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Prediction of the Mechanism of Epimedin C Promoting Tunneling Nanotubes Production Based on Network Pharmacology

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Abstract

Background: To predict the mechanism of Tunneling nanotubes (TNTs) formation promoted by Epimedin C based on network pharmacology. **Methods:** The targets of Epimedin C were obtained through the PubChem, Pharm Mapper, Swiss Target Prediction and UniProt databases. Using the Genome Annotation Database Platform (GeneCards) database, the targets of TNTs were identified. The common targets were identified after intersection, and the STRING database was applied to construct a Protein-Protein Interaction (PPI) Network. The DAVID database was used for Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. **Results:** Through network pharmacology, 11 key targets including ALB, IGF1, BMP2, ACHE, MMP2, LCN2, EGFR, CDK2, SRC, ANXA5, and BMP7 were screened. The GO analysis results involved signaling pathways such as the positive regulation of vascular smooth muscle cell proliferation, positive regulation of osteoblast differentiation, and epithelial to mesenchymal transition. The KEGG results were related to endocrine, bladder cancer, EGFR tyrosine kinase inhibitors of drug resistance, FoxO and other signaling pathways. **Conclusions:** Epimedin C may promote the generation of TNTs through the endocrine and EGFR tyrosine kinase inhibitor resistance signaling pathways. The findings of this study provide a theoretical basis for further investigation into the mechanism by which Epimedin C promotes TNTs generation.

1 Introduction

Epimedium brevicornu Maxim., also known as Sanzhi Jiuye Cao, Xianlingpi, or Gangqian, has leaves as its medicinal part. It has a warm nature, a pungent and sweet taste, and the effects of tonifying kidney yang, strengthening muscles and bones, and dispelling wind and dampness [1]. According to modern pharmacological research, *Epimedium brevicornu* Maxim. has regulatory effects on the cardiovascular system, immune system, blood lipids, and blood glucose, and has various pharmacological effects such as improving immune function, anti-osteoporosis, antioxidant, anti-inflammation, anti-tumor, reducing blood glucose, and antidepression. Among them, flavonoids are the main active ingredients [2]. In most types of *Epimedium brevicornu* Maxim. preparations, the content of flavonoid compounds follows the order: Epimedin C > Epimedin B > Icariin > Baohuoside I > Epimedin A > Icaritin [3]. Research has shown that total flavonoids of *Epimedium brevicornu* Maxim. can be used for anti-osteoporosis, anti-myocardial hypoxia, anti-tumor, and immunoregulation [4].

Tunneling nanotubes (TNTs) are one of the communication methods between animal cells, which was first observed by RUSTOM et al. in 2004 in rat pheochromocytoma cells (PC12), human embryonic kidney cells (HEK), and normal rat kidney cells (NRK) [5]. TNTs are an extremely fine membrane nanochannel capable of transferring substances such as proteins and mitochondria. TNTs are different from previous gap junctions and are a novel form of intercellular communication. Gap junctions are mostly responsible for short-range communication between cells, while TNTs can achieve long-distance and directional communication between cells. Currently, TNTs have been found in various types of cells such as nerve cells, immune cells, and tumor cells [6-8]. TNTs can mediate the transmission of genetic information and long-distance transmission of Ca^{2+}. For example, microRNA-155 can transfer between bladder cancer cells RT4 and T24 through TNTs, enhancing the invasion and proliferation of bladder cancer [9]. The TNTs formed between two independent pericytes in the capillary system are called interpericyte tunnelling nanotubes (IP-TNTs) and can form a functional TNTs network in the mouse retina [10]. In addition, due to their sufficiently large inner diameter, TNTs can facilitate the transfer of organelles such as mitochondria, Golgi apparatus, lysosomes, and intracellular vesicles between cells [11]. The main pathway for mitochondrial transfer between cells is the transport of TNTs [12]. Under stress conditions, TNTs can also act as a survival mechanism for cells. Melatonin can promote mitochondrial transfer between damaged HT22 cells via TNTs [13]. Mitochondrial transport via TNTs between certain cell types can also be bidirectional. The TNTs present between mesenchymal stem cells and umbilical vein endothelial cells can mediate the bidirectional transport of mitochondria between cells, facilitating cell osteogenic and angiogenic abilities [14,15]. In the field of bone tissue regeneration, the TNT-mediated intercellular mitochondrial transport can promote the process of bone regeneration [16].

