

基于UPLC-Q-TOF-MS/MS及网络药理学探讨活络效灵丹治疗糖尿病周围神经病变的物质基础研究

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摘要: 本实验通过超高效液相色谱-四极杆-飞行时间串联质谱(UPLC-Q-TOF-MS/MS)、网络药理学探讨活络效灵丹治疗糖尿病周围神经病变(diabetic peripheral neuropathy, DPN)的物质基础。利用UPLC-Q-TOF-MS/MS技术结合PeakView™ 1.2软件、Molecule Profiler™软件分析活络效灵丹的体外成分及正常大鼠与高脂饲料喂养联合小剂量链脲佐菌素注射所致DPN大鼠的入血成分,通过Swiss Target Prediction、GeneCards等数据库筛选成分对应靶点及疾病靶点,利用Venny 2.1平台获得交集靶点,导入STRING平台及Cytoscape 3.10.1软件构建蛋白互作网络及“成分-靶点”网络图分析其核心成分与核心靶点,对核心靶点进行GO和KEGG富集分析。实验获得南京中医药大学实验动物伦理委员会批准(批准号: 202310A023)。结果共鉴定出活络效灵丹提取物83个化学成分,包括丹参酮II_A等52个萜类、-Z-藜本内酯等11个苯酞类、阿魏酸等15个有机酸类及4个香豆素类、1个酚类成分。在正常大鼠血浆中鉴定出15个原型成分与29个代谢产物,在DPN大鼠血浆中鉴定出17个原型成分与32个代谢产物。网络药理学结果显示,3-乙酰基-11-酮基- β -乳香酸、11-酮基- β -乳香酸等5个核心成分可能通过TNF、IL6等27个核心靶点,调节AGE-RAGE、PI3K/Akt等信号通路发挥治疗DPN的作用。本研究通过UPLC-Q-TOF-MS/MS技术分析了活络效灵丹的体内化学成分及主要化合物的裂解规律,结合网络药理学分析其治疗DPN的核心成分,为阐明活络效灵丹治疗DPN的增效物质基础及作用机制研究提供科学依据。

关键词: 活络效灵丹; UPLC-Q-TOF-MS/MS; 网络药理学; 糖尿病周围神经病变; 化学成分; 入血成分

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Exploring the material basis of Huoluoxiaolingdan in the treatment of diabetic peripheral neuropathy based on UPLC-Q-TOF-MS/MS and network pharmacology

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Abstract: This study investigated the material basis of Huoluoxiaolingdan in treating diabetic peripheral

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neuropathy (DPN) through ultra performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) and network pharmacology. UPLC-Q-TOF-MS/MS combined with PeakView™ 1.2 software and Molecule Profiler™ software were used to analyze the chemical components of Huoluoxiaolingdan and blood-absorbed ingredients in normal rats and rats with DPN induced by high fat diet combined with low dose streptozotocin injection. Swiss Target Prediction database, GeneCards and other databases were used to search the corresponding targets of active ingredients and disease targets. The intersection targets were obtained using Venny 2.1 platform, which were then imported into the STRING platform and Cytoscape 3.10.1 software to construct the protein-protein interaction network and the "component-target" network relationship diagram, and the core components and core targets were analyzed. GO and KEGG pathway enrichment analysis of key targets were performed. All experiments were approved by the experimental Animal Ethics Committee from Nanjing University of Chinese Medicine (No. 202310A023). A total of 83 chemical components were identified, including 52 terpenoids such as tanshinone II_A, 11 phthalides such as *Z*-ligustilide, 15 organic acids such as ferulic acid, 4 coumarins and 1 phenol. Additionally, 15 prototype components and 29 metabolites were identified in normal rats' plasma, and 17 prototype components and 32 metabolites were identified in DPN rats' plasma. Network pharmacology results show that 5 core components such as 3-acetyl-11-keto- β -boswellic acid and 11-keto- β -boswellic acid may act through 27 core targets such as TNF and IL6 to regulate AGE-RAGE and PI3K/Akt and other signaling pathways so as to play a therapeutic role in treatment of DPN. The study efficiently analyzed the constituents *in vitro* and blood-absorbed ingredients of Huoluoxiaolingdan and the cracking regularity of the main compounds, and the core components of its treatment of DPN were analyzed in combination with network pharmacology, which can provide reference for further research of the pharmacodynamic substantial basis and mechanism of Huoluoxiaolingdan in the treatment of DPN.

Key words: Huoluoxiaolingdan; UPLC-Q-TOF-MS/MS; network pharmacology; diabetic peripheral neuropathy; chemical component; blood-absorbed ingredient

糖尿病周围神经病变 (diabetic peripheral neuropathy, DPN) 属于糖尿病常见的难治性并发症, 临床表现主要为针刺样、撕裂样疼痛、感觉异常, 最典型的表现“手套-袜子感”, 其发病率高, 起病缓慢, 患者不易察觉, 病情复杂且病程较长, 若不及时干预, 将引发足部溃疡、坏疽, 严重者需截肢, 甚至失去生命^[1]。DPN 发病机制与微循环障碍、神经营养因子失调、晚期糖基化产物 (AGEs) 过度积累、氧化应激及炎症等因素均有密切关系, 且目前临床还没有治疗 DPN 的特效药物, 一般是通过控制血糖、使用营养神经药物等来缓解, 疗效有限, 还可能出现不良反应^[2,3]。

中医认为 DPN 属“痹症”, 病因为气血凝滞, 经络气血运行受阻, 造成壅滞凝涩^[4]。活络效灵丹一方来源于名医张锡纯的《医学衷中参西录》, 由当归、丹参、乳香及没药各 15 g 组成, 合用有养血活血、舒经活络、行气止痛、消积除癥的功效^[5]; 现代药理研究表明活络效灵丹具有抗炎、消肿、镇痛及改善微循环的作用^[6], 临床上应用于治疗 DPN, 能明显提升患者的神经传导速度^[7]。目前对活络效灵丹的研究多停留在临床观察水平, 缺乏对全方化学成分的研究, 且方中四药皆“善入血分、通经络”, 但未见其入血成分的相关报道, 缺乏对正常状态及病理状态下动物体内成分的探究, 功效

物质基础尚不明确。

因此, 本实验建立 UPLC-Q-TOF-MS/MS 法, 分别鉴定活络效灵丹的化学成分及正常大鼠与 DPN 大鼠灌胃给药后体内的原型成分及代谢产物, 结合网络药理学分析活络效灵丹入血成分治疗 DPN 的活性成分、核心靶点与通路, 为后续探究活络效灵丹治疗 DPN 的功效物质基础及作用机制奠定基础。

材料与方法

仪器 高分辨液质联用仪配有 ExionLC 和液相色谱和 Zeno TOF 7600 质谱仪 (美国 AB Sciex 公司); Unique-R40 多功能超纯水系统 (厦门锐思捷水纯化技术有限公司); Labconco CentriVap 真空离心浓缩仪 (北京照生行仪器设备有限公司); BT125D 型电子天平 (赛多利斯科学仪器有限公司); GL-16G 型离心机 (上海安亭科学仪器厂); 微型涡旋混合仪 (上海沪西分析仪器厂有限公司); 三诺安稳+血糖仪 (批号: 2J01B240303, 三诺生物传感股份有限公司); Von-Frey 纤维细丝机械刺激针 (美国 Danmic Aesthesio 公司)。

药物及试剂 当归 (批号: 231011)、丹参 (批号: 230918)、乳香 (批号: 230821)、没药 (批号: 230920) 均购自苏州市天灵中药饮片有限公司, 经南京中医药大学

段金廛教授鉴定均为正品合格药材;对照品阿魏酸(批号:110773-201012)、隐丹参酮(批号:110852-200806)均购自中国食品药品检定研究院;11-羰基- β -乳香酸(批号:HS16311R2)、3 α -乙酰氧基-羊毛脂-8,24-二烯-21-酸(批号:HS15151B1)均购自宝鸡辰光生物科技有限公司;二氢丹参酮I(批号:MUST-15020102)购自成都曼斯特生物科技有限公司;Z-藁本内酯(批号:A26GB158725)、丹参素(批号:H09N10S102463)、丹酚酸A(批号:J26IB221287)、丹酚酸B(批号:S29GB162878)、丹参酮I(批号:K30D10B107208)、丹参酮II_A(批号:J10GB151070)、3-乙酰-11-羰基- β -乳香酸(批号:J20GB152181)、 α -乳香酸(批号:F16IB207144)、 β -乳香酸(批号:N17GB168325)均购自上海源叶生物科技有限公司;2-甲氧基-8,12-环氧吉马烷-1(10)7,11-三烯-6-酮(MCS134,批号:231640)购自美国TargetMol公司;2-甲氧基-5-乙酰基-呋喃吉马烷-1(10)-烯-6-酮(FSA)为实验室自制,纯度 $\geq 95\%$;乙腈为色谱级(德国Merck公司);甲酸色谱级(美国ThermoFisher Scientific公司);屈臣氏蒸馏水购自南京禾苗生物科技有限公司;无水乙醇(分析纯,批号:20240122,无锡市亚盛化工有限公司);链脲佐菌素(streptozotocin, STZ;批号:S0130,美国Sigma公司);柠檬酸钠缓冲液(0.1 mmol·L⁻¹, pH 4.5,批号:C-1013,北京索莱宝公司)。

实验动物 SPF级雄性SD大鼠,体重180~220 g,购于上海斯莱克实验动物有限责任公司,动物许可证号为SCXK(沪)2022-0004,南京中医药大学实验动物中心屏障环境内分笼饲养,室温控制在22℃左右,相对湿度40%~70%,保持采光通风良好,大鼠正常活动。本实验获得南京中医药大学动物实验伦理委员会审核批准(伦理批准号为202310A023),实验过程中普通饲料和垫料均购自南京中医药大学实验动物中心;高脂饲料(配方:维持基础料43.6%,猪油17.5%,蔗糖12%,全脂奶粉10%,酪蛋白10%,实验动物预混料2%,微晶纤维素1.9%,磷酸氢钙2%,石粉1%)购自书玉生物科技有限公司。

