基于网络药理学的复方酸枣仁汤的药效物质筛选及其含量测定

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摘要: 本文拟构建“化学成分-疾病靶点”网络筛选复方酸枣仁汤治疗失眠的药效物质, 并建立高效、快速的方法对其进行含量测定。采用液质联用技术鉴定了复方酸枣仁汤中的103个化合物, 针对其中27个潜在药效物质进行网络药理学分析, 选取成分度值最大的前6个成分: 新芒果苷、芒果苷、洋川芎内酯I、斯皮诺素、6'''-阿魏酰斯皮诺素、阿魏酸作为药效物质。建立UHPLC法对药效物质进行含量测定, 采用Waters CORTECS T3 (2.1 mm × 150 mm, 1.6 μm) 色谱柱, 流动相为0.1%磷酸水-乙腈, 分析方法验证结果表明所建立的方法专属性好, 各成分在考察范围内线性关系良好 (r > 0.999 0), 方法具有良好的灵敏度、精密度、准确度、耐用性和稳定性。结果表明该方法能够用于复方酸枣仁汤的药效物质的筛选和含量测定, 为基于药效的中药质量评价提供了依据。

关键词: 复方酸枣仁汤; 失眠; HPLC-Q-TOF MS/MS; 网络药理学; 含量测定

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Screening and determination of effective components in Compound Suanzaoren Decoction based on network pharmacology

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Abstract: This article intended to build a component-target network to screen the effective components of Compound Suanzaoren Decoction, and to establish an efficient and rapid method for the determination of effective components. 103 compounds in the Compound Suanzaoren Decoction were identified by HPLC-Q-TOF MS/MS. 27 potential effective substances were analyzed by network pharmacology. The first six components with the largest degree were selected as effective components, including neomangiferin, mangiferin, ferulic acid, senkyunolide I, spinosin, and 6'''-feruloylspinosin. A UHPLC method was established for determination. The chromatographic separation was performed on a Waters CORTECS T3 column (2.1 mm × 150 mm, 1.6 μm) with the mobile phase of 0.1% phosphoric acid solution and acetonitrile. The results showed that the specificity was good with no interference for the chromatographic peaks. The linear was excellent within the scope of investigation, with the values of r higher than 0.999 0 for all analytes. The method had been verified to have good sensitivity, precision, accuracy, durability and stability. Therefore, the results showed that the method established was suitable for the screening and determination of effective components in Compound Suanzaoren Decoction, which provided a basis for the quality evaluation of traditional Chinese medicine.

Key words: Compound Suanzaoren Decoction; insomnia; HPLC-Q-TOF MS/MS; network pharmacology; determination

失眠是影响身心健康的一大重要问题[1]。据统计, 全世界至少有6%的人遭受失眠和睡眠紊乱的困扰[2]。临床上常用镇静催眠类化学药治疗失眠, 但该类药物不良反应强, 依赖性高, 并存在戒断反应[3], 因此, 不良反应小、疗效好的中药越来越受到人们的关注。

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复方酸枣仁汤是治疗失眠的经典名方, 张仲景《金匮要略•血痹虚劳病脉证并治》[4]记载“虚劳虚烦不得眠, 酸枣仁汤主之”。该方剂临床疗效显著, 沿用至今。复方酸枣仁汤由酸枣仁、知母、茯苓、川芎、甘草五味药组成, 方中酸枣仁养血补肝, 为君药; 茯苓安神通阴, 知母滋阴润燥、清热除烦, 二者共为臣药; 川芎调肝血疏肝气, 为佐药; 甘草和中缓急, 调和诸药, 为使药[5], 各药味相辅相成, 能够起到养血补肝, 治疗失眠的功效。

复方酸枣仁汤成分繁多, 目前含量测定指标的选取未能很好地揭示药物与靶点、疾病之间的关联性, 不能体现中药复方“多靶点、多通路”的作用特点[6], 因此, 本研究采用HPLC-Q-TOF MS/MS法鉴定了复方酸枣仁汤的化学成分, 采用网络药理学方法建立了“化学成分-疾病靶点”相关性网络[7], 预测了复方酸枣仁汤的药效物质, 并根据传统水提取方法, 建立了基于药效物质的高效、快速的含量测定方法, 为复方酸枣仁汤质量评价体系的完善提供参考。