Research reported that the main flavonoid components of *Epimedium brevicornu* Maxim., such as Epimedin C, Epimedin B, Icariin, and Baohuoside I, can act on bone metabolism [17]. TNTs can boost bone regeneration by mediating intercellular mitochondrial transport. It can be reasonably inferred that the main flavonoids in *Epimedium brevicornu* Maxim. may directly or indirectly enhance the formation of TNTs in the process of exerting biological effects. Therefore, this study selected Epimedin C, the most abundant flavonoid in *Epimedium brevicornu* Maxim., as a representative compound to preliminarily explore its role and mechanism in promoting TNTs production.

Against the backdrop of complex biological networks, network pharmacology integrates compound structures, biological effects, and relevant targets to construct molecular interaction networks. This approach has emerged as a crucial research strategy for elucidating the effective components, targets, and mechanisms of traditional Chinese medicine. In recent years, there has been accumulated research on the morphological characteristics, formation mechanisms, regulatory factors, and functions of TNTs in the development of various diseases [10]. As a potential active ingredient in the prevention and treatment of osteoporosis, a systematic clarification of its stimulatory effect on TNT production and the underlying molecular mechanism will not only contribute to the development of a new approach for osteoporosis treatment but also provide a theoretical foundation for disease treatment strategies centered on the regulation of intercellular junctions. Therefore, in this study, we employed network pharmacology to systematically forecast the potential targets of Epimedin C in regulating TNT formation, establish a protein interaction network, and preliminarily investigate the mechanism by which Epimedin C promotes TNT formation. Aiming to offer innovative theoretical support for the in - depth research and clinical application of this component.

2 Materials and methods

2.1 The obtainment of targets of Epimedin C

Using PubChem database (https://pubchem.ncbi.nlm.nih.gov/), the mol file and SMILES of the chemical structure of Epimedin C were retrieved. Then the obtained mol file was uploaded to Pharm Mapper database (http://lilab-ecust.cn/pharmmapper/index. Html) to predict the action targets of Epimedin C. With Norm Fit > 0.5 as the screening criterion, the obtained target names were standardized through the UniProt database (https://www.uniprot.org/). The SMILES structural formula was also imported into the Swiss Target Prediction database (http://swisstargetprediction.ch/), and targets of Epimedin C were predicted with a probability threshold of > 0. The results from both databases were integrated to acquire common targets.

2.2 Screening of TNTs targets and common target genes

With “tunneling nanotubes” as the keyword in GeneCards database (https://www. genecards. org/), the targets of TNTs were identified. The targets of Epimedin C and TNTs were uploaded to the bioinformatics online platform (https://www.bioinformatics.com.cn/) to generate a Venn diagram, and their intersection was taken to obtain potential targets of Epimedin C promoting TNTs generation.

2.3 Protein-protein interaction (PPI) network construction

To further understand the functions of the selected target protein genes and their roles in signaling pathways, the common targets were imported into the Metascape database [18]. By inputting a list of target gene names and limiting the species to humans, a threshold of $p < 0.01$ was set for Gene Ontology (GO) biological process enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway enrichment analysis. Finally, the obtained results were analyzed.

2.4 GO functional enrichment analysis and KEGG pathway enrichment analysis

The David database (https://david.ncifcrf.gov/) was used to perform GO and KEGG enrichment analysis on intersecting genes. The top 10 results (all results were included if fewer than 10 were available) from the KEGG enrichment analysis and the GO analysis in Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) were uploaded to the

bioinformatics platform for visualization processing, with FDR as the X-axis, Term as the Y-axis, Count as the point size, and *p* Value as the colorbar condition. Corresponding enrichment bubble plots were plotted.

3 Results

3.1 Common targets of Epimedin C and TNTs

After screening the data from the Swiss Target Prediction database and Pharm Mapper database, we identified 16 and 232 targets of Epimedin C, respectively. Following merging and deduplication, a total of 243 targets of Epimedin C were obtained. A search in the GeneCards database yielded 157 targets related to TNTs. The intersection of the targets of Epimedin C and TNTs resulted in 11 intersection targets (Figure 1), and the detailed content of the intersection targets was recorded in Table 1.