对照品溶液的制备 精密称取Z-藁本内酯、阿魏酸、丹参素、丹酚酸A、丹酚酸B、隐丹参酮、丹参酮I、二氢丹参酮I、丹参酮II_A、11-羰基- β -乳香酸、3-乙酰-11-羰基- β -乳香酸、 α -乳香酸、 β -乳香酸、3 α -乙酰氧基-羊毛脂-8,24-二烯-21-酸、MCS134、FSA,用甲醇配制浓度为1 mg·mL⁻¹的单一对照品溶液;分别吸取0.05 mL单一对照品溶液于同一10 mL量瓶中,用甲醇定容,制成各成分浓度均为0.005 mg·mL⁻¹的混合对照品溶液。

活络效灵丹提取物制备 按1:1:1:1的配比称取适量当归、丹参、乳香、没药饮片,加入6倍量纯水浸泡

0.5 h,水蒸气蒸馏法回流提取8 h,静置1 h,收集挥发油,经无水硫酸钠干燥,水提液趁热过滤,滤液置于旋转蒸发仪中浓缩,剩余药渣加入10倍量80%乙醇,回流提取1 h,提取3次,滤液置于旋转蒸发仪中浓缩至无醇味,与水提液合并浓缩至相当于药材0.8 g·mL⁻¹,加入干燥后的挥发油混合均匀,待用。

活络效灵丹供试品溶液制备 吸取活络效灵丹提取物0.05 mL,加入0.95 mL色谱级乙腈,涡旋混匀,于4℃下13 000 r·min⁻¹离心15 min,取上清液经0.22 μ m孔径滤膜过滤,4℃冰箱保存待测。

糖尿病周围神经病变模型大鼠造模及给药 12只SD大鼠适应性喂养一周,随机分为空白组与模型组,每组6只,空白组喂养普通饲料,模型组大鼠喂养高脂饲料3周后,注射小剂量1% STZ建立糖尿病(diabetes mellitus, DM)大鼠:大鼠禁食12 h后,避光环境下单侧腹腔注射35 mg·kg⁻¹的STZ,72 h后测大鼠尾静脉空腹血糖 ≥ 11.1 mmol·L⁻¹视为DM造模成功。DM大鼠继续以高脂饲料喂养3周,检测大鼠机械痛阈值(mechanical withdrawal threshold, MWT),采用Up and down法检测^[8]:将大鼠置于底部为多孔钢丝的笼中自由活动30~60 min适应环境,从2.0 g的von Frey纤维针开始,垂直刺激大鼠后肢足底中部,至纤维丝弯曲为S形,持续6 s以上,重复3次,每次间隔1~3 min,当大鼠出现舔咬、抬脚或缩回,视为阳性表现,根据动物反应,选用克数更大或更小的纤维丝进行下一次刺激,直至产生6个有效测定数据。测得大鼠机械痛阈值下降10%的判定为DPN大鼠造模成功^[9,10]。

血浆样本的制备 提前禁食12 h,给药前取自身空白血浆0.5 mL,随即按生药量6.25 g·kg⁻¹灌胃给予活络效灵丹提取物,给药1 h后经眼底静脉丛取血0.5 mL于含肝素钠的离心管内静置2 h,于4℃、4 000 r·min⁻¹离心10 min,取上清液,-80℃冰箱存放备用。

血浆样本的预处理 取各组血浆样品于冰上化冻,吸取100 μ L血浆加入400 μ L预冷乙腈,涡旋混匀60 s,4℃、13 000 r·min⁻¹离心15 min,取上清液离心浓缩至干,残留物加入50 μ L预冷乙腈复溶,4℃、13 000 r·min⁻¹离心15 min,取上清用于入血成分检测分析。

液相条件 Waters ACQUITY UPLC BEH C₁₈色谱柱(100 mm \times 2.1 mm, 1.7 μ m);流动相:0.1%甲酸水(A)-乙腈(B);柱温:40℃;流速:0.3 mL·min⁻¹,进样量:2 μ L;梯度洗脱:0~3 min, 5%~20% B; 3~7 min, 20%~30% B; 7~13 min, 30%~80% B; 13~15 min, 80%~87% B; 15~17 min, 87%~90% B; 17~18 min, 90%~95% B; 18~19 min, 95%~5% B; 19~20 min, 5% B。

质谱条件 开启动态背景扣除 (DBS) 功能, 采用电喷雾离子源 (ESI), 正、负离子模式扫描, 正离子电喷雾电压: 5.5 kV, 负离子电喷雾电压: -4.5 kV; 离子源温度: 550 °C; 去簇电压: 80 V (正离子)/-80 V (负离子); 碰撞能量: 35 V (正离子)/-35 V (负离子); 质量扫描范围 m/z 80~1 000; 喷雾气和辅助气: 55 psi (1 psi ≈ 6.895 kPa); 气帘气 35 psi。

数据处理 采用 Graphpad Prism 8 软件对大鼠 FBG 及 MWT 进行统计分析, 数据用均值 ± 标准差 ($\bar{x} \pm s$) 表示, 组间各指标比较采用 Unpaired student's *t* 检验, $P < 0.05$ 表示具有统计学意义。质谱原始数据导入 PeakView™ 1.2 软件 (美国 AB Sciex 公司), 根据精确分子量及二级质谱裂解碎片, 结合对照品质谱图、参考文献及 PubChem 数据库进行比对, 结合化合物特有的质谱裂解规律鉴定体外成分及入血原型成分。借助 Molecule Profiler™ 软件 (美国 AB Sciex 公司), 通过多重质量亏损功能, 与空白样本比较, 在小于 5×10^{-6} 的误差范围预测代谢产物准分子离子峰, 根据参考文献结合质谱裂解规律鉴定代谢产物。

活络效灵丹入血成分靶点预测及疾病靶点筛选 将 DPN 大鼠体内鉴定出的 17 个成分输入 PubChem 数据库 (<https://pubchem.ncbi.nlm.nih.gov/>) 搜索下载结构文件, 选择 Swiss Target Prediction 数据库 (<http://www.swisstargetprediction.ch/>) 中 probability 值 > 0 的靶点, 合并删除重复值, 得到成分靶点。在 GeneCards 数据库 (<https://www.genecards.org/>)、OMIM 数据库 (<https://www.omim.org/>)、DrugBank 数据库 (<https://go.drugbank.com/>) 中, 以关键词“diabetic peripheral neuropathy”进行搜索, 保留 GeneCards 数据库前 25% 的靶点, 整合所有靶点并去重, 得到疾病靶点。使用 Venny 2.1 作图工具 (<https://bioinfogp.cnb.csic.es/tools/-venny/>) 将成分靶点与疾病靶点取交集, 得到活络效灵丹治疗 DPN 的潜在靶点。

蛋白互作网络 (protein-protein interaction, PPI) 与“成分-靶点”网络图构建 将潜在靶点输入 STRING 数据库 (<https://string-db.org/>), 物种选择“Homo sapiens”,

构建 PPI 互作网络图, 导入 Cytoscape 3.10.1 软件进行分析, 根据度值 (degree)、中介中心性 (betweenness centrality, BC)、接近中心性 (closeness centrality, CC) 筛选出核心靶点; 运用 Cytoscape 3.10.1 软件构建“成分-靶点”网络图, 根据度值筛选核心成分。

GO 和 KEGG 通路富集分析 将核心靶点导入 DAVID 数据库 (<https://david.ncifcrf.gov/>) 进行 GO 生物功能富集分析和 KEGG 通路富集分析, 设置阈值 $P < 0.05$ 进行筛选, 将结果导入微生信平台绘图。

结果

1 糖尿病周围神经病变大鼠模型制备

造模后, 空白组 (CON 组) 大鼠体重稳定上升, 毛色鲜亮, 精神状态良好; 模型组 (MOD 组) 大鼠体重减轻, 毛色发黄、脱毛, 多饮、多食、多尿, 空腹血糖值显著上升, 机械痛阈值明显下降, 与空白组相比具有显著性差异 ($P < 0.01$), 提示 DPN 大鼠造模成功。结果如图 1 所示。

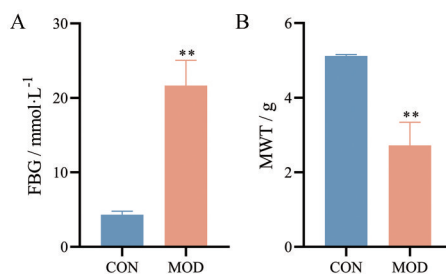


Figure 1 Comparison of fasting blood glucose (FBG, A) and mechanical withdrawal threshold (MWT, B) in each group. Model group (MOD) is induced by high fat diet combined with low dose streptozotocin injection. $n = 6$, $\bar{x} \pm s$. ** $P < 0.01$ vs control group (CON)

2 活络效灵丹体外化学成分鉴定

在活络效灵丹提取物中共鉴定出 83 种化合物, 其中萜类 52 种、苯酚类 11 种、有机酸类 15 种、香豆素类 4 种、酚类 1 种, 正负离子模式下的总离子流图如图 2 所示, 主要鉴定信息见表 1。

Table 1 Compounds identified from Huoluoxiaolingdan by UPLC-Q-TOF-MS/MS

| No. | t_R /min | Compound | Formula | m/z | Adduct | Error/ $\times 10^{-6}$ | MS/MS |
|-----|------------|-------------------|--|-----------|--------------------|-------------------------|--|
| 1 | 1.00 | Gallic acid | C ₇ H ₆ O ₅ | 169.014 0 | [M-H] ⁻ | -1.5 | 125.024 6, 107.014 0 |
| 2 | 2.42 | Caffeic acid | C ₉ H ₈ O ₄ | 179.034 8 | [M-H] ⁻ | -1.2 | 135.045 0, 117.032 4, 107.049 9 |
| 3 | 2.43 | Danshensu | C ₉ H ₁₀ O ₅ | 197.045 2 | [M-H] ⁻ | -2.0 | 179.034 7, 135.046 1, 123.046 0, 109.029 5 |
| 4 | 2.56 | Coumarate | C ₉ H ₈ O ₃ | 165.054 6 | [M+H] ⁺ | -0.2 | 119.085 6, 91.054 1 |
| 5 | 3.84 | Vanillic acid | C ₈ H ₈ O ₄ | 169.049 6 | [M+H] ⁺ | 0.3 | 125.060 1, 111.007 1, 95.066 8 |
| 6 | 5.06 | Ferulic acid | C ₁₀ H ₁₀ O ₄ | 195.065 2 | [M+H] ⁺ | -0.6 | 177.055 0, 149.059 2, 134.036 5 |
| 7 | 5.07 | 6-Methoxycoumarin | C ₁₀ H ₈ O ₃ | 177.054 7 | [M+H] ⁺ | 0.4 | 149.060 0, 145.028 7, 134.036 0, 117.033 8 |
| 8 | 5.09 | Isoferulic acid | C ₁₀ H ₁₀ O ₄ | 193.050 1 | [M-H] ⁻ | -2.9 | 178.027 1, 134.037 4 |