材料与方法

仪器　Agilent 1260 Infinity II (美国安捷伦公司)-Q-TOF 5600 (AB Sciex 公司)、Agilent 1290 Infinity II (美国安捷伦公司); PeakViewTM v 1.1 软件 (AB Sciex 公司); AB135-S 十万分之一天平 (Mettler TOLEDO 仪器有限公司); DZTW 电子调温加热套 (北京市永光明医疗仪器有限公司); TDZ24 WS 低速台式离心机 (湖南湘仪实验室仪器开发有限公司); EYELA N-1100 型旋转蒸发仪 (日本东京理化器械株式会社); SHZ-III型循环水真空泵 (上海亚荣生化仪器厂); KQ-300E 型超声波清洗器 (昆山市超声仪器有限公司)。

试剂　酸枣仁皂苷 A (批号 CHB191210); 酸枣仁皂苷 B (批号 CHB190125); 斯皮诺素 (批号 CHB171011); 6'''-阿魏酰斯皮诺素 (批号 CHB180324); 知母皂苷 BII (批号 CHB18115); 新芒果苷 (批号 CHB160910); 芒果苷 (批号 CHB160628); 阿魏酸 (批号 CHB180206); 洋川芎内酯 A (批号 CHB180615); 洋川芎内酯 I (批号 CHB170926); 甘草苷 (批号 CHB180608); 甘草酸 (CHB170606); 芹糖甘草苷 (批号 CHB180109)。所有的对照品均购自成都克洛玛生物有限公司, 含量质量分数均不低于 98.0%。纯净水 (杭州娃哈哈有限公司); 乙腈 (色谱纯, 美国 Fisher 有限公司); 甲酸 (色谱纯, 天津市博迪试剂有限公司); 甲醇 (色谱纯, 美国 Sigma-Aldrich 公司); 磷酸 (色谱纯, 天津市科密欧化学试剂有限公司)。

材料　酸枣仁、知母、茯苓、甘草饮片均由某企业提供, 川芎饮片购自沈阳市各大药房, 所有饮片均由沈阳药科大学中药学院王东教授鉴定, 分别为鼠李科植物酸枣 *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hu ex H. F. Chou 的干燥成熟种子、百合科植物知母 *Anemarrhena asphodeloides* Bge. 的干燥根茎、多孔菌科真菌茯苓 *Poria cocos* (Schw.) Wolf 的干燥菌核、豆科植物甘草 *Glycyrrhiza uralensis* Fisch. 的干燥根和根茎、伞形科植物川芎 *Ligusticum chuanxiong* Hort. 的干燥根茎。

化学成分鉴定

色谱条件　色谱柱: Aglient Zorbax Eclipse Plus C18 (150 mm × 4.6 mm, 3.5 μm); 流动相: 0.1% 甲酸水溶液 (A)-乙腈 (B); 流速为 1.0 mL·min^{-1}, 柱温 30 ℃, 梯度洗脱, 洗脱程序: 0～3 min, 7%～9% B; 3～8 min, 9%～15% B; 8～16 min, 15%～20% B; 16～28 min, 20%～55% B; 28～38 min, 55%～70% B; 38～48 min, 70%～90% B; 48～51 min, 90% B。进样量: 5 μL。

质谱条件　采用 AB Sciex Q TOF 系统, 离子源为电喷雾离子源 (ESI 源); 检测方式为正、负离子检测; 离子喷雾电压为-4 500 V 和 5 000 V; 离子源温度: 550 ℃; 雾化气 (N_2) 压力: 55 psi (1 psi ≈ 6.9 kPa); 辅助气 (N_2) 压力: 55 psi; 气帘气 (N_2) 压力: 35 psi; TOF MS 模式下扫描范围为 *m/z* 100～1 500 Da; 去簇电压 (DP): 60 V (正离子)/-60 V (负离子); 碰撞能 (CE): 10 V (正离子)/-10 V (负离子); TOF MS/MS 模式下扫描范围为 *m/z* 100～1 000 Da; 去簇电压 (DP): 60 V (正离子)/-60 V (负离子); 碰撞能 (CE): 35 V (正离子)/-35 V (负离子)。

