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Research article

Study on mechanism of transdermal administration of eugenol for pain treatment by network pharmacology and molecular docking technology

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ABSTRACT

The objective of this study was to explore the pharmacological mechanism of transdermal administration of eugenol (EUG) for pain treatment. Firstly, network pharmacology techniques were employed to identify the potential targets responsible for the analgesic effect of EUG. Subsequently, molecular docking technology was used to validate interactions between EUG and the crystal structure of the core target protein. Finally, the impact of EUG on the expression and activation of TRPV1 receptors in HaCaT cells was evaluated through *in vitro* experiments, thus confirming the analysis of network pharmacology. The study suggested that the transdermal administration of EUG for pain treatment might target the TRPV1 receptor. Molecular docking revealed that EUG could spontaneously bind to the TRPV1 receptor with a high binding ability. The analysis of Western blot (WB) and intracellular Ca^{2+} levels demonstrated that EUG could increase the expression of TRPV1 in HaCaT cells, activating TRPV1 to induce intracellular Ca^{2+} influx ($P < 0.05$). These findings suggested that the initial application of EUG would cause a brief stimulation of TRPV1 receptors and upregulation of TRPV1 expression. Upon continued exposure, EUG would act as a TRPV1 agonist, increasing intracellular Ca^{2+} levels that might be associated with desensitization of pain sensations.

1. Introduction

Chronic pain is a sensory disorder that has long been a formidable obstacle in the field of medicine, profoundly impacting individuals' overall well-being. In the realm of clinical practice, opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently employed for pain management. Nevertheless, opioids possess potent addictive and tolerance-inducing properties [1], while NSAIDs can give rise to gastrointestinal adverse reactions [2]. Therefore, the pursuit of new pharmaceuticals and understanding

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their mechanisms of action are becoming increasingly imperative, with minimal side effects to the human body being an important requirement.

Traditional Chinese medicine (TCM) has beneficial clinical applications, and it is an efficacious approach to obtain effective analgesic drugs from commonly utilized Chinese herbs in clinical practice. Ground cloves are utilized for chronic pain by topical transdermal treatment in TCM. Due to its significant therapeutic effects and diminished adverse reactions, it has attracted increasing attention. Eugenol (EUG), the predominant phenolic compound obtained from clove oil, possesses considerable medicinal potential [3]. The chemical structure of EUG is similar to capsaicin (CAP), which has been demonstrated to interact with the TPRV1 receptor in

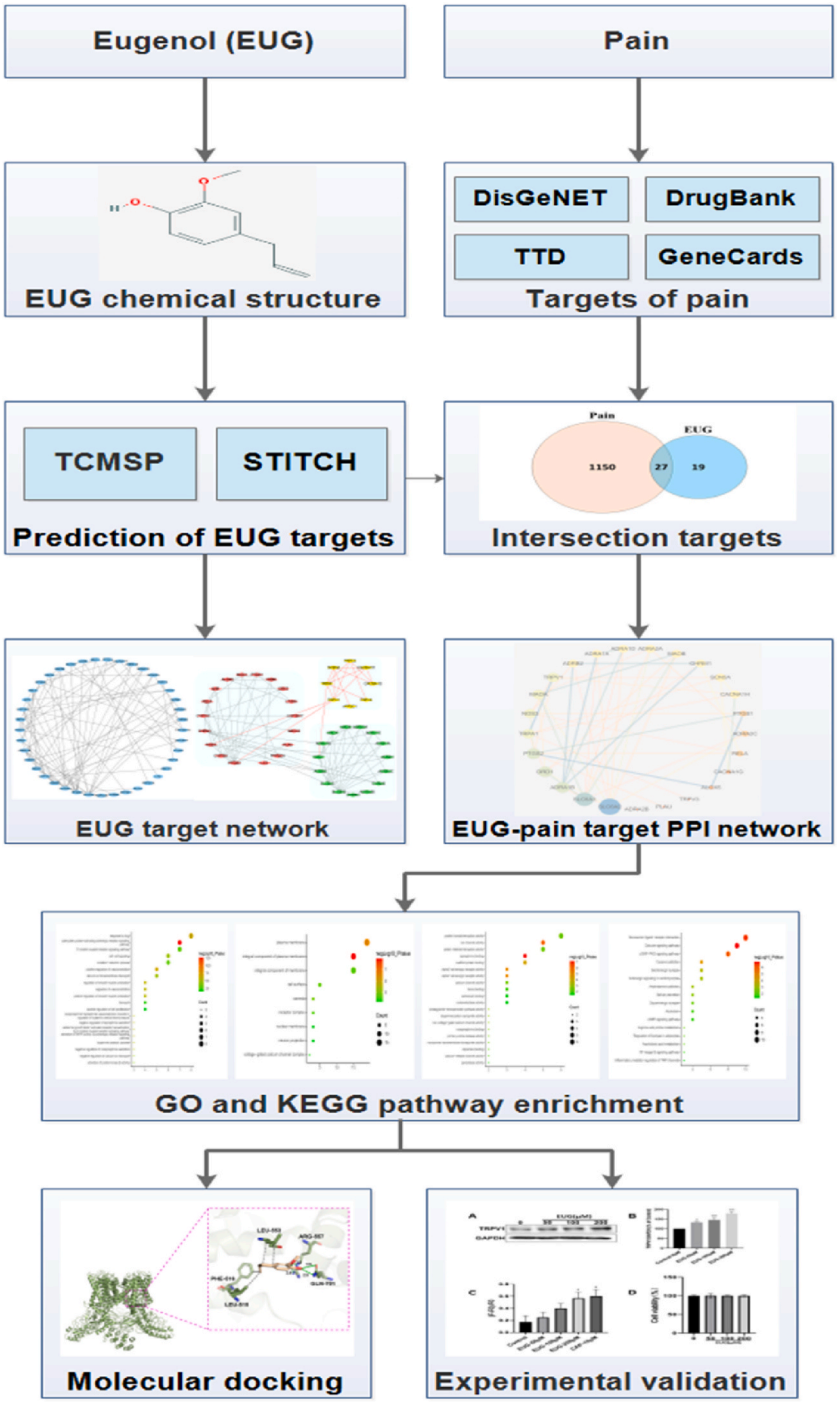


Fig. 1. Flowchart of investigating transdermal administration of EUG for pain treatment.

mammals and produce an analgesic response. TPRV1 serves as a pivotal transduction molecule for nociceptors and functions as a versatile molecular pain receptor. As a cation channel, TPRV1 plays a critical role in the molecular pathways that contribute to pain sensitization and injury-induced hyperalgesia [4–6]. Consequently, the stimulation of TPRV1 channels has been recognized as a significant approach for pain management.