Continued

| No. | t_R /min | Compound | Formula | m/z | Adduct | Error/ $\times 10^{-6}$ | MS/MS |
|-----|------------|---|---|-----------|---------------------|-------------------------|--|
| 9 | 5.39 | 3,4-Dicaffeoylquinic acid | C ₂₉ H ₂₄ O ₁₂ | 515.119 1 | [M-H] ⁻ | -0.8 | 353.087 1, 191.055 7, 179.034 1 |
| 10 | 5.51 | Salvianolic acid D | C ₂₀ H ₁₈ O ₁₀ | 417.081 6 | [M-H] ⁻ | -2.8 | 197.045 3, 135.044 9 |
| 11 | 6.16 | 7-Hydroxycoumarin | C ₉ H ₆ O ₃ | 163.039 0 | [M+H] ⁺ | 0.2 | 135.044 7, 117.034 0 |
| 12 | 6.16 | Rosmarinic acid | C ₁₈ H ₁₆ O ₈ | 359.076 5 | [M-H] ⁻ | -1.9 | 179.035 3, 161.026 3 |
| 13 | 6.17 | Isomer of umbelliferide | C ₉ H ₆ O ₃ | 161.023 9 | [M-H] ⁻ | -3.0 | 133.028 7, 117.039 1 |
| 14 | 6.28 | Azelaic acid | C ₉ H ₁₆ O ₄ | 187.097 2 | [M-H] ⁻ | -2.0 | 125.096 7, 123.081 4 |
| 15 | 6.51 | Isomer of senkyunolide I | C ₁₂ H ₁₆ O ₄ | 225.112 1 | [M+H] ⁺ | -0.3 | 207.101 8, 179.106 3, 165.090 5 |
| 16 | 6.54 | Senkyunolide I | C ₁₂ H ₁₆ O ₄ | 225.112 1 | [M+H] ⁺ | -0.3 | 207.101 8, 189.090 5, 179.078 9 |
| 17 | 6.78 | Salvianolic acid B | C ₃₆ H ₃₀ O ₁₆ | 717.143 0 | [M-H] ⁻ | -4.3 | 519.093 3, 339.049 5, 321.040 2, 295.061 8 |
| 18 | 6.98 | Senkyunolide F | C ₁₂ H ₁₄ O ₃ | 207.101 7 | [M+H] ⁺ | 0.4 | 189.091 3, 179.106 0, 123.044 3 |
| 19 | 6.99 | Senkyunolide H | C ₁₂ H ₁₆ O ₄ | 225.112 1 | [M+H] ⁺ | -0.3 | 207.101 5, 189.091 1, 179.106 9, 165.089 5, 161.095 7, 117.069 4 |
| 20 | 7.09 | New osthol lactone | C ₁₂ H ₁₈ O ₂ | 195.137 9 | [M+H] ⁺ | -0.5 | 177.127 3, 159.117 1, 117.070 0 |
| 21 | 7.42 | Salvianolic acid A | C ₂₆ H ₂₂ O ₁₀ | 493.113 0 | [M-H] ⁻ | -2.0 | 313.071 8, 295.062 9, 203.034 9, 185.024 7, 159.045 2, 109.030 3 |
| 22 | 9.04 | Myrrhanolide B | C ₁₅ H ₁₈ O ₄ | 261.112 6 | [M-H] ⁻ | -2.5 | 201.092 1, 187.076 4, 175.112 7 |
| 23 | 9.29 | Coniferyl ferulate | C ₂₀ H ₂₀ O ₆ | 355.117 0 | [M-H] ⁻ | -4.8 | 311.128 1, 296.104 6 |
| 24 | 9.44 | Atractylenolide III | C ₁₅ H ₂₀ O ₃ | 249.148 5 | [M+H] ⁺ | 0.0 | 135.080 6, 107.084 8 |
| 25 | 9.50 | Myrrheterpenoid H | C ₁₆ H ₂₀ O ₄ | 277.143 6 | [M+H] ⁺ | 0.4 | 245.120 1, 121.066 8 |
| 26 | 9.63 | Commipholinone | C ₁₄ H ₁₆ O ₃ | 233.117 5 | [M+H] ⁺ | 1.1 | 119.085 4, 105.069 9 |
| 27 | 9.76 | Commiterpene D | C ₁₈ H ₂₂ O ₅ | 319.154 3 | [M+H] ⁺ | 1.0 | 227.107 9, 156.093 4 |
| 28 | 9.89 | 9,10-Secoisoahydro-xyaconido lactone | C ₁₅ H ₁₈ O ₃ | 247.132 9 | [M+H] ⁺ | 0.2 | 159.081 8, 91.055 0 |
| 29 | 10.02 | Isopimpinellin | C ₁₃ H ₁₀ O ₅ | 247.059 4 | [M+H] ⁺ | -3.1 | 189.091 0, 161.095 7, 133.064 9 |
| 30 | 10.59 | MCS134 | C ₁₆ H ₂₂ O ₃ | 285.147 5 | [M+Na] ⁺ | 4.9 | 187.038 6, 171.116 7, 159.116 4, 145.101 3 |
| 31 | 10.89 | Przewaquinone A | C ₁₉ H ₁₈ O ₄ | 311.127 8 | [M+H] ⁺ | 0.0 | 283.132 4, 237.056 3 |
| 32 | 10.91 | Nortanshinone | C ₁₇ H ₁₂ O ₄ | 281.080 3 | [M+H] ⁺ | -1.8 | 253.086 2, 225.092 4 |
| 33 | 11.06 | Tanshinone II _B | C ₁₉ H ₁₈ O ₄ | 311.127 8 | [M+H] ⁺ | 0.0 | 293.117 1, 283.132 9, 275.106 7, 250.106 9, 247.111 0 |
| 34 | 11.06 | 1S-Hydroxy-anhydride of 16R cryptotanshinone | C ₁₉ H ₂₀ O ₅ | 327.122 5 | [M-H] ⁻ | -4.0 | 283.133 4, 239.143 7 |
| 35 | 11.38 | Senkyunolide A | C ₁₂ H ₁₆ O ₂ | 193.122 2 | [M+H] ⁺ | -0.5 | 147.117 8, 137.061 0, 119.084 9, 105.070 0, 93.070 7 |
| 36 | 11.47 | Tanshialdehyde | C ₁₉ H ₁₆ O ₄ | 309.112 2 | [M+H] ⁺ | 0.2 | 265.123 2, 250.098 6, 235.076 3 |
| 37 | 11.49 | Canangaterpene VI | C ₁₅ H ₂₂ O ₂ | 223.169 0 | [M+H] ⁺ | -1.0 | 119.085 7, 91.054 6 |
| 38 | 11.91 | N-Butylphthalide | C ₁₂ H ₁₄ O ₂ | 191.106 6 | [M+H] ⁺ | -0.1 | 173.097 3, 145.100 8 |
| 39 | 12.05 | FSA | C ₁₈ H ₂₄ O ₅ | 321.169 5 | [M+H] ⁺ | -0.4 | 185.096 7, 168.095 3, 159.083 4, 145.076 3, 131.086 0 |
| 40 | 12.06 | Commiterpene B | C ₁₈ H ₂₂ O ₄ | 303.159 4 | [M+H] ⁺ | 1.0 | 211.112 5, 181.065 7 |
| 41 | 12.09 | Dihydrotanshinone I | C ₁₈ H ₁₄ O ₃ | 279.101 4 | [M+H] ⁺ | -0.5 | 261.094 1, 233.099 0, 205.103 5 |
| 42 | 12.17 | Z-Ligustilide | C ₁₂ H ₁₄ O ₂ | 191.106 6 | [M+H] ⁺ | -0.1 | 173.096 3, 163.111 6, 145.100 8, 117.070 0, 105.069 8, 91.054 5 |
| 43 | 12.27 | (1E)-8,12-Epoxy-germacra-1,7,10,11-tetraen-6-one | C ₁₅ H ₁₈ O ₂ | 231.138 1 | [M+H] ⁺ | 0.7 | 145.065 1, 105.070 0, 91.054 4 |
| 44 | 12.37 | 2-Methoxy-furanogermacra-1(10),4-diene | C ₁₆ H ₂₀ O ₃ | 261.148 3 | [M+H] ⁺ | -0.5 | 175.112 3, 161.135 2, 91.053 9 |
| 45 | 12.39 | Danshenxinkun B | C ₁₈ H ₁₆ O ₃ | 281.117 1 | [M+H] ⁺ | -0.6 | 253.158 7, 235.113 9 |
| 46 | 12.46 | Epicurzerenone | C ₁₅ H ₂₀ O ₂ | 233.153 4 | [M+H] ⁺ | -0.9 | 145.102 5, 131.087 3, 95.086 0 |
| 47 | 12.92 | 9-Methylmyrrhone | C ₁₆ H ₁₈ O ₃ | 259.132 8 | [M+H] ⁺ | -0.3 | 211.074 5, 189.092 1, 174.101 8, 161.060 2 |
| 48 | 12.96 | Myrrhone | C ₁₅ H ₁₆ O ₂ | 229.122 2 | [M+H] ⁺ | -0.5 | 159.086 6, 145.066 9 |
| 49 | 13.03 | Tanshinone I | C ₁₈ H ₁₂ O ₃ | 277.085 7 | [M+H] ⁺ | -1.0 | 262.063 2, 249.096 1, 221.096 3 |
| 50 | 13.08 | Cryptotanshinone | C ₁₉ H ₂₀ O ₃ | 297.148 3 | [M+H] ⁺ | -0.6 | 282.126 4, 279.140 0, 268.111 1, 251.144 4, 237.097 3, 227.071 4 |
| 51 | 13.17 | Rel-3R-methoxy-4S-furanogermacra-1E,10(15)-dien-6-one | C ₁₆ H ₂₀ O ₃ | 261.148 4 | [M+H] ⁺ | -0.4 | 175.076 7, 161.060 4, 145.085 2, 131.086 1 |
| 52 | 13.18 | Myrrheterpenoid J | C ₁₆ H ₂₀ O ₃ | 261.148 4 | [M+H] ⁺ | -0.6 | 229.125 3, 201.110 2, 196.088 8 |
| 53 | 13.36 | Incense oxide | C ₂₀ H ₃₄ O ₃ | 323.257 8 | [M+H] ⁺ | -0.7 | 163.147 1, 149.133 0, 121.102 3 |
| 54 | 13.46 | Methylenetanshinquinone | C ₁₈ H ₁₄ O ₃ | 279.101 4 | [M+H] ⁺ | -0.5 | 261.096 7, 233.099 6, 205.103 1 |