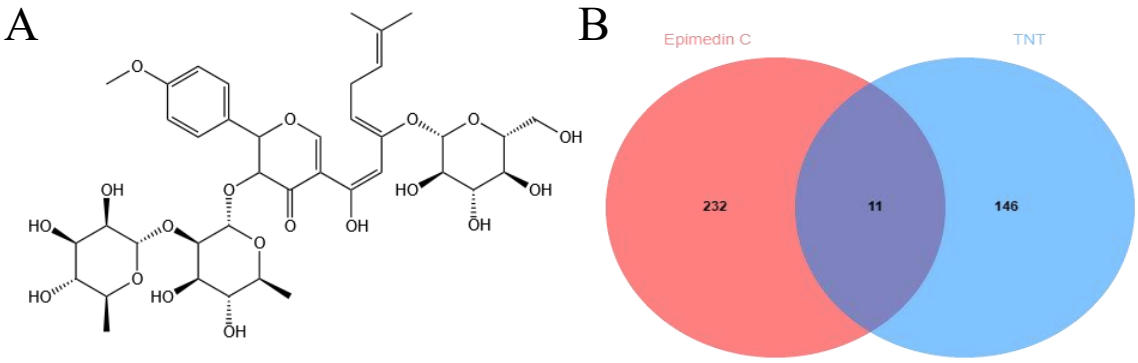

Figure 1 Prediction of common targets for Epimedin C in TNTs formation. (A) the chemical structure of Epimedin C. (B) Common targets of Epimedin C and TNTs.

Table 1 Common targets of Epimedin C and TNTs.

Abbreviation of intersection target	Full name	Uniprot ID
ALB	Albumin	P02768
IGF1	Insulin Like Growth Factor 1	P05019
BMP2	Bone Morphogenetic Protein 2	P12643
ACHE	Acetylcholinesterase (Cartwright Blood Group)	P22303
MMP2	Matrix Metallopeptidase 2	P08253
LCN2	Lipocalin 2	P80188
EGFR	Epidermal Growth Factor Receptor	P00533
CDK2	Cyclin Dependent Kinase 2	P24941
SRC	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase	P12931
ANXA5	Annexin A5	P08758
BMP7	Bone Morphogenetic Protein 7	P18075

3.2 Construction of the PPI network for intersection targets of Epimedin C and TNTs

11 intersection targets were imported into a String database to construct a PPI network. The results showed that the network contained a total of 11 nodes, 33 edges, an average of 6 nodes, and an average local clustering coefficient of 0.841 (Figure 2, Table S1). According to the connectivity between the targets, each target was divided into three parts: red, blue, and green. The PPI network indicated that BMP2 and BMP7 were closely correlated, and EGFR, IGF1, MMP2, and SRC might interact with one another. ALB may be

associated with LCN2, ACHE, ANXA5, and CDK2, and the subsequent study on the pathway mechanism offers some assistance.

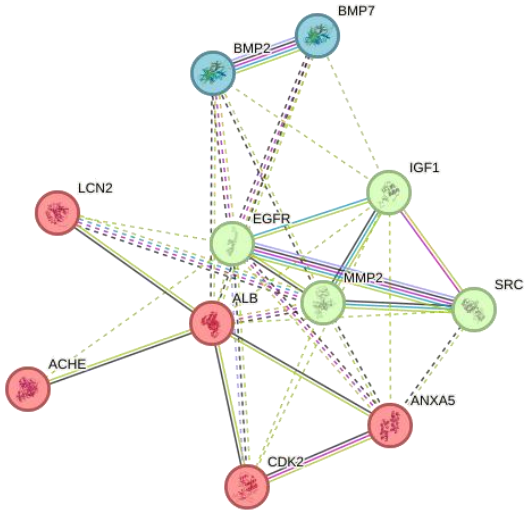

Figure 2 PPI network for intersection targets of Epimedin C and TNTs.

3.3 GO functional enrichment analysis and KEGG pathway enrichment analysis of intersection targets

11 intersection targets were uploaded to the David database for GO functional analysis and KEGG enrichment analysis. The results mainly revealed that the association between Epimedin C and TNTs involved 10 BP, 8 CC, and 10 MF in GO functional analysis. BP mainly involved the positive regulation of transcription, epithelial to mesenchymal transition, and the positive regulation of osteoblast differentiation (Figure 3, Table S2). CC primarily included components such as extracellular space, focal adhesion, and platelet alpha granule lumen (Figure 4, Table S3). MF was primarily associated with functions such as BMP receptor binding, binding energy, and protein binding (Figure 5, Table S4). KEGG pathway enrichment analysis revealed the action pathways of calycosin C on TNTs. The intersection targets mainly involve endocrine, bladder cancer, EGFR tyrosine kinase inhibitor resistance, and FoxO signaling pathway. (Figure 6, Table S5). These pathways and functions may play a key role in the process of Epimedin C promoting the generation of TNTs.