It has been reported that EUG has the ability to induce the expression of TRPV1 in trigeminal ganglion neurons [7]. Furthermore, previous studies have demonstrated that EUG possesses anesthetic properties in rodents [8,9] and can mitigate neuropathic pain in rats [10]. The analgesic and local anesthetic attributes of EUG have been harnessed for the development of topical therapeutic formulations [3], particularly in the field of dentistry [11–14].

However, in TCM, research on the mechanism of clove power for pain treatment through transdermal administration has not yet been studied, and its impact on skin cells has not been investigated. As previously discussed, the activation of TRPV1 by EUG plays a significant role in its analgesic properties [7]. Based on all the information mentioned earlier, we speculated that EUG may also act on TRPV1 receptors in HaCaT cells to exert its transdermal analgesic effect. Therefore, our study used network pharmacology, molecular docking and *in vitro* validation experiments to preliminarily explore the mechanism of transdermal administration of EUG for pain treatment.

Network pharmacology has been widely used in drug research due to its holistic, systematic, and effective approach of using bioinformatics, molecular biology and databases to systematically study the interrelationships between “drugs-targets-pathways-diseases” [15,16]. In this study, we planned to initially determine the correlation between the analgesic effect of EUG through transdermal administration and TRPV1 using network pharmacology methods, and then verified our hypothesis through *in vitro* experiments, that is, EUG acting on the TRPV1 receptor plays a crucial role in the analgesic efficacy of transdermal administration.

In this study, we used network pharmacology, molecular docking and *in vitro* verification for the first time to identify the potential targets of pain relief though local transdermal administration of EUG, providing experimental evidence for its clinical application. A flowchart of the experimental procedure is shown in Fig. 1.

2. Materials and methods

2.1. EUG chemical structure

PubChem (https://pubchem.ncbi.nlm.nih.gov/) is an open database of chemical that offers exhaustive information on their respective compounds structures and descriptor data. Utilizing the PubChem database as a reference, the 2D chemical structure of EUG was retrievable (Fig. 2).

2.2. Prediction of EUG targets

TCMSP (https://old.tcmsp-e.com/tcmsp.php) represents a distinct system pharmacology platform of Chinese herbal medicines designed to elucidate the intricate relationships between drugs, targets, and diseases. STITCH (http://stitch.embl.de/) is a valuable resource aimed at exploring both known and predicted interactions of chemicals and proteins. Chemicals are linked to other chemicals and proteins via evidential support derived from experiments, databases and the scholarly literature.

TCMSP and STITCH databases were employed to predict the potential targets of EUG. The chemical name "Eugenol" was used as a keyword to search for targets related to EUG in the TCMSP database. The SMILES string "COC1═C(C═CC(═C1)CC═C)O″ of EUG was uploaded to the STITCH database, and "Homo sapiens" was selected as the organism to search for targets related to EUG. The corresponding targets of EUG were then selected from the two aforementioned databases and standardized in the UniProt database, with the attributes set to “Reviewed” and “Human” [17]. Following this, these standardized targets were consolidated and any duplicate items were eliminated, thus identifying the potential targets of EUG.

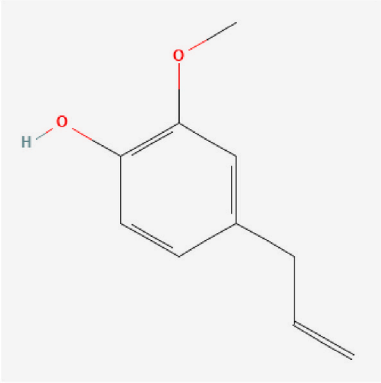


Fig. 2. EUG chemical structure.

2.3. Prediction of targets of EUG for pain treatment

DisGeNET (https://www.disgenet.org/) is a discovery platform which includes an extensive collections of genes and variants correlated to human diseases. DrugBank Online (https://go.drugbank.com/) is an extensive, open-access database housing comprehensive data pertaining to drugs and their corresponding therapeutic targets. TTD (http://db.idrblab.net/ttd/) serves as a database of comprehensive information pertaining to the known and explored therapeutic protein and nucleic acid targets, along with detailed data regarding the targeted disease. GeneCards (https://www.genecards.org/) is a database for the human genome, transcriptome, and proteome, providing functional information for all known and putative human genes.

Using the term "pain" as a keyword, the targets associated with pain were identified in the DisGeNET, DrugBank, TTD, and GeneCards databases. The targets collected from these four databases were standardized by using the UniProt database, with duplicate targets and those lacking gene information being removed. The potential targets of EUG were compared with the relevant targets involved in pain using the ImageGP platform (https://www.bic.ac.cn/ImageGP), in order to determine the predictive targets of EUG related to pain treatment.

2.4. Network construction and analysis

STRING (https://string-db.org/) is a database for storing known and predicted information about protein-protein interactions, including direct and indirect associations between proteins. The potential targets of EUG and the predictive targets of EUG related to pain treatment were uploaded to the STRING database, with the "Organisms" option selected as "Homo sapiens". Finally, we identified the EUG target network and the EUG-pain target network, which were imported into Cytoscape 3.6.1 software for visualizing Protein-Protein Interaction Networks (PPI).

2.5. Gene ontology (GO) and KEGG pathway enrichment analysis

DAVID (https://david.ncifcrf.gov/home.jsp) is a bioinformatics database that integrates biological data and analysis tools, providing systematic and comprehensive biological function annotation for large-scale gene or protein lists to help users extract biological information.

The predictive targets of EUG for pain treatment were imported into the DAVID database and set the "Organisms" as "Homo sapiens" for GO enrichment analysis and KEGG pathway enrichment analysis. Only items with P-values <0.05 were selected, and finally, GO enrichment analysis and KEGG pathway enrichment analysis results were obtained.

2.6. Molecular docking

The "sdf" file of EUG and CAP were downloaded from the PubChem database, and its mechanical structure was optimized using Open Babel 3.1.1. Then, an appropriate 3D structure "pdb" file of TRPV1 (PDB ID: 8GF8) [18] was downloaded from the RCSB Protein Data Bank (http://www.rcsb.org/). Then were removed the water molecules and molecule ligands from the target protein receptor using PyMOL 2.5.0. AutoDockTools 1.5.6 was used for molecular docking to investigate combination patterns and interactions between the target and ligands. In order to explore the potential binding sites between ligands and TRPV1 receptors, the grid box size of $112 \times 112 \times 110$ points with a spacing of 1.0 Å between grid points was set to cover the entire protein structure. The central coordinates of the grid box are 105.59, 105.597, 117.198. The binding conformation was visualized by PyMOL 2.5.0.