Continued

| No. | t_R /min | Compound | Formula | m/z | Adduct | Error/ $\times 10^{-6}$ | MS/MS |
|-----|------------|--|-------------------|-----------|-----------|-------------------------|--|
| 55 | 13.66 | Levistolide A | $C_{24}H_{28}O_4$ | 381.205 7 | $[M+H]^+$ | -0.7 | 191.110 8, 173.096 4, 149.059 5, 145.100 8, 135.043 8, 117.070 0 |
| 56 | 13.75 | Angelicide | $C_{24}H_{28}O_4$ | 381.205 7 | $[M+H]^+$ | -0.7 | 335.200 6, 191.112 2 |
| 57 | 13.81 | Olibanumol I | $C_{29}H_{46}O_2$ | 427.356 5 | $[M+H]^+$ | -1.2 | 163.147 7, 149.131 9 |
| 58 | 13.82 | Senkyunolide P | $C_{24}H_{28}O_4$ | 381.205 8 | $[M+H]^+$ | -0.6 | 335.201 5, 279.136 7, 191.113 4 |
| 59 | 13.85 | Dehydromiltirone | $C_{19}H_{20}O_2$ | 281.153 3 | $[M+H]^+$ | -1.1 | 266.130 4, 253.158 5 |
| 60 | 13.90 | Alismol | $C_{15}H_{24}O$ | 221.189 9 | $[M+H]^+$ | -0.6 | 147.116 7, 107.085 4, 103.054 0 |
| 61 | 14.04 | Tanshinone II _A | $C_{19}H_{18}O_3$ | 295.132 7 | $[M+H]^+$ | -0.6 | 280.110 4, 277.125 9, 266.094 9, 262.100 8, 249.127 6 |
| 62 | 14.19 | (-)- α -Cubebene | $C_{15}H_{24}$ | 205.194 8 | $[M+H]^+$ | -1.2 | 105.070 8, 91.057 8 |
| 63 | 14.22 | Dihydro-cryptotanshinone | $C_{19}H_{22}O_3$ | 299.163 9 | $[M+H]^+$ | -1.0 | 281.153 8, 253.158 9 |
| 64 | 14.30 | Miltirone | $C_{19}H_{22}O_2$ | 283.169 1 | $[M+H]^+$ | -0.7 | 268.145 3, 265.159 0 |
| 65 | 14.46 | Lupan-20(29)-ene-3 α ,30-diol | $C_{30}H_{50}O_2$ | 443.387 6 | $[M+H]^+$ | -1.8 | 297.247 6, 217.195 1 |
| 66 | 14.68 | 11-Oxo-24-norursa-3,9(11),12-triene | $C_{29}H_{44}O$ | 409.346 2 | $[M+H]^+$ | -0.6 | 219.174 8, 175.148 2 |
| 67 | 14.69 | Tsugaric acid A | $C_{32}H_{50}O_4$ | 499.377 3 | $[M+H]^+$ | -1.8 | 439.358 2, 369.273 4, 329.276 2, 255.212 5 |
| 68 | 14.76 | 11-Carbonyl- β -boswellic acid | $C_{30}H_{46}O_4$ | 469.330 6 | $[M-H]^-$ | -3.7 | 407.331 2, 391.300 0 |
| 69 | 14.79 | Lupeolic acid | $C_{30}H_{46}O_4$ | 455.351 7 | $[M+H]^+$ | -0.7 | 437.341 9, 271.241 6 |
| 70 | 15.01 | Boscartol C | $C_{20}H_{32}O_2$ | 305.247 2 | $[M+H]^+$ | -0.9 | 175.147 0, 159.116 5, 145.101 2 |
| 71 | 15.23 | Incensole | $C_{20}H_{34}O_2$ | 307.263 0 | $[M+H]^+$ | -0.6 | 123.118 1, 109.102 8, 95.088 4 |
| 72 | 15.36 | Boscartin X | $C_{22}H_{36}O_4$ | 365.268 2 | $[M+H]^+$ | -1.2 | 305.248 5, 175.147 1, 151.111 6, 121.101 2 |
| 73 | 15.79 | β -Hydroxyman-subin-13(17)-ene-11-ketone | $C_{22}H_{34}O_2$ | 331.262 9 | $[M+H]^+$ | -0.8 | 189.163 6, 121.101 0 |
| 74 | 16.36 | α -Boswellic acid | $C_{30}H_{48}O_3$ | 455.350 8 | $[M-H]^-$ | -5.0 | 437.351 8, 409.350 9, 361.063 9 |
| 75 | 16.45 | 3-Acetyl-11-keto- β -boswellic acid | $C_{32}H_{48}O_5$ | 513.357 3 | $[M+H]^+$ | -0.3 | 497.812 6, 495.347 9, 470.005 9, 453.339 3, 437.328 0 |
| 76 | 16.51 | Verticiol | $C_{20}H_{34}O$ | 291.267 9 | $[M+H]^+$ | -1.2 | 107.085 3, 93.069 9, 81.070 5 |
| 77 | 16.53 | 3 α -Acetyl-9,11-deoxy- β -boswellic acid | $C_{32}H_{48}O_4$ | 497.362 2 | $[M+H]^+$ | -0.7 | 437.342 1, 391.335 8 |
| 78 | 16.67 | β -Boswellic acid | $C_{30}H_{48}O_3$ | 455.350 8 | $[M-H]^-$ | -5.0 | 409.310 5, 367.391 6, 283.712 5 |
| 79 | 16.91 | Cycloartenol | $C_{30}H_{50}O$ | 427.392 8 | $[M+H]^+$ | -1.5 | 189.163 6, 173.131 8 |
| 80 | 17.44 | Commiphorane G2 | $C_{22}H_{34}O$ | 315.268 0 | $[M+H]^+$ | -0.8 | 145.100 5, 105.069 9 |
| 81 | 17.70 | Epilupeol | $C_{30}H_{48}O$ | 425.377 0 | $[M+H]^+$ | -1.9 | 187.147 5, 135.116 7 |
| 82 | 17.72 | Lupenone | $C_{30}H_{48}O$ | 425.376 7 | $[M+H]^+$ | -2.3 | 203.179 2, 189.163 1, 147.116 6, 105.069 5, 81.070 0 |
| 83 | 17.85 | Incensole acetate | $C_{22}H_{36}O_3$ | 349.273 4 | $[M+H]^+$ | -0.9 | 203.179 9, 163.148 2 |

2.1 萜类化合物 活络效灵丹提取物中共鉴定出52种萜类化合物,包括19种倍半萜类、20种二萜类及13种三萜类化合物,主要来自丹参、乳香、没药。二萜类化合物在正离子模式下的信号响应较高,准分子离子峰主要以 $[M+H]^+$ 形式呈现,其中丹参中的二萜醌类主要丢失 H_2O 、 CH_3 和CO等中性分子^[11]。

例如化合物**61** ($t_R = 14.04$ min),正离子模式下准分子离子峰为 m/z 295.132 7 $[M+H]^+$,推断化合物的分子式为 $C_{19}H_{18}O_3$ (误差为 -0.6×10^{-6}),二级质谱图中出现的碎片离子为 m/z 280.110 4、277.125 9、266.094 9、262.100 8、249.127 6,其中碎片离子 m/z 280.110 4为该化合物失去1分子 CH_3 所形成,进一步脱去1分子 H_2O 形成 m/z 262.100 8 $[M+H-CH_3-H_2O]^+$ 的碎片离子;母离子失去醛基形成 m/z 266.094 9 $[M+H-CHO]^+$,脱去1分子 H_2O 产生 m/z 277.125 9 $[M+H-H_2O]^+$,继续脱去

1分子CO得到 m/z 249.127 6 $[M+H-H_2O-CO]^+$,结合文献^[12]数据及与对照品比对,确定该化合物为丹参酮II_A,可能的裂解途径见图3A。

三萜类化合物主要构型有齐墩果烷型及乌苏烷型,母核结构不易开裂,裂解方式主要为脱去 H_2O 、乙酰基及 CH_3COOH 等侧链取代基。以化合物**75** ($t_R = 16.45$ min)为例,正离子模式下准分子离子峰为 m/z 513.357 3,推断化合物分子式为 $C_{32}H_{48}O_5$ (误差为 -0.3×10^{-6}),主要的二级碎片离子为 m/z 497.812 6、495.347 9、470.005 9、453.339 3、437.328 0,碎片离子 m/z 497.812 6、495.347 9、470.005 9、453.339 3分别为分子离子峰失去1分子O、 H_2O 、乙酰基及 CH_3COOH 形成的碎片离子, m/z 453.339 3进一步脱去O形成 m/z 437.328 0 $[M+H-CH_3COOH-O]^+$ 。结合裂解规律及与文献^[13]、对照品比对,鉴定该化合物为3-乙酰基-11-酮