对照品溶液的制备　分别取酸枣仁皂苷 A、酸枣仁皂苷 B、斯皮诺素、6'''-阿魏酰斯皮诺素、知母皂苷 BII、芒果苷、新芒果苷、阿魏酸、洋川芎内酯 I、洋川芎内酯 A、甘草苷、甘草酸、芹糖甘草苷 13 种对照品适量, 用甲醇溶解, 摇匀配制成浓度均为 25 μg·mL^{-1} 的混合对照品溶液。

供试品溶液的制备　将收集的酸枣仁、知母、茯苓、川芎、甘草多批饮片按复方酸枣仁汤处方比例, 随机组合成 10 批次样品。精密称取各味饮片于圆底烧瓶中, 加入 8 倍体积的水, 浸泡 80 min, 加热回流 30 min, 采用七号药筛滤过。向药渣中继续加入 6 倍体积的水, 加热回流 25 min, 滤过, 合并两次滤液, 减压浓缩至 0.83 g·mL^{-1} (生药材), 即得复方酸枣仁汤。精密量取复方酸枣仁汤 2.0 mL 于 10 mL 量瓶, 加入甲醇适量, 超声 20 min, 放冷, 定容, 摇匀, 4 000 r·min^{-1} 离心 5 min, 取上清, 过 0.22 μm 微孔滤膜, 取续滤液即得。

按照上述色谱条件和质谱条件, 进样分析, 通过与对照品保留时间、高分辨准分子离子及碎片离子等信息进行比对, 并参考文献, 进行结构鉴定。

网络药理学分析流程　通过查阅文献, 在已鉴定

出来的化合物中, 寻找复方酸枣仁汤组方饮片水提入血成分, 作为潜在的活性成分。通过PubChem[8]查询潜在活性成分的3D结构, 导入PharmMapper数据库[9]预测成分的靶点 (仅选择“人源基因”)。通过GeneCards数据库和OMIM数据库, 以“insomnia”和“sleepless”为关键词, 搜索与失眠有关的靶点。成分靶点与疾病靶点取交集得到共同靶点, 将这些共同靶点视为复方酸枣仁汤治疗失眠的关键靶点, 利用Cytoscape 3.7.2软件[10]构建“化学成分−疾病靶点”网络并进行通路富集分析, 筛选药效物质。

UHPLC法测定6种药效物质

UHPLC色谱条件　色谱柱: Waters Cortecs T3 (2.1 mm × 150 mm, 1.6 μm); 流动相: 0.1%磷酸水溶液 (A)−乙腈 (B); 流速为0.3 mL·min^{-1}, 柱温30 ℃, 梯度洗脱, 洗脱程序: 0～3 min, 7%～8% B; 3～8 min, 8%～8.5% B; 8～13 min, 8.5% B; 13～16 min, 8.5%～9% B; 16～20 min, 9% B; 20～23 min, 9%～10% B; 23～27 min, 10%～11% B; 27～40 min, 11%～15% B; 40～55 min, 15%～17% B; 55～60 min, 17%～19% B; 60～62 min, 19%～22% B; 62～66 min, 22%～90% B。进样量1 μL, 检测波长275 nm。

混合对照品溶液的制备　分别取新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素、6'''-阿魏酰斯皮诺素对照品适量, 精密称定, 用甲醇溶解、稀释定容, 配制成质量浓度分别为386.9、271.9、44.32、28.74、93.15和33.87 μg·mL^{-1}的混合对照品溶液。