2.7. Cultivation of HaCaT cells

HaCaT cells (The immortalized human keratinocyte cell line) were obtained from Suzhou Haixing Biosciences Co., Ltd. (Jiangsu, China). HaCaT cells were cultured in DMEM medium (Servicebio, China) containing 1 % Penicillin-Streptomycin (Gibco, USA) and 10 % fetal bovine serum (FBS) (LONSERA, China), and incubated at 37 °C in an atmosphere of 5 % CO_2.

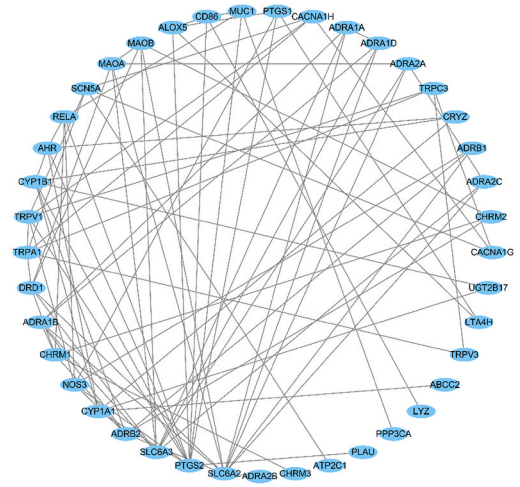
2.8. Cell viability assay

EUG (MCE, USA) was dissolved in DMSO and additional dilutions were done with culture medium. Cell viability was assessed by measuring the absorbance using the CCK-8 assay. HaCaT cells (5×10^3 cells/well) were seeded into a 96-well microplate and treated with 0 , 50 , 100 and 200 μM EUG for 48 h 10 μL of CCK-8 solution (Beyotime, China) was added to each well and the plate was incubated for an additional 2 h at 37 °C under 5 % CO_2 atmosphere. Cell viability was evaluated by measuring the absorbance at 450 nm using a multimode microplate reader (Tecan, Switzerland).

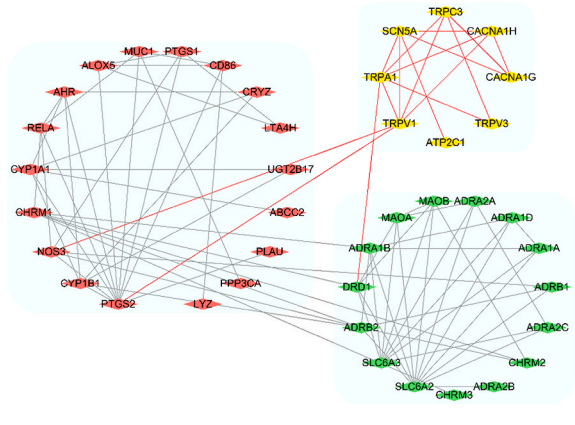
2.9. Western blot

HaCaT cells were seeded into 6-well plates at a density of 5×10^6 cells/well and incubated for 24 h at 37 °C and 5 % CO_2. After treatment with 0, 50, 100, and 200 μM EUG for 24 h, HaCaT cells were washed twice with cold PBS (Servicebio, China) and then lysed with 200 μL RIPA lysis buffer (Beyotime, China). The protein concentration was quantified using the BCA protein assay kit (Beyotime, China). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto

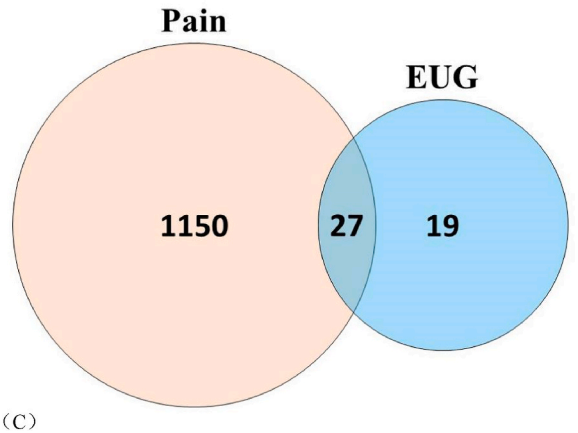
polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5 % BSA and then incubated overnight at 4 °C with TRPV1 mouse antibody (1 : 2000, Proteintech, USA). After washing the membranes, they were incubated with HRP-conjugated secondary antibodies (Biosharp, China) and developed using a chemiluminescence instrument (Bio-Rad, USA).



(A)



(B)



（C）

Fig. 3. EUG targets and EUG-pain targets. (A)EUG target PPI network. (B) EUG target PPI subnetwork was clustered using the k-means clustering method. All nodes in network and subnetwork were organized by node degree. (C) Target genes matching of pain and EUG.

2.10. Measurement of intracellular Ca^{2+} levels

Fura-2 AM (Calcium ion fluorescent probes) (Beyotime，China) was used for the measurement of intracellular Ca^{2+} levels. HaCaT cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h at 37 °C and 5 % CO_2. Next, HaCaT cells were washed twice with Krebs-HEPES buffer (120 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1 mM NaH_2PO_4 and 10 mM HEPES (pH 7.4)) and subsequently incubated with 2 μM Fura-2 AM at 37 °C for 1 h. Then, HaCaT cells were washed twice with Krebs-HEPES buffer and treated with 0，50，100 and 200 μM EUG and 10 μM CAP (MACKLIN, China) for 30 min. Fluorescence intensity was detected using a multimode microplate reader at an excitation/emission wavelength of 340nm/510 nm. Finally, the results were visualized as fluorescence changes relative to an untreated control, expressed as $(F–F_0)/F_0$, where F and F_0 represented the fluorescence intensity of the samples treated with EUG and CAP, and the untreated control, respectively.

2.11. Statistical analysis

All data were expressed as means ± standard errors of the means. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were used to determine statistically significant differences. These tests were performed using Prism 8.0 (GraphPad Software, CA, USA). *P* Values < 0.05 were considered statistically significant.