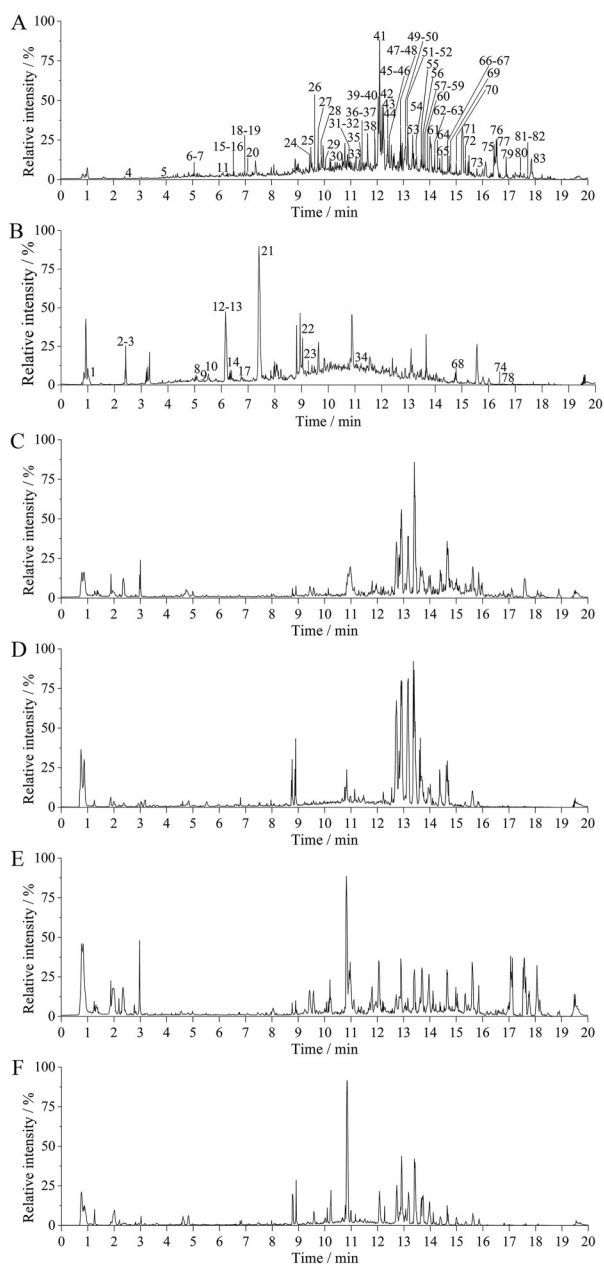


Figure 2 Total ion current chromatograms in positive (A, C, E) and negative (B, D, F) ion modes. A, B: Extract of Huoluoxiaolingdan; C, D: Normal rats' plasma after administration; E, F: Diabetic peripheral neuropathy (DPN) rats' plasma after administration. The peak number is the same as the number in the Table 1

基- β -乳香酸,可能的裂解途径见图3B。

2.2 苯酐类化合物 鉴定出11种苯酐类化合物,主要存在于当归中,分为简单苯酐与苯酐二聚体。苯酐类化合物在裂解时易脱去 H_2O 、 CO 、 CO_2 ,或失去侧链上的烯基结构^[14]。

以化合物42($t_{\text{R}} = 12.17 \text{ min}$)为例,正离子模式下该化合物准分子离子峰为 m/z 191.106 6,判断化合物的分子式为 $\text{C}_{12}\text{H}_{14}\text{O}_2$ (误差为 -0.1×10^{-6}),二级质谱的

主要碎片离子有 m/z 173.096 3、163.111 6、145.100 8、117.070 0、105.069 8、91.054 5等,分子离子峰丢失2分子 CH_2 形成 m/z 163.111 6的碎片离子, m/z 173.096 3、145.100 8、117.070 0、105.069 8为准分子离子峰连续丢失1分子 H_2O 、1分子 CO 、2分子 CH_2 、1分子 C_3H_4 形成的碎片离子,在 m/z 105.069 8的基础上丢失1分子 CH_2 产生 m/z 91.054 5 $[\text{M}+\text{H}-\text{H}_2\text{O}-\text{CO}-\text{C}_4\text{H}_6]^+$ 。结合参考文献^[15]并与对照品比对,判定该化合物为Z-藁本内酯,其裂解途径见图3C。

2.3 有机酸类化合物 共鉴定出15种有机酸类化合物,主要来源于当归、丹参,其裂解规律为容易丢失 CO 、 CO_2 、 COOH 、 H_2O 等小分子。例如化合物6($t_{\text{R}} = 5.06 \text{ min}$),正离子模式下显示准分子离子峰为 m/z 195.065 2,判断化合物的分子式为 $\text{C}_{10}\text{H}_{10}\text{O}_4$ (误差为 -0.6×10^{-6}),主要的二级质谱的碎片离子包括 m/z 177.055 0、149.059 2、134.036 5,其中碎片离子 m/z 177.055 0是由准分子离子峰脱去1分子 H_2O 所形成,继而丢失1分子 CO 形成 m/z 149.059 2 $[\text{M}+\text{H}-\text{H}_2\text{O}-\text{CO}]^+$,在此基础上丢失1分子 CH_3 形成 m/z 134.036 5 $[\text{M}+\text{H}-\text{H}_2\text{O}-\text{CO}-\text{CH}_3]^+$ 。依据参考文献^[16]结合对照品比对,推断该化合物为阿魏酸,可能的裂解途径见图3D。

2.4 其他类化合物 除上述3种化合物外,还在活络效灵丹提取物中检测出4种香豆素类化合物及1种酚类化合物,其中香豆素类化合物来源于当归,酚类化合物来源于乳香。

3 活络效灵丹入血成分分析

3.1 活络效灵丹入血原型成分分析 各组血浆处理后检测得到正负离子模式下的总离子流图,见图2;处理质谱数据,结合体外成分质谱信息及文献查阅,分别扣除各组空白血浆中的内源性成分,于空白给药组(SL组)中鉴定出15种原型成分,在模型给药组(BL组)中鉴定出17种原型成分,具体信息见表2。共检测出9个相同的原型成分,主要为有机酸类、苯酐类及萜类化合物,其中有机酸类来自当归、丹参中的阿魏酸与异阿魏酸,苯酐类主要来源于当归中的洋川芎内酯F,萜类来源于丹参中的丹参酮II_B、隐丹参酮、二氢丹参酮I,乳香中的3-乙酰基-11-酮基- β -乳香酸及没药中的myrrhanolide B。鉴定出的成分大多为药材中的特征性成分,提示这些成分可能为活络效灵丹改善DPN的功效物质。可能因为不同状态大鼠药物吸收程度存在差异,检测出SL组及BL组的原型成分种类不同,SL组中还检测出丹参醛、丹参素等成分,BL组中鉴定出SL组中没有的Z-藁本内酯、MCS134及11-羧基- β -乳香酸等成分。

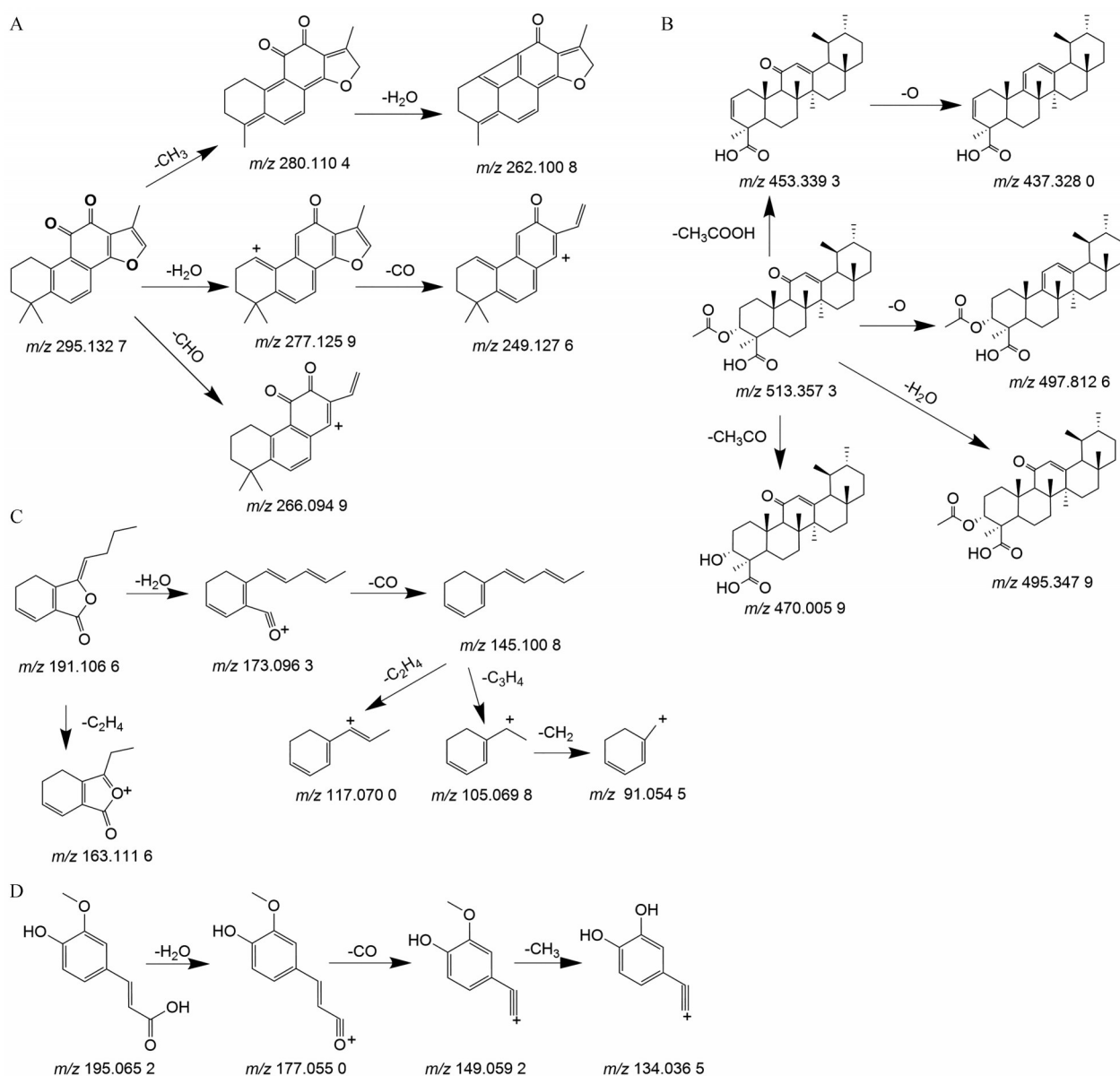


Figure 3 Proposed fragmentation pathways of ingredients of Huoluoxiaolingdan extracts. A: Tanshinone II_A; B: 3-Acetyl-11-keto-β-boswellic acid; C: Z-Ligustilide; D: Ferulic acid