供试品溶液的制备　按照“化学成分鉴定”项下“供试品溶液的制备”方法, 制备供试品溶液。

缺味对照溶液的制备　按照“化学成分鉴定”项下“供试品溶液的制备”方法, 分别制备缺少酸枣仁饮片、缺少知母饮片、缺少川芎饮片的缺味对照溶液。

系统适用性　取混合对照品溶液, 按照“UHPLC色谱条件”, 连续进样6次, 记录理论塔板数 (n)、相邻色谱峰的分离度 (R)、各色谱峰信噪比、拖尾因子、计算各待测成分峰面积的RSD。

分析方法验证

专属性　分别取混合对照品溶液、供试品溶液、各缺味对照溶液, 按照“UHPLC色谱条件”, 进样分析, 比较色谱图。

线性与范围　分别精密量取上述混合对照品溶液0.5、1.0、2.0、5.0和7.5 mL置于10 mL量瓶中, 用甲醇定容至刻度, 将这5种溶液及“混合对照品溶液的制备”项下的溶液作为标准系列溶液。按照“UHPLC色谱条件”进样分析, 记录峰面积。

检测限和定量限　取混合对照品溶液, 用甲醇进行逐步稀释, 以信噪比为3∶1和10∶1为参考, 配制溶液, 分别测得各化合物的检测限和定量限。

稳定性　取供试品溶液, 4 ℃放置, 分别于0、2、4、8、12和24 h进样分析, 计算各待测成分峰面积的RSD。

精密度　精密量取同一批复方酸枣仁汤1.0、2.0和3.0 mL, 按“化学成分鉴定”项下“供试品溶液的制备”方法, 制备低、中、高3个浓度的供试品溶液, 每一种浓度平行制备3份, 按照“UHPLC色谱条件”进样分析, 计算各成分含量, 并计算RSD, 作为重复性实验。精密量取同一批复方酸枣仁汤2.0 mL, 按“化学成分鉴定”项下“供试品溶液的制备”方法, 每天制备相同浓度的供试品溶液3份, 平行制备3天, 按照“UHPLC色谱条件”连续3天进样分析, 计算各成分含量, 并计算RSD, 作为中间精密度实验。

准确度　取已知各待测成分含量的复方酸枣仁汤9份, 精密量取1.0 mL, 分别按约为已知含量的50%、100%和150%等3个水平加入对照品, 制备低、中、高3种不同浓度的供试品溶液, 每一种浓度平行制备3份, 按照“UHPLC色谱条件”进样分析。计算各待测成分的平均回收率及RSD。

耐用性　分别考察色谱条件在不同进样体积 (0.9、1和1.1 μL), 不同检测波长 (274、275和276 nm), 不同体积分数的磷酸溶液流动相 (体积分数为0.09%磷酸溶液、体积分数为0.10%磷酸溶液和体积分数为0.11%磷酸溶液), 不同柱温 (29、30和31 ℃) 的条件下, 测定待测成分的含量, 并计算不同条件的RSD。

含量测定　分别取混合对照品溶液, 按供试品溶液制备方法制备10批不同批次饮片提取的复方酸枣仁汤供试品, 按“UHPLC色谱条件”进样分析, 记录各待测成分峰面积, 采用外标法计算各待测成分的含量。

结果

1　化学成分鉴定

在复方酸枣仁汤中初步鉴定了103个化合物, 通过与对照品保留时间、高分辨准分子离子及碎片离子等信息进行比对, 准确鉴定了13个化合物, 所有化合物的质量误差均小于5.0×10^{-6}。鉴定结果及成分信息如表1[11-18]所示。对103个化合物的来源进行归属[16-18], 发现有27个化合物来源于酸枣仁, 14个来自知母, 5个来自茯苓, 20个来自川芎, 33个来源于甘草, 化合物100、101、102、103为酸枣仁和甘草两种饮片的共有成分。

Table 1　Identification of 103 chemical compounds in Compound Suanzaoren Decoction by HPLC-Q-TOF MS/MS. [a]Identified with reference standard. SZR: Ziziphi Spinosae semen; ZM: Anemarrhenae Rhizoma; FL: Poria; CX: Chuanxiong Rhizoma; GC: Glycyrrhizae Radix et Rhizoma