3. Results

3.1. EUG target PPI network

The TCMSP and STITCH databases were used to predict the targets of EUG. The targets were screened and standardized based on the UniProt database. After retaining "Human" targets and removing duplicate targets, 46 corresponding targets of EUG were selected. These targets were imported into the STRING database, and the resulting protein interactions were saved as a "tsv" file. This file was imported into Cytoscape 3.6.1 software to generate the EUG target PPI network diagram (Fig. 3A). This network depicted the regulatory connections among the EUG targets. The nodes in the diagram symbolized the target points, while the edges represented the relationships between these targets. The network consisted of 41 important nodes and 87 edges, and the average node degree was 4.24. The degree of a node referred to the number of edges between nodes in the network. The greater the degree of a node is, the more nodes are connected to it and the more important in the network. And we utilized the degree values for assessing the node's importance in the network. The top 20 targets of EUG network were shown in Table 1. Table 1 also showed the following values: Average Shortest Path Length (ASPL), Betweenness Centrality (BC), Closeness Centrality (CC), and Clustering Coefficient. Average shortest path length represents the minimum number of links required to connect the entire network. In a network, a smaller average shortest path length indicates more efficient signal propagation through the nodes. Betweenness centrality represents the likelihood of a signal passing through a node, with higher betweenness centrality values indicating greater node importance. Closeness centrality and clustering coefficient can indicate network density, with denser networks exhibiting higher efficiency [19,20]. The EUG target PPI network was clustered using the k-means clustering method, resulting in the formation of 3 clusters (Fig. 3B), with PTGS2, SLC6A2, TRPV1 being the nodes with the greatest degrees in the 3 subnetworks. Meanwhile, PTGS2, SLC6A2, and TRPV1 exhibited a high betweenness centrality with corresponding values of 0.318, 0.195, and 0.180 respectively, indicating that the three key nodes were of great importance in the

Table 1
Top 20 targets of the EUG target PPI network.

Target name	Abbreviation	ASPL	BC	CC	Clustering coefficient	Degree
Sodium-dependent noradrenaline transporter	SLC6A2	2.600	0.195	0.385	0.182	11
Prostaglandin G/H synthase 2	PTGS2	2.325	0.318	0.430	0.200	11
Sodium-dependent dopamine transporter	SLC6A3	3.075	0.037	0.325	0.286	8
Beta-2 adrenergic receptor	ADRB2	2.325	0.200	0.430	0.238	7
Cytochrome P450 1A1	CYP1A1	2.800	0.071	0.357	0.400	6
Nitric oxide synthase	NOS3	2.300	0.141	0.435	0.267	6
Muscarinic acetylcholine receptor M1	CHRM1	2.500	0.129	0.400	0.133	6
Alpha-1B adrenergic receptor	ADRA1B	2.800	0.059	0.357	0.467	6
D(1A) dopamine receptor	DRD1	2.650	0.153	0.377	0.333	6
Transient receptor potential cation channel subfamily A member 1	TRPA1	2.625	0.139	0.381	0.333	6
Transient receptor potential cation channel subfamily V member 1	TRPV1	2.475	0.180	0.404	0.333	6
Cytochrome P450 1B1	CYP1B1	2.425	0.130	0.412	0.400	6
Aryl hydrocarbon receptor	AHR	2.750	0.016	0.364	0.600	5
Transcription factor p65	RELA	2.525	0.091	0.396	0.300	5
Sodium channel protein type 5 subunit alpha	SCN5A	2.975	0.065	0.336	0.400	5
Amine oxidase [flavin-containing] A	MAOA	3.100	0.006	0.323	0.700	5
Amine oxidase [flavin-containing] B	MAOB	3.100	0.011	0.323	0.500	5
Arachidonate 5-lipoxygenase	ALOX5	3.175	0.015	0.315	0.500	4
T-lymphocyte activation antigen CD86	CD86	3.150	0.052	0.317	0.333	4
Mucin-1 subunit alpha	MUC1	2.925	0.058	0.342	0.333	4

Note: ASPL: Average Shortest Path Length; BC: Betweenness Centrality; CC: Closeness Centrality.

network.

3.2. Targets of EUG for pain treatment

We collected targets with score ≥0.2 from the DisGeNET database, targets with a relevance score >8 from the GeneCards database [21], and all related targets retrieved from the DrugBank and TTD databases. Integrating the retrieval results from DisGeNET, GeneCards, DrugBank, and TTD databases, pain-related targets were obtained. The potential targets of EUG were mapped to pain-related targets using the ImageGP platform, resulting in a total of 27 potential targets of EUG related to pain treatment (Table 2), and a Venn diagram was drawn (Fig. 3C).

3.3. EUG-pain target PPI network

By importing the potential targets of EUG related to pain treatment into the STRING database, the obtained protein-protein interaction data were visualized as a PPI network using Cytoscape 3.6.1 software (Fig. 4). The PPI network consisted of 25 nodes (target genes) and 52 edges (interaction relationships). The nodes were arranged based on the degree, where larger nodes indicated greater degree. The strength of the interactions between nodes was represented by the thickness of the edges, with thicker edges indicating higher combine score. The top 10 targets ranked by degree were SLC6A2, SLC6A3, ADRA1B, DRD1, PTGS2, TRPA1, NOS3, MAOA, TRPV1, ADRB2.

3.4. Enrichment analysis

The process of GO enrichment analysis comprised three distinct categories, namely Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The biological process analysis resulted in 62 enrichment results, primarily inclusive of Response to drug, Adenylate cyclase-activating adrenergic receptor signaling pathway, G-protein coupled receptor signaling pathway, Cell-cell signaling, Oxidation-reduction process, Positive regulation of vasoconstriction, Calcium ion transmembrane transport, Regulation of smooth muscle contraction, Regulation of vasoconstriction, Positive regulation of smooth muscle contraction, and others (Fig. 5A). The cellular component analysis produced 9 enrichment results, primarily inclusive of Plasma membrane, Integral component of plasma membrane, Integral component of membrane, Cell surface, Caveola, Receptor complex, Nuclear membrane, Neuron projection and Voltage-gated calcium channel complex (Fig. 5B). The molecular function analysis resulted in 20 enrichment results, primarily inclusive of Protein homodimerization activity, Ion channel activity, Protein heterodimerization activity, Epinephrine binding, Scaffold protein binding, Alpha1-adrenergic receptor activity, Alpha2-adrenergic receptor activity, Calcium channel activity, Heme binding, Calmodulin binding, and others (Fig. 5C). The KEGG pathway enrichment analysis resulted in 16 pathways, primarily inclusive of Neuroactive ligand-receptor interaction, Calcium signaling pathway, cGMP-PKG signaling pathway, Cocaine addiction, Serotonergic

Table 2
27 potential targets of EUG related to pain treatment.