Table 2 UPLC-Q-TOF-MS/MS analysis of components in the blood of Huoluoxiaolingdan. 1-23: Prototype components; M1-M38: Metabolites; SL: Normal rats' plasma after administration; BL: DPN rats' plasma after administration; +: Detected; -: Not detected

| No. | <i>t_R</i> /min | Formula | <i>m/z</i> | Adduct | Error / $\times 10^{-6}$ | MS/MS | Compound | Phase | SL | BL |
|-----|------------------------------|--|------------|--------------------|-----------------------------|--|--|-------|----|----|
| 1 | 1.89 | C ₉ H ₈ O ₃ | 165.054 7 | [M+H] ⁺ | 0.3 | 119.050 9, 91.054 9 | Coumarate | - | + | + |
| 2 | 2.41 | C ₉ H ₁₀ O ₅ | 197.044 9 | [M-H] ⁻ | -3.4 | 179.034 4, 135.045 6, 123.045 3, 109.030 2 | Danshensu | - | + | - |
| 3 | 3.49 | C ₁₀ H ₁₀ O ₄ | 193.049 8 | [M-H] ⁻ | -4.4 | 178.025 6, 134.037 9 | Isoferulic acid | - | + | + |
| 4 | 6.34 | C ₁₀ H ₁₀ O ₄ | 195.065 1 | [M+H] ⁺ | -0.6 | 177.163 5, 149.050 9, 134.033 9 | Ferulic acid | - | + | + |
| 5 | 6.96 | C ₁₂ H ₁₄ O ₃ | 207.101 5 | [M+H] ⁺ | -0.4 | 189.090 9, 179.106 8, 123.044 8 | Senkyunolide F | - | + | + |
| 6 | 8.15 | C ₁₅ H ₁₈ O ₃ | 247.132 8 | [M+H] ⁺ | -0.5 | 159.080 7, 91.054 2 | 9,10-Secoiso-hydroxyaconido lactone | - | - | + |
| 7 | 8.90 | C ₁₃ H ₁₈ O ₄ | 261.113 3 | [M-H] ⁻ | 0.1 | 201.092 8, 187.079 4 | Myrrhanolide B | - | + | + |
| 8 | 9.60 | C ₁₆ H ₂₀ O ₃ | 261.148 5 | [M+H] ⁺ | 0.0 | 175.112 1, 161.095 6, 91.054 3 | 2-Methoxy-furanogermacra-1(10),4-diene | - | + | - |
| 9 | 9.87 | C ₁₅ H ₂₀ O ₃ | 249.148 5 | [M+H] ⁺ | 0.1 | 135.648 5, 107.084 1 | Atractylenolide III | - | + | - |

Continued

| No. | t_R /min | Formula | m/z | Adduct | Error / $\times 10^{-6}$ | MS/MS | Compound | Phase | SL | BL |
|-----|---------------|---|-----------|--------------------|-----------------------------|--|---|-------|----|----|
| 10 | 10.14 | C ₁₄ H ₁₆ O ₃ | 233.117 4 | [M+H] ⁺ | 0.6 | 119.085 1, 105.069 3 | Commipholinone | - | - | + |
| 11 | 10.77 | C ₁₅ H ₁₆ O ₂ | 229.122 1 | [M+H] ⁺ | -0.8 | 159.080 5, 145.101 1 | Myrrhone | - | - | + |
| 12 | 11.00 | C ₁₉ H ₁₈ O ₄ | 311.127 5 | [M+H] ⁺ | -1.1 | 293.116 0, 283.131 1, 275.099 7, 250.095 3, 247.119 6 | Tanshinone II _B | - | + | + |
| 13 | 11.39 | C ₁₉ H ₁₆ O ₄ | 309.112 0 | [M+H] ⁺ | -0.4 | 265.122 3, 250.098 9, 235.111 6 | Tanshialdehyde | - | + | - |
| 14 | 11.44 | C ₁₈ H ₁₄ O ₃ | 279.101 7 | [M+H] ⁺ | 0.3 | 261.092 1, 233.095 9, 205.100 4 | Dihydrotanshinone I | - | + | + |
| 15 | 11.68 | C ₁₉ H ₂₀ O ₃ | 297.148 5 | [M+H] ⁺ | -0.2 | 251.142 6, 237.091 1, 227.105 4 | Cryptotanshinone | - | + | + |
| 16 | 11.87 | C ₁₂ H ₁₄ O ₂ | 191.106 7 | [M+H] ⁺ | 0.0 | 173.098 6, 163.112 1, 145.062 5, 117.069 1, 105.067 9, 91.054 1 | Z-Ligustilide | - | - | + |
| 17 | 12.43 | C ₁₆ H ₂₂ O ₃ | 263.163 6 | [M+H] ⁺ | -2.0 | 187.074 9, 171.117 6, 159.077 6, 145.100 6 | MCS134 | - | - | + |
| 18 | 14.19 | C ₃₀ H ₄₆ O ₃ | 455.351 5 | [M+H] ⁺ | -1.0 | 437.341 3, 271.204 3 | Lupeolic acid | - | + | - |
| 19 | 14.61 | C ₃₀ H ₄₆ O ₄ | 469.331 7 | [M-H] ⁻ | -1.4 | 407.222 2, 391.952 6 | 11-Carbonyl- β -boswellic acid | - | - | + |
| 20 | 15.85 | C ₁₅ H ₂₄ O | 221.189 9 | [M+H] ⁺ | -0.5 | 147.117 7, 107.085 8, 103.055 2 | Alismol | - | - | + |
| 21 | 16.32 | C ₃₂ H ₄₈ O ₅ | 513.357 2 | [M+H] ⁺ | -0.5 | 495.354 1, 453.340 2 | 3 α -Acetyl-9,11-deoxy- β -boswellic acid | - | + | + |
| 22 | 16.40 | C ₃₀ H ₄₈ O | 425.376 2 | [M+H] ⁺ | -3.7 | 203.178 6, 189.164 1, 147.116 3, 105.069 3, 81.069 3 | Lupenone | - | + | - |
| 23 | 17.60 | C ₁₄ H ₂₂ O ₂ | 223.169 0 | [M+H] ⁺ | -1.3 | 119.086 0, 91.053 7 | Canangaterpene VI | - | - | + |
| M1 | 2.29 | C ₁₈ H ₁₄ O ₃ | 279.101 0 | [M+H] ⁺ | -1.9 | 279.100 2, 205.087 4 | Reduction of tanshinone I | I | - | + |
| M2 | 2.38 | C ₉ H ₁₀ O ₈ S | 277.001 9 | [M-H] ⁻ | -1.6 | 197.045 8, 179.035 0, 135.045 2 | Sulfate danshensu | II | + | + |
| M3 | 2.92 | C ₉ H ₁₀ O ₄ | 181.050 3 | [M-H] ⁻ | -1.9 | 163.040 2, 135.045 3, 119.050 5, 107.050 3 | Dehydroxyl danshensu | I | - | + |
| M4 | 3.32 | C ₁₂ H ₁₆ O ₂ | 193.122 4 | [M+H] ⁺ | 0.3 | 165.127 3, 123.079 7 | Dedihydroxylation of senkyunolide I/H | I | + | + |
| M5 | 3.35 | C ₁₀ H ₁₂ O ₅ | 211.060 9 | [M-H] ⁻ | -1.3 | 193.050 7, 178.027 2, 149.024 4, 134.037 5 | Methyl danshensu | II | + | + |
| M6 | 3.90 | C ₁₅ H ₂₂ O | 219.174 0 | [M+H] ⁺ | -1.5 | 145.101 2, 105.069 5 | Dehydrogenation of alismol | I | + | + |
| M7 | 4.99 | C ₁₂ H ₁₄ O ₂ | 191.106 7 | [M+H] ⁺ | 0.0 | 173.095 1, 145.100 7, 135.080 6 | Dehydrogenation of senkyunolide A | I | + | + |
| M8 | 5.10 | C ₁₂ H ₁₄ O ₃ | 207.101 7 | [M+H] ⁺ | 0.7 | 189.092 0, 165.055 3, 161.094 7, 133.101 2 | Ketone formation of senkyunolide A | I | + | + |
| M9 | 5.17 | C ₁₅ H ₂₂ O ₄ | 267.159 0 | [M+H] ⁺ | -0.3 | 249.147 9, 231.137 6, 203.142 5, 185.132 1, 119.085 6, 105.069 9 | Internal hydrolysis of atractylenolide III | I | + | + |
| M10 | 5.53 | C ₁₅ H ₂₀ O ₄ | 265.143 6 | [M+H] ⁺ | 0.4 | 247.133 8, 229.123 6, 105.069 9 | Oxidation of atractylenolide III | I | + | + |
| M11 | 5.89 | C ₁₅ H ₁₈ O ₄ | 263.128 2 | [M+H] ⁺ | 1.5 | 245.117 1, 203.118 5, 175.111 1 | Ketone formation of atractylenolide III | I | + | + |
| M12 | 6.95 | C ₁₂ H ₁₄ O ₃ | 207.101 4 | [M+H] ⁺ | -0.6 | 189.091 8, 179.108 4, 161.096 9, 133.101 2, 121.100 6, 107.085 4 | Hydroxyl Z-ligustilide | I | + | + |
| M13 | 7.06 | C ₁₂ H ₁₈ O ₂ | 195.137 4 | [M+H] ⁺ | -2.7 | 177.127 5, 153.051 1, 149.132 5, 139.110 4 | Hydrogenation of senkyunolide A | I | + | - |
| M14 | 7.10 | C ₁₁ H ₁₂ O ₄ | 209.080 9 | [M+H] ⁺ | 0.4 | 165.067 5, 121.065 4, 109.063 5 | Methyl ferulic acid | II | + | - |
| M15 | 7.65 | C ₁₂ H ₁₆ O ₂ | 193.122 1 | [M+H] ⁺ | -0.8 | 165.125 7, 147.114 2, 119.085 1, 107.086 2, 93.068 9 | Hydrogenation of Z-ligustilide | I | + | + |
| M16 | 8.90 | C ₁₅ H ₁₈ O ₄ | 263.128 1 | [M+H] ⁺ | 1.0 | 245.123 3, 217.127 0 | Demethylation and methylene to ketone of MCS134 | I | + | + |
| M17 | 8.98 | C ₁₂ H ₁₆ O ₃ | 209.117 0 | [M+H] ⁺ | -1.3 | 153.089 4, 121.062 6, 107.085 4 | Dehydroxyl senkyunolide I/H | I | + | - |
| M18 | 9.08 | C ₁₁ H ₁₂ O ₃ | 193.085 9 | [M+H] ⁺ | 0.0 | 175.073 8, 151.109 4, 147.076 7 | Demethylation and methylene to ketone of senkyunolide A | I | + | + |
| M19 | 9.16 | C ₁₆ H ₂₂ O ₃ | 263.164 2 | [M+H] ⁺ | 0.0 | 189.096 0, 161.062 1 | Methyl atractylenolide III | II | - | + |
| M20 | 9.32 | C ₁₆ H ₂₀ O ₅ | 293.138 6 | [M+H] ⁺ | 0.7 | 275.127 8, 247.132 0, 187.111 5 | Demethylation to carboxylic acid of MCS134 | I | + | + |
| M21 | 9.54 | C ₁₉ H ₂₀ O ₄ | 313.143 6 | [M+H] ⁺ | 0.6 | 271.093 3, 269.152 4, 267.138 1, 254.094 1, 253.085 6, 249.109 4 | Hydroxyl cryptotanshinone | I | + | - |
| M22 | 9.82 | C ₁₉ H ₁₆ O ₄ | 309.112 4 | [M+H] ⁺ | 0.8 | 265.121 9, 223.075 4 | Hydroxylation and oxidation of tanshinone II _A | I | + | - |
| M23 | 9.99 | C ₁₂ H ₁₂ O ₂ | 189.090 9 | [M+H] ⁺ | -0.5 | 171.080 5, 161.092 0, 143.085 9, 115.053 4 | Dehydrogenation of Z-ligustilide | I | + | + |
| M24 | 10.10 | C ₁₂ H ₁₄ O ₂ | 191.106 6 | [M+H] ⁺ | -0.4 | 173.093 2, 163.086 2, 107.087 3 | Dehydrogenation of senkyunolide F | I | + | + |
| M25 | 10.11 | C ₁₈ H ₁₂ O ₃ | 293.080 9 | [M+H] ⁺ | 0.1 | 293.079 6, 249.091 4 | Hydroxyl tanshinone I | I | + | + |

