and persistence of NeP [49]. Based on a thorough review of the existing literature and our network pharmacology analysis, we hypothesised that MFXD exerts its analgesic effects by modulating multiple signalling pathways, including the cAMP and calcium signalling pathways, LTP, inflammatory mediator regulation of TRP channels, and glutamatergic synapse. To identify the most crucial signalling pathway regulated by MFXD, we employed the MCODE algorithm to extract core targets from a network of key targets. MCODE (Motif Cluster Detection) is a graph-based algorithm that identifies densely connected subgraphs within large PPI networks. It is a widely used method for detecting protein complexes and groups of proteins that interact extensively. MCODE iteratively removes nodes from the network until a subgraph with a high density of interactions is identified [50]. Using the MCODE algorithm, we identified five significant clusters of core MFXD targets against NeP, with LTP being the most enriched signaling pathway among these clusters. These findings suggest that LTP plays a central role in the analgesic mechanism of MFXD.

Through molecular docking experiments, we discovered that several MFXD compounds exhibited strong binding affinities for targets associated with the LTP pathway. For example, mesaconitine, rosmarinic acid, and delgrandine demonstrate high binding affinities for GRIN2B (also known as NMDAR2B) and ERK. Additionally, mesaconitine, rosmarinic acid, and delgrandine displayed a high affinity for CAMK2A (also known as CaMKIIα). Based on the high correlation between mesaconitine, rosmarinic acid, and delgrandine from MFXD and the key targets of the LTP pathway, our next work will focus on investigating whether mesaconitine, rosmarinic acid, and delgrandine have ameliorative effects on CCI-induced NeP and explore their direct interactions with key targets such as NMDAR2B, CaMKIIα, and ERK. We further validated these findings by examining the expression of key LTP proteins in the spinal cord of CCI rats. The results demonstrated that MFXD significantly suppressed phosphorylation levels of NMDAR2B, CaMKIIα, ERK, and CREB, indicating that MFXD exerts its analgesic effect, at least partially, by inhibiting these targets via mesaconitine, rosmarinic acid, and delgrandine, thereby suppressing LTP. NMDAR interact with other nociceptive units and secondary messenger systems, contributing to NeP development. Aurora kinases have been implicated in the regulation of kinesin family member 17 (KIF17), which is associated with NMDA receptors activation. Tozasertib, a pan-aurora kinase inhibitor, effectively reduces evoked and chronic ongoing pain in nerve-injured rats by decreasing KIF17-mediated NMDAR2B activation. These findings suggest that targeting the KIF17-NMDAR2B crosstalk holds promise for NeP treatment [51,52]. *Sida cordifolia* L., a herbal medicine, has also been shown to attenuate pain hypersensitivity in the dorsal root ganglia and spinal cord of CCI rats by inhibiting KIF17-mediated NMDAR2B signalling. This further corroborated the potential of targeting the KIF17-NMDAR2B interaction during NeP treatment [53]. The erythropoietin-producing hepatoma (Eph) receptor, a tyrosine kinase receptor, interacts with various molecular switches, including NMDAR and MAPK, to regulate the pathophysiology of chronic pain [54]. Bergenin, a natural compound, mitigated chemotherapy-induced NeP by suppressing TRP channels and NMDAR2B expression [55]. Collectively, these findings highlight NMDAR2B as a pivotal protein involved in NeP and a promising therapeutic target for the effective management of NeP. In future studies, we will further investigate whether upstream regulatory factors of NMDAR2B, such as TRP channels (TRPV1 and TRPA1), Aurora kinases, or interacting molecules, such as KIF17 and Eph, mediate the analgesic effects of MFXD and their relationship with NMDAR2B.

Given the presence of multiple active compounds in MFXD, LTP may not be the sole signalling pathway responsible for MFXD's analgesic effects. Transient receptor potential (TRP) channels are sensors of a variety of cellular and environmental signals. Specific TRP channels, such as TRPV1, TRPA1, and TRPM2, are sensitive to heat, irritants, and inflammatory mediators. Their activation

triggers pain signals, which are sent to the brain [56]. TRPA1 plays a role in various forms of pain, including neuropathic cold pain, inflammatory pain, NeP and hereditary episodic pain syndromes and has been pursued as a promising drug target. In 2015, the TRPA1 antagonist ODM108 (an acetylene-containing compound) entered a phase I clinical trial for NeP. However, complex nonlinear pharmacokinetics emerged, and the clinical trial was discontinued in 2016 [56,57]. Dermorphin [D-Arg2, Lys4] (1–4) amide (DALDA), a preferential peripherally acting mu-opioid receptor agonist, significantly ameliorates paclitaxel-induced evoked and spontaneous ongoing pain in rats by downregulating TRPA1 and NMDAR2B expressions, concomitant with the inhibition of microglial activation [58]. DALDA also mitigated frostbite-induced chronic pain by reducing the expression of TRP channels (TRPA1, TRPV1, and TRPM8), glial cell activation, and neuroinflammation in the sciatic nerve, dorsal root ganglion, and spinal cord of frostbite-injured rats [59]. Additional research is needed to clarify the potential involvement of other signalling pathways, such as the cAMP signalling pathway, inflammatory mediator regulation of TRP channels and calcium signalling pathway, and other targets, such as TRPA1 and TRPV1, in MFXD's analgesic mechanism (Fig. 9). This study suggests us with the insight that treatments involving multiple components, targets, and signalling pathways of TCM may be more effective in treating NeP than approaches involving a single component, target, and signalling pathway. Clinically, a combination of different target antagonists or agonists may be an effective treatment strategy for NeP. Future studies should focus on identifying the active compounds within MFXD and exploring the molecular mechanisms underlying NeP suppression. These investigations will undoubtedly contribute to the discovery of novel therapeutic agents for the treatment of NeP.

5. Conclusion

In conclusion, this study represents a significant advancement in NeP treatment by demonstrating the analgesic efficacy of MFXD and elucidating its potential mechanism of action. This study opens new doors for exploring TCMs as an effective therapeutic strategy for NeP and paves the way for the development of novel and improved treatment options for this debilitating condition.

Data availability statement

Data is contained within the article and is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

This study was conducted in accordance with ARRIVE guidelines and approved by the Animal Experimentation Ethics Committee at The First Affiliated Hospital of Guangzhou University of Chinese Medicine (License No. GZTCMF1-2021100).

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CRediT authorship contribution statement

Yihui Chai: Validation, Investigation, Formal analysis. **Siyu He:** Writing – original draft, Validation, Software, Methodology, Investigation. **Dayi Liang:** Software, Investigation. **Chunsong Gu:** Investigation. **Qian Gong:** Validation. **Ling Long:** Investigation. **Peng Chen:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Long Wang:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35970.

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