No.	Identification	t_R/min	Formula	Ion adduction	Measured mass (*m*/*z*)	Error/ 10^{-6}	Fragment (*m*/*z*)	Source
1	3*S*-1-*N*-*β*-*D*-Glucopyranosyl-3-hydroxy-indole-3-acetic acid/3*R*-1-*N*-*β*-*D*-glucopyranosyl-3-hydroxy-indole-3-acetic acid[11]	4.54	$C_{16}H_{19}NO_9$	$[M+H]^+$	370.113 0	−0.7	212.067 4, 188.069 6, 146.059 2, 128.049 3	SZR
2	Protocatechuic acid[11]	5.20	$C_7H_6O_4$	$[M-H]^-$	153.019 5	0.8	107.015 9, 124.016 5	SZR
3	6-Glu-coclaurine[12]	7.13	$C_{23}H_{29}NO_8$	$[M+H]^+$	448.196 7	−1.0	286.142 8, 269.115 8, 175.074 6, 107.049 5	SZR
4	4-Hydroxybenzoic acid[12]	8.94	$C_7H_6O_3$	$[M-H]^-$	137.025 0	4.5	108.022 9, 136.016 7	SZR
5	Epicatechin[13]	9.80	$C_{15}H_{14}O_6$	$[M-H]^-$	289.071 6	−0.5	221.081 2, 203.071 1, 123.044 9, 109.030 1	SZR
6	Coclaurine[11,12]	10.61	$C_{17}H_{19}NO_3$	$[M+H]^+$	286.143 9	0.4	107.049 6, 175.075 3	SZR
7	Magnoflorine[12]	11.52	$C_{20}H_{23}NO_4$	$[M+H]^+$	342.169 7	−0.7	297.109 7, 282.086 2, 265.083 2, 222.088 7	SZR
8	Catechine[13]	12.00	$C_{15}H_{14}O_6$	$[M-H]^-$	289.071 6	−0.7	221.082 6, 203.070 7, 187.039 2, 109.030 2	SZR
9	Hovetrichoside C[11]	12.44	$C_{21}H_{22}O_{11}$	$[M-H]^-$	449.108 5	−0.9	259.060 0, 269.046 2, 125.023 9, 287.056 5	SZR
10	Kaempferol-3-*O*-rutinoside[11,14]	14.50	$C_{27}H_{30}O_{15}$	$[M+H]^+$	595.164 6	−1.9	433.111 9, 337.068 9, 313.069 2, 283.058 3	SZR
11	Puerarin[11]	14.70	$C_{21}H_{20}O_9$	$[M-H]^-$	415.103 7	−0.9	252.041 3, 25.053 1,	SZR
12	Spinosin[a][11,14,15]	15.33	$C_{28}H_{32}O_{15}$	$[M+H]^+$	609.180 1	−2.2	327.085 2, 351.085 9, 297.075 3, 447.127 9, 285.075 3, 411.107 5, 609.178 6	SZR
13	Camelliaside B[11]	15.45	$C_{32}H_{38}O_{19}$	$[M-H]^-$	725.194 9	1.9	284.033 4, 575.153 3	SZR
14	Caaverine[11]	15.78	$C_{17}H_{17}NO_2$	$[M+H]^+$	268.133 1	−0.3	251.105 4, 236.082 6, 219.079 4, 208.087 4, 191.084 5, 190.076 6, 189.068 8, 165.069 6	SZR