Target name	Abbreviation
Sodium-dependent noradrenaline transporter	SLC6A2
Sodium-dependent dopamine transporter	SLC6A3
Alpha-1B adrenergic receptor	ADRA1B
D(1A) dopamine receptor	DRD1
Prostaglandin G/H synthase 2	PTGS2
Transient receptor potential cation channel subfamily A member 1	TRPA1
Nitric oxide synthase	NOS3
Amine oxidase [flavin-containing] A	MAOA
Transient receptor potential cation channel subfamily V member 1	TRPV1
Beta-2 adrenergic receptor	ADRB2
Alpha-1A adrenergic receptor	ADRA1A
Alpha-1D adrenergic receptor	ADRA1D
Alpha-2A adrenergic receptor	ADRA2A
Amine oxidase [flavin-containing] B	MAOB
Muscarinic acetylcholine receptor M1	CHRM1
Sodium channel protein type 5 subunit alpha	SCN5A
Voltage-dependent T-type calcium channel subunit alpha-1H	CACNA1H
Prostaglandin G/H synthase 1	PTGS1
Alpha-2C adrenergic receptor	ADRA2C
Transcription factor p65	RELA
Voltage-dependent T-type calcium channel subunit alpha-1G	CACNA1G
Arachidonate 5-lipoxygenase	ALOX5
Transient receptor potential cation channel subfamily V member 3	TRPV3
Urokinase-type plasminogen activator short chain A	PLAU
Alpha-2B adrenergic receptor	ADRA2B
Alpha-2B adrenergic receptor	ABCC2
Pre-mRNA 3′-end-processing factor FIP1	FIP1L1

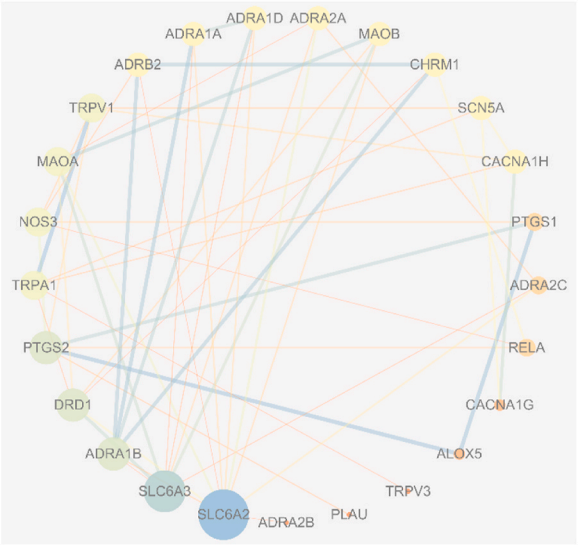


Fig. 4. EUG-pain target PPI network. All nodes were organized by node degree, where larger nodes indicated greater degree. The strength of the interactions between nodes was represented by the thickness of the edges, with thicker edges indicating higher combine score.

synapse, Adrenergic signaling in cardiomyocytes, Amphetamine addiction, Salivary secretion, Dopaminergic synapse, Alcoholism, and others (Fig. 6).

3.5. Molecular docking

Based on the PPI networks and enrichment analysis results, we selected TRPV1 (PDB ID: 8GF8) as the receptor for molecular docking. The molecular docking was conducted to investigate the interaction between ligands and the core target TRPV1, and the docking results were subsequently analyzed. It is generally believed that when the conformation of the ligand and the receptor is stable, the lower the energy, the greater the likelihood of interaction. Typically, if the binding energy is less than −5 kJ/mol (−1.2 kcal/mol), then the docking result is considered acceptable [22]. As shown in Fig. 7A, EUG established hydrogen bonds with the ARG-557and GLN-701residues of TRPV1 receptor. In addition, the LEU-515, PHE-516 and LEU-553 residues of TRPV1 receptor were found to engage in hydrophobic interactions with EUG. The analysis of molecular docking results revealed that the binding energy between EUG and TRPV1 receptor was −4.56 kcal/mol, further demonstrated the strong binding capacity. Similarly, we performed molecular docking of CAP and TRPV1 receptor, revealing that CAP formed hydrogen bonds with SER512 and ARG557 residues of TRPV1 receptor. Additionally, CAP formed hydrophobic interactions with LEU515, PHE543, ALA546, LEU547, THR550, PHE591, ILE662, and LEU663 residues of TRPV1 receptor, and exhibited a binding mode similar to that of EUG. The analysis of molecular docking results showed that the binding energy between CAP and TRPV1 receptor was −5.02 kcal/mol (Fig. 7B).

3.6. Effects of EUG on TRPV1 receptors in HaCaT cells

In order to verify the effect of EUG on TRPV1 receptors in HaCaT cells, CCK-8 assay was firstly conducted to investigate the effect of EUG on cell viability. The drug concentration was determined based on cell viability. As shown in Fig. 8D, treatment with EUG (50，100，200 μM) for 48 h did not affect cell viability, therefore, 50, 100, and 200 μM EUG were used in further studies. The effect of EUG on the expression of TRPV1 receptors in HaCaT cells was examined by Western blot (WB). As presented in Fig. 8(A and B) and Fig. S1, all tested concentrations of EUG promoted the upregulation of the expression of TRPV1 receptors in HaCaT cells. As is well known, CAP is an agonist of the TRPV1 receptor, which can increase Ca^{2+} influx [23], and our experiment also confirmed this result. In order to evaluate the activation of TRPV1 receptors by EUG, Fura-2 AM was used to measure Ca^{2+} levels. The results showed that EUG increased the level of cellular Ca^{2+}, and that the effect of EUG at the highest concentration was similar to CAP ($P < 0.05$) (Fig. 8C).