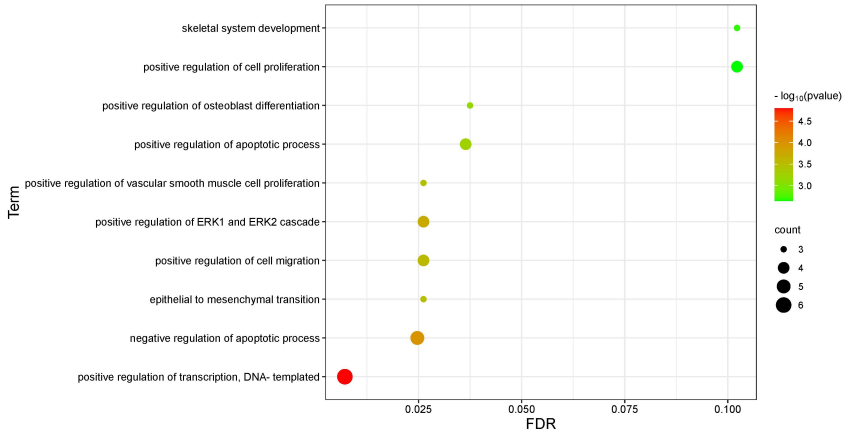

Figure 3 GO functional analysis of intersection targets (BP).

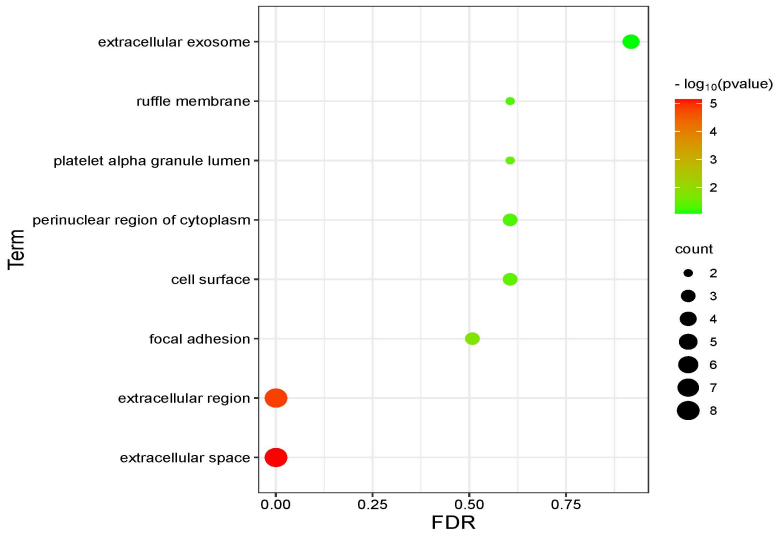

Figure 4 GO functional analysis of intersection targets (CC).

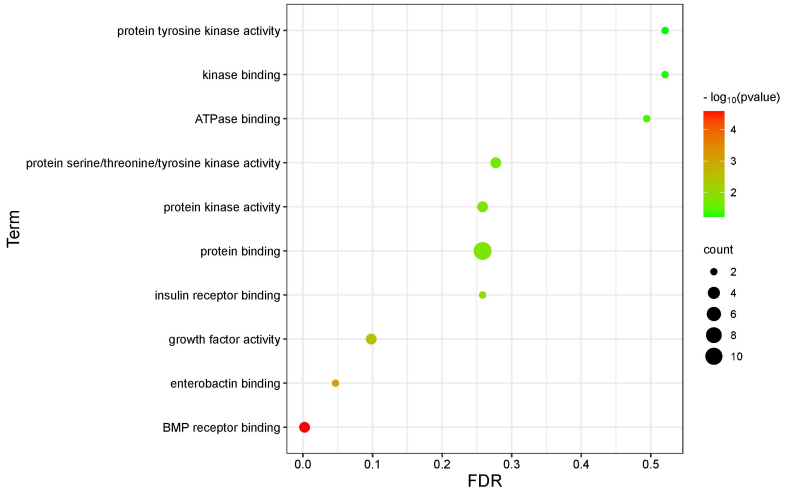

Figure 5 GO functional analysis of intersection targets (MF).