15	6‴-Vanilloylspinosin[11,13]	17.57	$C_{36}H_{38}O_{18}$	$[M+H]^+$	759.211 7	−1.8	639.168 3, 429.117 7, 351.085 2, 327.085 4, 297.075 4	SZR
16	6‴-*p*-Hydroxylbenzoyspinosin[11-13]	18.39	$C_{35}H_{36}O_{17}$	$[M+H]^+$	729.200 8	−2.4	609.165 7, 429.117 1, 351.086 0, 327.084 9, 297.074 0, 121.028 5	SZR
17	Vitexin-4"-*O*-glucoside[11-14]	18.43	$C_{27}H_{30}O_{15}$	$[M+H]^+$	595.164 2	−2.6	287.054 9, 595.163 7	SZR
18	6‴-*O*-(3*R*-1-*N*-*β*-*D*-Glucopyranosyl-2-oxo-3-hydroxy-indole-3-acetyl) spinosin[11]	19.06	$C_{44}H_{49}NO_{23}$	$[M-H]^-$	958.266 5	4.5	649.177 4	SZR
19	6‴-Sinapoylspinosin[11-13]	19.39	$C_{39}H_{42}O_{19}$	$[M+H]^+$	815.237 3	−2.5	635.170 3, 447.127 3, 429.118 6, 393.094 2, 351.088 6, 327.085 4, 297.075 5, 207.063 0, 175.038 6	SZR
20	6‴-Feruloylspinosin[a][11-14,16]	19.91	$C_{38}H_{40}O_{18}$	$[M+H]^+$	785.227 6	−1.5	327.084 6, 351.084 5, 177.053 7, 297.074 3, 429.116 5, 665.184 6, 785.223 7	SZR
21	6‴-*p*-Coumaroylspinosin[11-13]	19.97	$C_{37}H_{38}O_{17}$	$[M+H]^+$	755.217 4	−1.0	447.127 5, 429.116 5, 351.085 4, 327.085 7, 297.074 9, 147.044 3	SZR
22	*epi*-6‴-(*N*-*β*-*D*-Glucopyranosyl)-2‴‴, 3‴‴-dihydro-2‴‴-oxo-3‴‴-yl-acetate spinosin[11]	20.22	$C_{44}H_{49}NO_{22}$	$[M+H]^+$	944.279 9	−2.1	782.225 5, 764.216 2, 489.137 7, 393.095 6, 351.085 5, 327.085 2	SZR
23	*N*-Nornuciferine[11]	21.10	$C_{18}H_{19}NO_2$	$[M+H]^+$	282.148 6	−0.9	265.120 6, 250.097 2, 235.073 9, 234.101 9, 191.084 4, 189.068 5, 178.076 4	SZR
24	6"-*O*-Feruloylspinosin[11-14,16]	21.19	$C_{38}H_{40}O_{18}$	$[M+H]^+$	785.226 6	−2.8	489.141 2, 447.127 1, 327.085 3, 285.074 3	SZR
25	Amphibine D/Lotusine B[12]	22.46	$C_{36}H_{49}N_5O_5$	$[M+H]^+$	632.379 0	−2.6	148.112 1, 289.190 4, 261.196 0, 316.199 6, 344.196 0, 632.377 1	SZR
26	Jujuboside A[a][11-14,16]	23.30	$C_{58}H_{94}O_{26}$	$[M-H]^-$	1 205.600 2	3.4	911.527 3	SZR
27	Jujuboside B[a][11-14,16]	24.15	$C_{52}H_{84}O_{21}$	$[M-H]^-$	1 043.549 3	5.0	911.505 5	SZR