4. Discussion

In TCM, clove powder is employed for topical transdermal administration to treat chronic pain, demonstrating significant efficacy and negligible side effects. The analgesic effect of this transdermal therapy is typically determined by the content of clove essential oil, of which EUG is the main active ingredient [3]. In order to elucidate its mechanism, network pharmacology was employed to predict the potential targets responsible for the analgesic effect of EUG. Based on the analysis of EUG-pain target PPI network, the top 10 targets with a great degree of connectivity were identified, which might represent the core targets involved in mediating the analgesic effects of EUG. One of the targets that we have identified is TRPV1, a non-selective cation channel with high permeability to Ca^{2+}. It

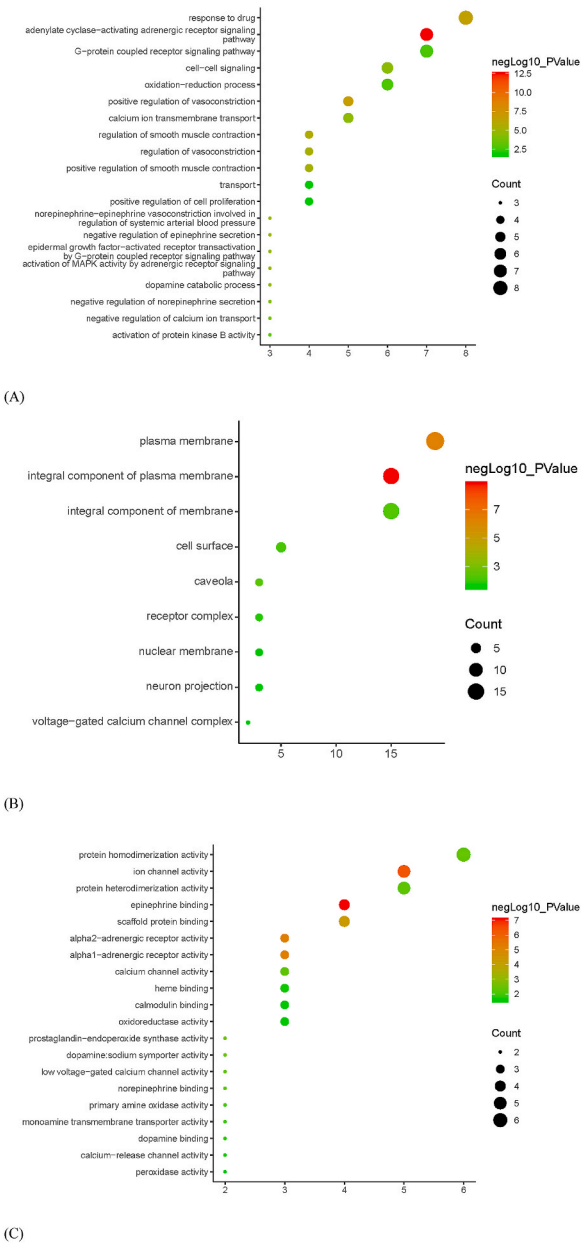


Fig. 5. GO enrichment analysis of potential targets of EUG related to pain treatment: (A)Biological Process (BP), (B)Cellular Component (CC), and (C)Molecular Function (MF).

was cloned from the dorsal root ganglia of rats in 1997 [5]. Minke's team was the first to establish a link between trp mutants and Ca^{2+} [24]. Shortly thereafter, Minke and Selinger confirmed that the trp protein is a plasma membrane component (or part of a plasma membrane component) that oscillates between Ca^{2+} transport and non-transport states [25]. The TRPV1 ion channel is activated by vanilloids CAP and resiniferatoxin [26]. The TRPV1 nociceptor functions as a multi-modal detector of harmful signals, responding to various harmful chemical and physical stimuli, such as heat ($\geq$43 °C), protons (low pH $<$ 6.0), bradykinins, lipoxygenase products of arachidonic acid, the endogenous lipid cannabinoid ligands N-arachidonoyl-ethanolamine (anandamide) and N-arachidonoyldopamine [5,27]. The function of TRPV1 channels is controlled by ligands that induce the opening of ion channels. It has become an attractive drug target for the treatment of pain. Pharmaceutical companies have developed many TRPV1 small molecule inhibitors as potential analgesics [28]. Nonetheless, the majority of these inhibitors have proven unsuccessful in preclinical and clinical trials due to serious side effects, including hyperthermia and alteration of thermal sensation [29]. Therefore, to circumvent the adverse effects associated with TRPV1 inhibitors, researchers are exploring the utilization of TRPV1 agonists for pain relief. Upon the administration of a significant or repetitive quantity of TRPV1 agonists, such as CAP, it powerfully eliminates neurons that express TRPV1 at axon ends

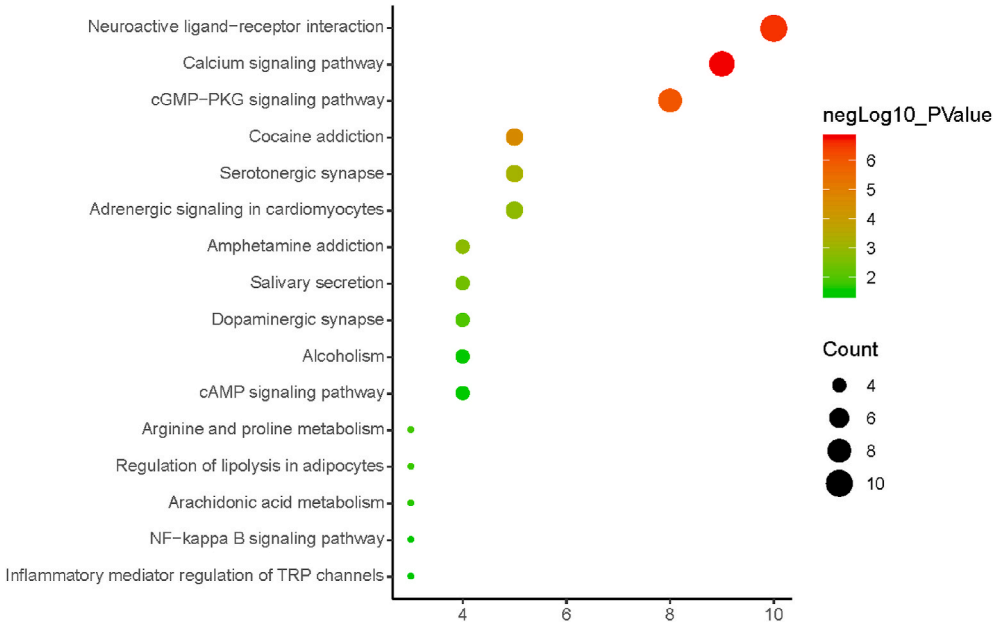


Fig. 6. KEGG pathway enrichment analysis of potential targets of EUG related to pain treatment.

by inducing calcium overload in said neurons which reduces the sensitivity of TRPV1 to pain and ultimately yields analgesic benefits [30,31].