Continued

| No. | t_R /min | Formula | m/z | Adduct | Error $\times 10^{-6}$ | MS/MS | Compound | Phase | SL | BL |
|-----|---------------|--|-----------|--------------------|---------------------------|--|--|-------|----|----|
| M26 | 10.43 | C ₁₅ H ₂₀ O ₂ | 233.153 3 | [M+H] ⁺ | -1.5 | 119.085 9, 91.053 7 | Dehydrogenation of atractylenolide III | I | - | + |
| M27 | 10.47 | C ₁₅ H ₂₄ O ₂ | 237.184 4 | [M+H] ⁺ | -2.1 | 163.112 2, 123.116 5, 119.086 2 | Oxidation of alismol | I | - | + |
| M28 | 10.79 | C ₁₅ H ₂₂ O ₃ | 251.164 0 | [M+H] ⁺ | -0.8 | 233.152 3, 205.157 6, 187.149 8, 159.119 6, 133.101 4, 105.069 7 | Hydrogenation of atractylenolide III | I | + | + |
| M29 | 10.99 | C ₁₉ H ₁₈ O ₄ | 311.127 2 | [M+H] ⁺ | -1.8 | 293.115 2, 267.138 8, 265.086 5, 247.115 5 | Hydroxylation and dehydrogenation of cryptotanshinone | I | + | - |
| M30 | 11.29 | C ₃₀ H ₄₆ O ₄ | 471.346 8 | [M+H] ⁺ | -0.2 | 453.334 6, 409.356 9 | Deacetylation of 3-acetyl-11-keto- β -boswellic acid | I | + | + |
| M31 | 11.39 | C ₁₉ H ₁₆ O ₄ | 309.112 4 | [M+H] ⁺ | 0.9 | 291.101 0, 281.117 0 | Dehydrogenation of tanshinone II _B | I | + | + |
| M32 | 11.66 | C ₁₉ H ₂₀ O ₃ | 297.148 4 | [M+H] ⁺ | -0.3 | 251.144 0, 237.090 8 | Hydrogenation of tanshinone II _A | I | + | + |
| M33 | 12.64 | C ₁₂ H ₁₆ O ₃ | 209.117 0 | [M+H] ⁺ | -1.0 | 191.106 8, 173.132 8, 165.067 8 | Oxidation of senkyunolide A | I | - | + |
| M34 | 12.68 | C ₁₃ H ₁₆ O ₃ | 221.117 1 | [M+H] ⁺ | -0.8 | 193.124 7, 137.059 6 | Methyl senkyunolide F | II | + | + |
| M35 | 12.83 | C ₃₀ H ₄₈ O ₄ | 471.345 9 | [M-H] ⁻ | -4.4 | 471.346 5, 393.314 7 | Hydrogenation of 11-carbonyl- β -boswellic acid | I | - | + |
| M36 | 14.01 | C ₃₀ H ₄₆ O ₅ | 485.325 4 | [M-H] ⁻ | -3.8 | 485.326 9, 423.326 8 | Hydroxyl 11-carbonyl- β -boswellic acid | I | + | + |
| M37 | 14.57 | C ₃₂ H ₄₈ O ₆ | 529.350 8 | [M+H] ⁺ | -2.9 | 511.342 2, 469.333 7, 423.326 3 | Hydroxyl 3-acetyl-11-keto- β -boswellic acid | I | - | + |
| M38 | 16.38 | C ₃₂ H ₄₈ O ₄ | 497.361 5 | [M+H] ⁺ | -2.1 | 479.364 3, 437.340 8 | Dehydroxyl 3-acetyl-11-keto- β -boswellic acid | I | - | + |

3.2 活络效灵丹入血代谢产物鉴定 在SL组大鼠体内检测出29个代谢产物, BL组大鼠体内检测出32个代谢产物, 多发生羟基化、脱氢化、氧化等I相代谢反应与甲基化、硫酸化等II相代谢反应, 具体代谢产物信息见表2。

在BL组大鼠体内检测到代谢产物M2、M3及M5, 在SL组大鼠体内检测到代谢产物M2及M5。化合物M2 ($t_R = 2.38$ min), 负离子模式下准分子离子峰为 m/z 277.001 9 [M-H]⁻, 比原型成分丹参素的准分子离子峰 m/z 197.044 9 增加 80, 预测分子式为 C₉H₁₀O₈S (误差为 -1.6×10^{-6}), 二级裂解产生 m/z 197.045 8 [M-H-SO₃]⁻、179.035 0 [M-H-SO₃-H₂O]⁻、135.045 2 [M-H-SO₃-H₂O-CO]⁻ 等碎片离子, 推测 M2 是丹参素的硫酸化产物^[17]。化合物 M3 ($t_R = 2.92$ min) 及 M5 ($t_R = 3.35$ min), 准分子离子峰分别为 m/z 181.050 3 与 m/z 211.060 9, 判断分子式分别为 C₉H₁₀O₄ (误差为 -1.9×10^{-6}) 与 C₁₀H₁₂O₅ (误差为 -1.3×10^{-6}), M3 裂解产生 m/z 163.040 2 [M-H-H₂O]⁻、135.045 3 [M-H-HCOOH]⁻、119.050 5 [M-H-HCOOH-CH₂]⁻、107.050 3 [M-H-HCOOH-CO]⁻ 等碎片离子, M5 的二级质谱特征碎片包括 m/z 193.050 7 [M-H-H₂O]⁻、178.027 2 [M-H-H₂O-CH₃]⁻、149.024 4 [M-H-H₂O-CO₂]⁻、134.037 5 [M-H-H₂O-CH₃-CO₂]⁻, 推测 M3 是丹参素的去羟基化产物, M5 是丹参素的甲基化产物^[18]。具体代谢途径见图4。

4 活络效灵丹入血成分治疗糖尿病周围神经病变的网络药理学分析

4.1 活络效灵丹入血成分靶点预测及疾病靶点筛选 Swiss Target Prediction 数据库预测的靶点经去重

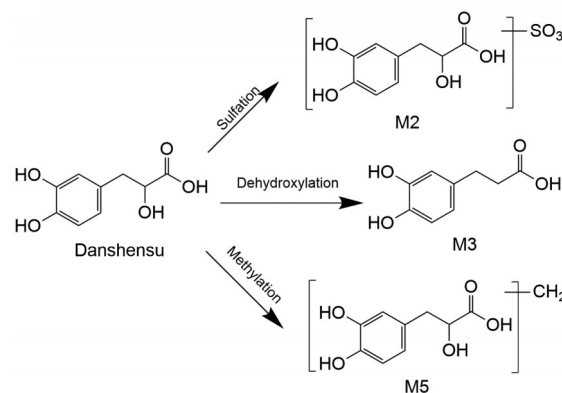


Figure 4 Metabolic pathway of danshensu

后得到入血成分对应靶点 447 个; GeneCards 等数据库获取的靶点筛选去重后得到疾病靶点 2 285 个。对成分靶点与疾病靶点取交集, 得到 160 个活络效灵丹的潜在作用靶点。

4.2 PPI 与“成分-靶点”图构建 将潜在靶点输入 STRING 数据库获得蛋白间相互作用关系, 导入 Cytoscape 3.10.1 软件分析并构建 PPI 互作网络图 (图 5A), 取度值、BC、CC 均值以上的靶点作为核心靶点, 得到 TNF、IL6、PPARG 等 27 个核心靶点; 将成分与交集靶点导入 Cytoscape 3.10.1 软件绘制“成分-靶点”图 (图 5B), 该网络图共有节点 177 个, 边 339 条, 其中度值排名为前 5 的活性成分为 3-乙酰基-11-酮基- β -乳香酸、11-酮基- β -乳香酸、丹参酮 II_B、洋川芎内酯 F、阿魏酸, 可能为活络效灵丹治疗 DPN 的核心成分。