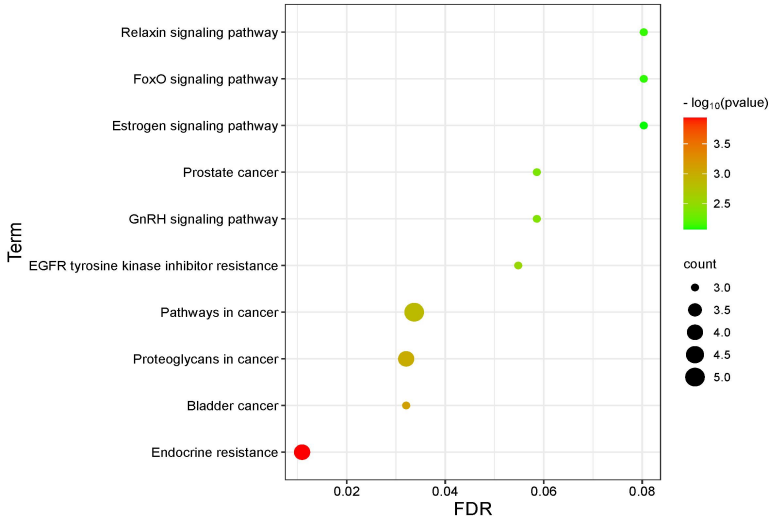

Figure 6 KEGG pathway enrichment analysis.

4 Discussion

This study preliminarily investigates the mechanism by which Epimedin C promotes the formation of TNTs using network pharmacology, providing a research direction for further exploration of corresponding mechanism. Total flavonoids are the main active ingredients of *Epimedium brevicornu* Maxim., in which Epimedin C accounts for the highest proportion. Modern research has shown that total flavonoids of *Epimedium brevicornu* Maxim. have beneficial effects on the immune system, endocrine tissues, bones and tumors, as well as the cardiovascular and cerebrovascular systems [16]. Total flavonoids of *Epimedium brevicornu* Maxim. can impact bone metabolism. The TNTs-mediated intercellular mitochondrial transport can promote bone regeneration. We can speculate that total flavonoids of *Epimedium brevicornu* Maxim. can promote the production of TNTs, but the relative mechanism remains obscure. Therefore, this study preliminarily explores the mechanism of Epimedin C boosting the generation of TNTs, with the aim of unveiling the role of Epimedin C in TNTs generation and providing a theoretical basis for subsequent research on Epimedin C-induced promotion of TNTs generation.

TNTs are mainly composed of intracellular skeletons and cell membranes such as F-actin and tubulin, which suspend above the substrates and connect different cells. TNTs contain single or multiple bundles of iTNT, each containing parallel actin filaments enveloped by a plasma membrane and connected by N-cadherin [10]. After treatment with actin inhibitors, the growth of TNTs in PC12 cells is significantly inhibited [5]. There are currently two known mechanisms for the formation of TNTs. One involves the extension of filopodia-like membrane protrusions, where multiple membrane protrusions protrude from the cell membrane. When one protrusion comes into contact with other cells, all but one protrusion retracts, leaving only one to form TNTs [19,20]. The other mechanism involves cell translocation, where two cells make direct contact, and membrane fusion occurs at the contact site. When the cells separate, the contact site is stretched and deformed, forming TNTs [21]. The duration of contact is the key to the formation of stable TNTs.

Through the PPI network diagram and KEGG enrichment analysis table of common targets, we can observe that the four targets in the green group and two targets in the blue group are closely related to Epimedin C and TNTs. These six targets are epidermal growth factor receptor (EGFR), insulin-like growth factor 1 (IGF1), matrix metalloproteinase 2 (MMP2), SRC oncogene (SRC), and bone morphogenetic protein 2/7 (BMP2/7). Upon combination with epidermal growth factor (EGF), EGFR can activate relevant genes in the nucleus, thereby promoting cell division and proliferation. During the process of cell proliferation, TNTs are also generated. IGF1 can bind to insulin-like growth factor 1 receptor (IGF-IR), which can regulate animal development and also plays an important role in muscle and bone growth, lymphocyte generation, and immunoregulation [22]. MMP2 is a critical member of the matrix metalloproteinase family, which is instrumental in angiogenesis along with extracellular matrix metalloproteinase inducer (EMMPRIN) [23]. SRC may participate in mediating embryonic development and cell growth [24]. BMP2/7 can affect mesenchymal stem cells and promote their osteogenic differentiation [25]. In summary, Epimedin C may promote the generation of TNTs by regulating targets such as EGFR, IGF1, MMP2, SRC, and BMP2/7.