It is noteworthy that the structure of the EUG under investigation bears resemblance to CAP [32]. In addition, it has been reported that EUG is a type of herbaceous compound that is less irritating than CAP. CAP induces pain, whereas EUG has not been reported to have this effect [7]. The milder nature of EUG compared to CAP is attributed to a shorter fatty acid chain at the vanillyl C4 position [33]. TRPV1 has become one of the most studied targets of EUG research in the realm of pain management, particularly within the dentistry [34]. However, there is a dearth of relevant research on transdermal administration. Therefore, we have chosen TRPV1 as the primary receptor for our research. In order to further explore the multiple mechanisms underlying the analgesic effects of EUG, we conducted functional enrichment analysis, indicating that the analgesic effect of EUG responded in Calcium ion transmembrane transport (BP), Integral component of plasma membrane (CC), Ion channel activity (MF) in GO terms and Neuroactive ligand-receptor interaction in the KEGG terms. This further validated our previous hypothesis: TRPV1 was speculated to be one of the core targets for the analgesic effects of EUG.

In TCM transdermal treatment of clove powder to treat chronic pain, the EUG volatile oil abundant in cloves easily penetrates the mechanical barrier of the skin. Keratinocytes constitute a vital part of the epidermis and form a mechanical barrier that protects the organisms. Keratinocytes have been shown to be involved in skin neuroimmune processes [35–37]. Keratinocytes also express the TRPV1 receptors [38]. The chemical structure of EUG is similar to that of CAP, suggesting that the two compounds may interact with the same receptor, i.e., transient receptor potential vanillic acid 1 (TRPV1) in mammals [7]. Molecular docking studies are a widely used computational method to study the binding patterns and interactions between ligands and receptor binding sites, so we used molecular docking to study the binding patterns of EUG to TRPV1 [39]. Based on molecular docking results, we observed that EUG can spontaneously bind to the TRPV1 receptor, and formed hydrogen bonds to ARG-557 and GLN-701 Studies have shown that ARG-577 residues are important for the binding of CAP, and this mutation will silence the activation of CAP [40,41], which is consistent with the docking position of EUG, and may be related to the activation of TRPV1 receptor by EUG. To confirm the activation of TRPV1 by EUG, *in vitro* validation experiments were conducted, and the results indicated that EUG could increase the influx of Ca^{2+} ($P < 0.05$). The lower binding energy of CAP as compared to that of EUG supported our results that CAP exhibited stronger activation of Ca^{2+} influx than EUG. Previous studies have demonstrated that the influx of Ca^{2+} is crucial in the desensitization of pain sensory, which is dependent on the elevation of intracellular Ca^{2+} over time [42–45]. EUG and CAP share part of the chemical structure of vanilloid compounds and can both activate the TRPV1 receptor [5,46], and the expression of TRPV1 plays a crucial role in regulating various symptoms such as pain, skin irritation [47–49]. Similar to CAP, EUG exhibits a dual pharmacological action with an initial pungent effect followed by a delayed analgesic effect [7,33]. This delayed analgesic effect is associated with receptor desensitization caused by repeated exposure to high concentrations of TRPV1 agonist stimulation [4,50]. Research by Shtaywy et al. has shown that the activation of TRPV1 by vanilloids leads to an increase in intracellular free Ca^{2+} levels and induces desensitization [51].

In this study, in order to validate the credibility of the TRPV1 receptor results obtained through network pharmacology methods, we first used EUG to act on HaCaT cells and conducted a WB experiment. The results showed that EUG enhanced the expression of TRPV1 receptor in HaCaT cells, which may be related to the initial pungent effect of EUG. Concurrently, the intracellular Ca^{2+} levels in HaCaT cells were assessed using Fura-2 AM, and the results indicated that the impact of EUG led to elevated intracellular Ca^{2+} levels,