4.3 GO 和 KEGG 通路富集分析 核心靶点在 DAVID

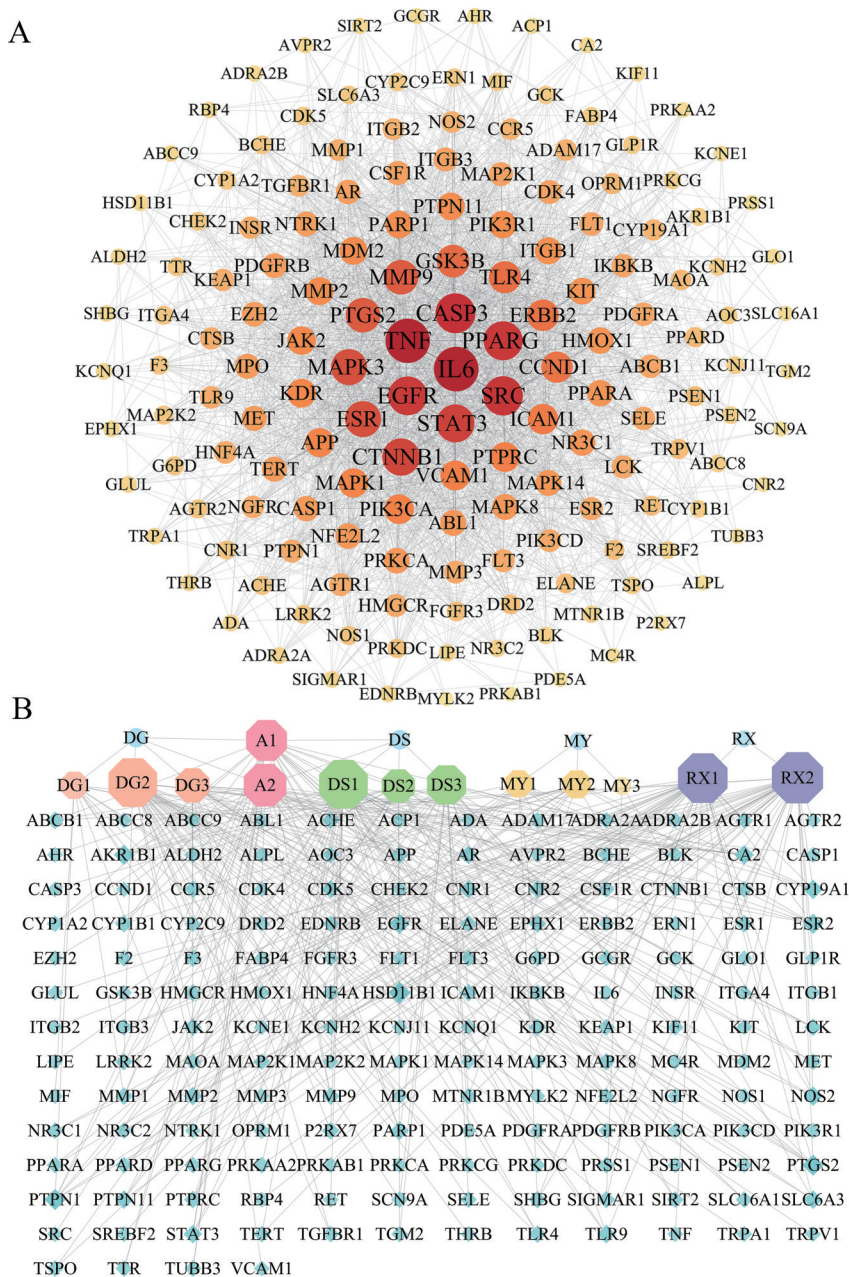


Figure 5 Protein-protein interaction network and compound-target network of Huoluoxiaolingdan against DPN. A: Protein-protein interaction network of related protein targets; B: Compound-target network of Huoluoxiaolingdan against DPN (octagon: Active components; rhomboid: Disease-related targets; DG: *Angelica sinensis*; DS: *Salvia miltiorrhiza*; RX: *Olibanum*; MY: *Myrrh*; A: Common ingredients of *Angelica sinensis* and *Salvia miltiorrhiza*. The area represents the degree)

数据库中进行 GO 与 KEGG 富集分析, 筛选得到活络效灵丹入血成分治疗 DPN 的 GO 分析结果中生物过程 (biological process, BP) 条目 219 个, 主要参与 RNA 聚合酶 II 启动子转录的正调控、磷酸化、ERK1 和 ERK2 级联的正向调控等; 细胞组分 (cellular component, CC) 条目 38 个, 主要包括细胞质膜、细胞质、细胞核等; 分子功能 (molecular function, MF) 条目 41 个, 主要涉及蛋白结合、相同蛋白结合、ATP 结合、酶结合等 (图

6A)。KEGG 富集分析得到 134 条通路, 结果显示, 活络效灵丹治疗 DPN 主要涉及 AGE-RAGE 信号通路、PI3K/Akt 信号通路、丝裂原活化蛋白激酶 MAPK 信号通路、白介素 IL-17 信号通路、肿瘤坏死因子 TNF 信号通路等 (图 6B)。

讨论

活络效灵丹为临床常用祛瘀止痛的方剂, 方中当

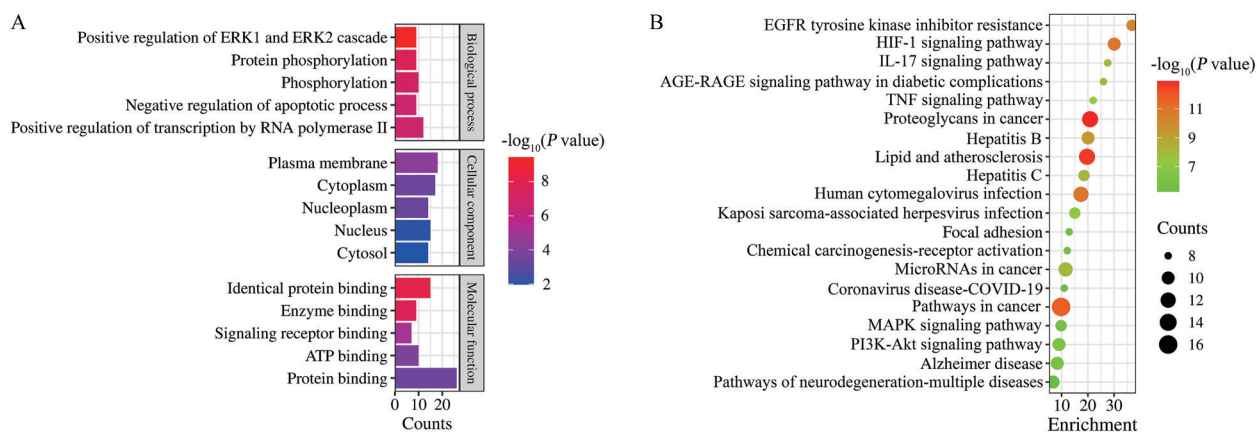


Figure 6 GO and KEGG pathway analysis at intersection targets. A: GO functional enrichment analysis; B: KEGG pathway enrichment analysis

归能补血止痛,为生血活血之主药,丹参能凉血消痈、祛瘀生新,乳香与没药相须为用,发挥活血生肌、止痛散瘀的作用^[5]。本研究采用UPLC-Q-TOF-MS/MS技术检测活络效灵丹提取物的体外成分,共鉴定出83个化学成分:24个来源于当归,23个来源于丹参,18个来源于乳香,22个来源于没药,其中,当归与丹参有4个相同的成分。

中药复方中的成分非常复杂,一般认为药物经口服后,真正吸收入血的才可能是中药发挥药效的成分。本实验在空白给药组与模型给药组大鼠体内检测到9种共有的入血原型成分,在SL组大鼠体内检测到15种原型成分与29种代谢产物,BL组大鼠体内检测到17种原型成分及32种代谢产物,与SL组相比,原型成分及代谢产物种类增多,可能由于正常动物与病理状态的动物体内微环境不同,且DPN病程较长,机体气血受损,持续高血糖环境下促进AGEs形成,炎症反应与氧化应激损伤加重,导致外周神经系统的微血管环境改变与代谢功能紊乱,血液循环减慢,进而使得机体在正常状态与病理状态下对药物吸收与代谢效率存在差异^[19,20]。

网络药理学预测得到排名前5的核心成分为3-乙酰基-11-酮基- β -乳香酸、11-酮基- β -乳香酸、丹参酮II_B、洋川芎内酯F及阿魏酸,其中阿魏酸能通过抑制神经炎症、促进坐骨神经修复以发挥镇痛作用^[21];11-酮基- β -乳香酸能显著降低STZ诱导的糖尿病大鼠血糖与血脂水平,改善胰腺受损情况^[22];丹参酮II_B可以通过抑制NF- κ B信号通路以发挥抗炎作用^[23];洋川芎内酯F可能是通过改善线粒体功能障碍与调节巨噬细胞极化以缓解动脉粥样硬化^[24];3-乙酰基-11-酮基- β -乳香酸能减轻1型糖尿病大鼠的高血糖,抑制STZ诱导的氧化应激、炎症反应及细胞凋亡^[25]。结果显示,活络效灵

丹可能是作用于TNF、IL6、PPARG、SRC、CTNBN1、CASP3等27个核心靶点,通过调控AGE-RAGE、PI3K/Akt等信号通路发挥治疗DPN的作用,其中TNF是一种促炎细胞因子,表达后导致细胞凋亡并激活炎症级联反应,引发炎症组织损伤与疼痛行为,并伴有痛觉过敏^[26,27];IL6是活化后的小胶质细胞释放的炎症因子,与小胶质细胞之间存在正向反馈,导致神经性疼痛时间增加,且可能发展为痛觉过敏或痛觉异常^[28]。PPARG是一种内源性抗炎因子,主要通过抑制NF- κ B信号通路以改善炎症,通常用于调节巨噬细胞浸润及降低炎症部位促炎分子产生以发挥抗炎镇痛作用^[29]。

综上所述,本研究鉴定了活络效灵丹的体外成分及正常大鼠和DPN大鼠的入血原型成分与代谢产物,分析了各类化合物的质谱裂解规律,通过网络药理学筛选活络效灵丹治疗DPN的核心成分与核心靶点,预测其可能的作用机制,推测其核心成分为活络效灵丹治疗DPN的潜在功效物质,为后续质量标准、功效物质基础及作用机制研究提供了科学依据。

作者贡献: 本文由宿树兰、段金彪负责课题总体设计及文章修改;张喆负责实验操作、数据处理及论文撰写;李玉琴、施亚宁、范若颖协助实验完成;尚尔鑫、朱悦负责实验技术指导。

利益冲突: 所有作者均声明不存在利益冲突。

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