Continued

No.	Identification	t_R/min	Formula	Ion adduction	Measured mass (*m*/*z*)	Error/ 10^{-6}	Fragment (*m*/*z*)	Source
28	Neomangiferin[a] [11,14,15]	8.35	$C_{25}H_{28}O_{16}$	$[M-H]^-$	583.130 6	0.2	301.034 4, 331.044 6, 463.086 2, 493.096 8	ZM
29	Mangiferin[a] [11,14-16]	11.22	$C_{19}H_{18}O_{11}$	$[M-H]^-$	421.077 3	−0.8	301.034 4, 331.0450, 259.025 2, 271.024 4, 421.075 6	ZM
30	Timosaponin D[11,14,16]	19.55	$C_{45}H_{74}O_{19}$	$[M+H]^+$	919.488 0	−1.9	289.214 9, 271.204 8, 433.329 1, 757.433 5	ZM
31	Timosaponin N[11,17]	19.63	$C_{45}H_{76}O_{20}$	$[M+HCOO]^-$	981.494 1	4.0	773.442 0	ZM
32	Timosaponin E1[11,17]	20.29	$C_{45}H_{76}O_{20}$	$[M+HCOO]^-$	981.495 0	4.9	773.436 5, 360.571 1	ZM
33	Timosaponin BII[a] [11,16,17]	20.70	$C_{45}H_{76}O_{19}$	$[M-H]^-$	919.490 8	3.0	919.493 6	ZM
34	Gurilioside H[11]	20.76	$C_{51}H_{86}O_{23}$	$[M+HCOO]^-$	1 111.556 2	2.8	919.490 3, 757.446 4	ZM
35	Timosaponin BIII[11,14,16]	20.88	$C_{45}H_{74}O_{18}$	$[M+H]^+$	903.492 1	−3.0	273.218 5, 255.208 3, 417.332 3, 399.322 2, 579.384 3, 741.433 6	ZM
36	Timosaponin AI[14]	20.89	$C_{33}H_{54}O_8$	$[M+H]^+$	579.387 6	−2.8	273.220 6, 255.210 6, 417.336 3, 301.142 8	ZM
37	(25*S*)-26-*O*-*β*-*D*-Glucopyranosyl-22-hydroxy-5*β*-furostanol alkenyl-3*β*,26-diol3-*O*-*β*-*D*-glucopyranosyl-(1-2)-*O*-*β*-*D*-galactopyranoside[11,14,16]	21.52	$C_{45}H_{74}O_{19}$	$[M-H]^-$	917.478 3	3.4	755.422 7, 917.477 7	ZM
38	Timosaponin BIV[11,17]	22.65	$C_{51}H_{84}O_{23}$	$[M+HCOO]^-$	1 109.541 9	4.1	901.492 6, 739.438 6	ZM
39	2,6,4'-Trihydroxy-4-methoxybenzophenone[11]	22.82	$C_{14}H_{12}O_5$	$[M-H]^-$	259.061 1	−0.6	165.018 6, 149.995 3	ZM
40	Timosaponin AIII[11,14,17]	23.74	$C_{39}H_{64}O_{13}$	$[M+H]^+$	741.439 9	−2.8	381.314 0, 399.325 4	ZM
41	Anemarrhenasaponin II[11, 7]	24.01	$C_{39}H_{66}O_{14}$	$[M+HCOO]^-$	803.445 1	3.5	757.439 2, 595.385 5, 757.439 2	ZM
42	3*β*,16*α*-Dihydroxy-lanosta-7,9(11),24-trien-21-oic acid[11,14,16]	24.17	$C_{30}H_{46}O_4$	$[M+H]^+$	471.346 2	−1.4	317.208 5, 453.347 6, 471.269 2	FL
43	Poricoic acid A[11,14]	28.55	$C_{31}H_{46}O_5$	$[M-H]^-$	497.327 4	0.3	479.313 3, 497.326 6	FL
44	Poricoic acid B[11,14]	32.75	$C_{30}H_{44}O_5$	$[M-H]^-$	483.311 2	−0.8	409.272 3, 483.310 2	FL
45	Polyporenic acid C[11,14]	38.58	$C_{31}H_{46}O_4$	$[M-H]^-$	481.332 3	0.0	421.305 1, 481.330 3	FL
46	Pachymic acid[11,14]	47.55	$C_{33}H_{52}O_5$	$[M+H]^+$	529.386 3	−4.6	451.353 8, 295.243 2	FL