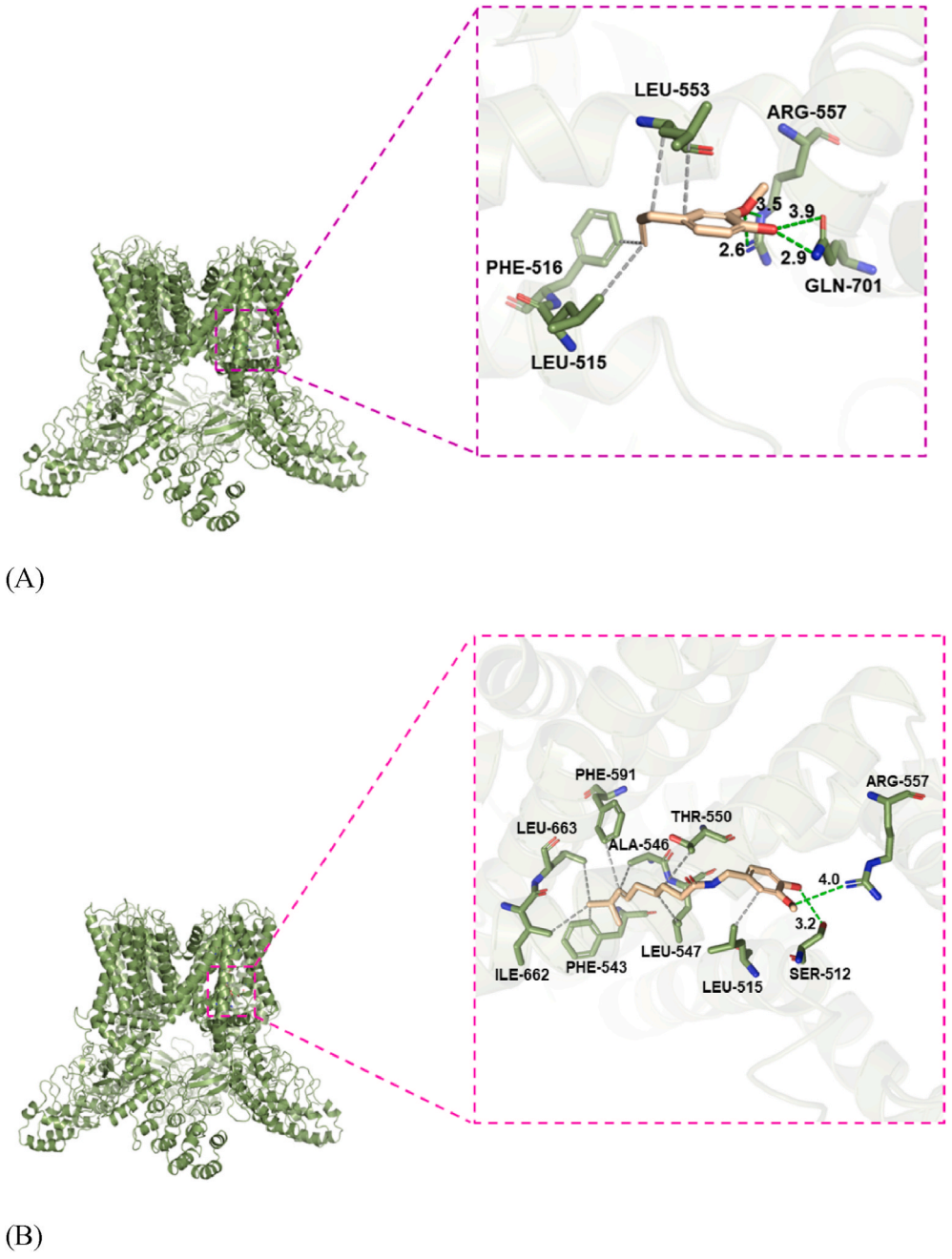


Fig. 7. Docking results for ligands and receptor: (A) Molecular docking model of EUG and TRPV1 receptor. (B) Molecular docking model of CAP and TRPV1 receptor.

potentially linked to its delayed analgesic effect. The activation of TRPV1 channels can be considered an attractive strategy for pain treatment. This study represented the first exploration of the interaction of EUG with TRPV1 in HaCaT cells. By understanding the mechanism of action of transdermal delivery of EUG in the treatment of pain, it could establish the groundwork for the future clinical application of EUG. However, this study only preliminarily explored the interaction between EUG and TRPV1 and compared EUG with CAP, and did not explore other core targets listed in the study. This constitutes a limitation of the study and will be a focal point for our forthcoming research.

5. Conclusion

In summary, our analysis of network pharmacology has uncovered that the TRPV1 receptor serves as a crucial target for the

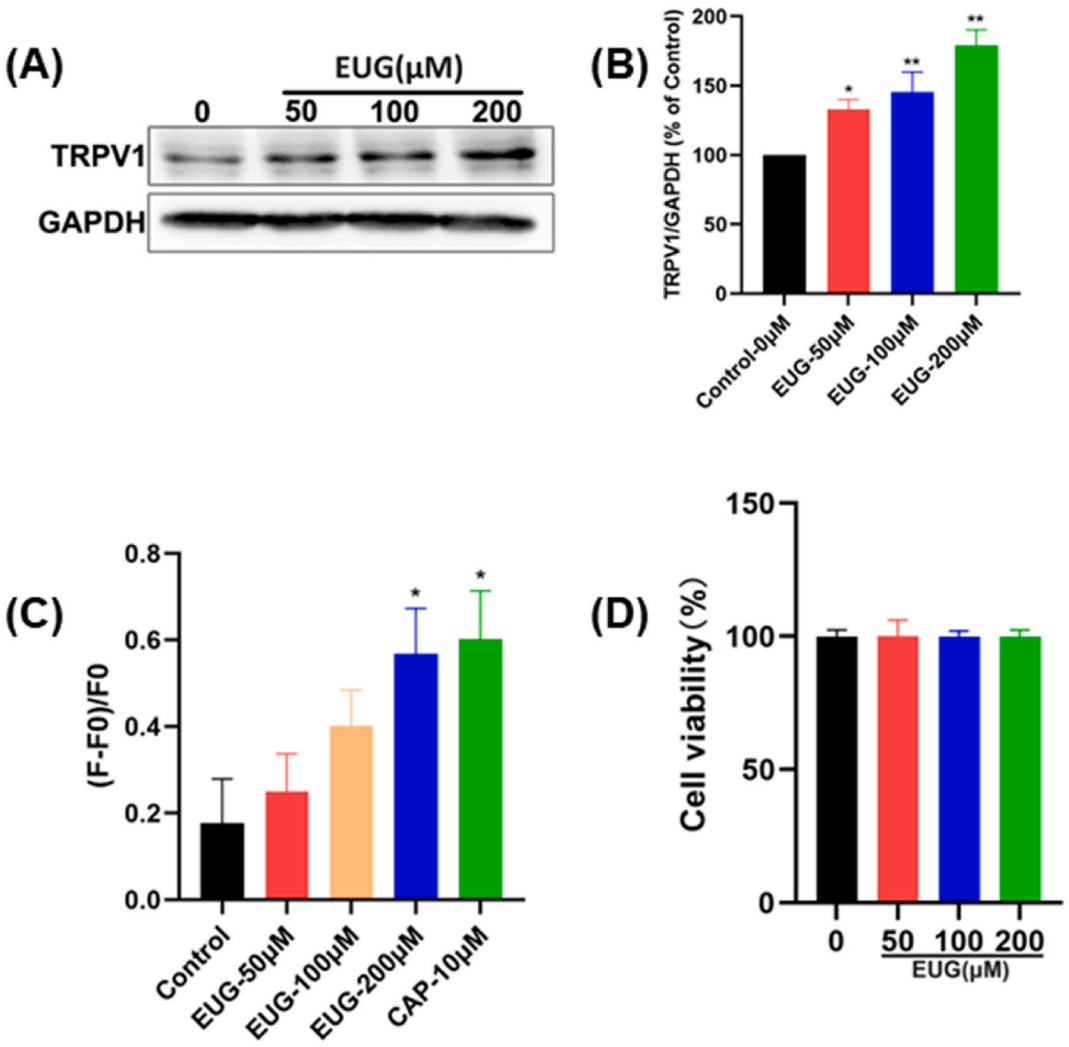


Fig. 8. Effects of EUG on TRPV1 receptors in HaCaT cells. (A & B) Effects of EUG on the expression of TRPV1 receptors in HaCaT cells. (C) Effects of EUG on Ca^{2+} influx in HaCaT cells. (D) Cell viability. $^{*}P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control. All experiments are repeated three times.

analgesic effects of transdermal administration of EUG. Based on molecular docking and *in vitro* experimental validation, EUG could spontaneously bind to the TRPV1 receptor and form a hydrogen bond with the ARG-557 and GLN-701 residues of TRPV1 receptor. Importantly, EUG acted as a TRPV1 agonist to increase intracellular Ca^{2+} levels, which might be associated with desensitization of pain sensations. These findings offered novel evidence for the topical transdermal application of EUG in pain treatment.

CRediT authorship contribution statement

Haoting Ye: Writing – original draft, Validation. **Qiuxiao Lin:** Data curation. **Qinghua Mei:** Writing – review & editing. **Qiuqiong Liu:** Project administration. **Siwei Cao:** Project administration.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29722.

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