GO analysis data show that the intersection targets mainly participate in biological processes such as transcriptional positive regulation, negative regulation of apoptosis, positive regulation of ERK1 and ERK2 cascade, cell proliferation and osteoblast

differentiation, as well as epithelial to mesenchymal transition. KEGG analysis results reveal that the intersection targets are mainly enriched in signaling pathways such as endocrine, bladder cancer, resistance to EGFR tyrosine kinase inhibitors and FoxO signaling pathways. Epimedin C may may enhance EGFR expression in endocrine, prolonging the contact time between membrane protrusions and other cells [26,27] and thus boosting the generation of TNTs. The effect of EGFR tyrosine kinase inhibitor resistance on TNTs is also achieved by changing the duration of contact between the membrane protrusions and other cells. Bladder cancer mainly regulates the production of TNTs by influencing the remodeling process of F-actin [28,29]. The binding of CDK2 and IGF1 in the FoxO signaling pathway affects the remodeling ability of the plasma membrane, which then interacts with cytoskeletal effectors to facilitate the formation of membrane ruffling and protrusions [30], thereby promoting the generation of TNTs.

The regulatory factors that impact the generation of TNTs include epidermal growth factor receptor pathway 8 (Eps8), exocyte complex [31], Fyn/ROCK/p-paxillin signaling pathway [29], Wnt/Ca^{2+} pathway [32], and myosin X (Myo10) [33]. According to the target analysis of each signaling pathway in Table S5, Epimedin C mainly enhances the generation of TNTs through two targets, EGFR and IGF1. Therefore, we surmise that endocrine and EGFR tyrosine kinase inhibitor resistance are the main signaling pathways through which Epimedin C promotes the generation of TNTs. Further experimental studies are required to determine which of these pathways has a more significant impact.

This study integrates network databases and computational prediction methods to obtain target information, screens intersection targets, and constructs a PPI network. Subsequently, GO functional enrichment analysis and KEGG pathway analysis are performed on target genes to preliminarily explore the mechanism of Epimedin C promoting TNTs generation. The research results can provide theoretical support for subsequent experimental studies on the promotion of TNTs production by Epimedin C, and also offer new insights into the application of Epimedin C in the treatment of TNTs-related diseases such as osteoporosis.

Although this study preliminarily predicted the potential targets and pathways of Epimedin C in regulating the generation of TNTs, there are still the following limitations: first, the network pharmacology analysis mainly relies on public databases and computational predictions, and the targets and pathways obtained have not been verified by *in vitro* and *in viv*o experiments, and their biological reality needs to be further confirmed; Second, the potential contribution of its metabolites was not considered for target prediction based on its prototype compound, which may undergo metabolic transformation *in vivo*. Thirdly, the database itself has update lag and species differences, and some target and pathway annotations may not be fully applicable to the mechanisms related to human osteoporosis. In the future, it is necessary to integrate experimental verification and in-depth mechanism research to more fully explain the regulatory effect of Epimedin C on TNTs.

Acknowledgements

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

L.X.: writing-original draft, conceptualization. G.C.: methodology, validation, data curation. M.Z.: writing-review and editing. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work

ensuring integrity and accuracy.

Ethics Approval and Consent to Participate

No ethical approval was required for this article.

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Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Supplementary

The following supporting information can be downloaded at: https://ojs.exploverpub.com/index.php/jecacm/article/view/326/sup. Supplementary Table S1: PPI network for intersection targets of Epimedin C and TNTs. Supplementary Table S2: GO functional analysis of intersection targets (BP). Supplementary Table S3: GO functional analysis of intersection targets (CC). Supplementary Table S4: GO functional analysis of intersection targets (MF). Supplementary Table S5: KEGG pathway enrichment analysis.

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