47	Adenine[11]	1.69	$C_5H_5N_5$	$[M+H]^+$	136.061 7	−0.7	119.036 4, 136.063 0	CX
48	Vanillic acid[11]	4.69	$C_8H_8O_4$	$[M+H]^+$	169.049 5	−1.7	151.033 8, 105.034 7	CX
49	Neochlorogenic acid/Chlorogenic acid[11]	9.43	$C_{16}H_{18}O_9$	$[M-H]^-$	353.087 8	0.1	191.056 2, 179.035 3	CX
50	Caffeic acid[11]	11.31	$C_9H_8O_4$	$[M-H]^-$	179.034 8	−0.8	135.044 8, 179.034 7	CX
51	Ferulic acid[a][11,14,15]	16.65	$C_{10}H_{10}O_4$	$[M-H]^-$	193.050 7	0.1	134.036 7, 178.026 5, 149.058 0, 193.050 2	CX
52	Senkyunolide J[11,14]	17.86	$C_{12}H_{18}O_4$	$[M+H]^+$	227.127 7	−0.2	153.053 8, 163.110 6, 191.106 2, 209.116 4	CX
53	Dicaffeoylquinic acid[14]	19.80	$C_{25}H_{24}O_{12}$	$[M-H]^-$	515.119 3	−0.4	191.055 6, 353.087 5, 179.035 1, 515.119 0	CX
54	Senkyunolide D[11,14]	20.11	$C_{12}H_{14}O_4$	$[M+H]^+$	223.096 3	−1.0	205.085 3, 223.089 1, 105.034 7	CX
55	Naringenin[11]	20.27	$C_{15}H_{12}O_5$	$[M+H]^+$	273.075 5	−0.8	153.018 9, 147.045 8, 119.047 8	CX
56	Senkyunolide I[a][11,14]	20.83	$C_{12}H_{16}O_4$	$[M+H]^+$	225.111 8	0.5	225.111 8, 207.102 5	CX
57	Senkyunolide F[11,14]	20.84	$C_{12}H_{14}O_3$	$[M+H]^+$	207.101 4	−1.0	207.101 9, 189.091 3, 119.085 7, 105.071 1, 133.064 8, 128.061 8, 161.096 9, 128.061 8, 179.107 0	CX
58	3-Butyl-4-hydroxyphthalide[11,14]	21.45	$C_{12}H_{14}O_3$	$[M+H]^+$	207.101 4	−1.0	207.101 9, 189.091 3, 161.096 9, 128.061 8, 179.107 0	CX
59	Daidzein[11]	21.81	$C_{15}H_{10}O_4$	$[M+H]^+$	255.065 0	−0.9	145.029 1, 137.023 0, 119.051 3	CX
60	*E*-Butylidenephthalide[11,14]	23.78	$C_{12}H_{12}O_2$	$[M+H]^+$	189.091 0	−0.1	115.055 2, 171.080 4, 189.091 6	CX
61	Butylidenephthalide[11,14]	25.77	$C_{12}H_{12}O_2$	$[M+H]^+$	189.091 0	−0.5	171.079 5, 189.089 0	CX
62	Senkyunolide A[a] [11,14]	28.70	$C_{12}H_{16}O_2$	$[M+H]^+$	193.122 1	−0.4	137.059 1, 119.085 5, 147.115 9, 175.111 2, 193.121 7, 109.064 5	CX
63	Cnidilide[14,18]	30.73	$C_{12}H_{18}O_2$	$[M+H]^+$	195.137 9	−0.1	107.085 9, 125.059 0, 149.132 8, 177.126 4	CX
64	*Z*/*E*-Ligustilide or 3-butylphthalide[11,14]	31.00	$C_{12}H_{14}O_2$	$[M+H]^+$	191.106 5	−0.7	105.070 5, 115.054 5, 117.070 1, 145.101 1, 173.095 4, 149.060 0	CX
65	Ligustilide dimer[11]	35.90	$C_{24}H_{30}O_4$	$[M+H]^+$	383.221 3	−1.0	191.106 7, 135.042 9	CX

Continued

No.	Identification	t_R/min	Formula	Ion adduction	Measured mass (*m*/*z*)	Error/ 10^{-6}	Fragment (*m*/*z*)	Source
66	Riligustilide/*Z*,*Z*′-6,8',7,3'-Diligustilide[14]	39.83	$C_{24}H_{28}O_4$	$[M+H]^+$	381.205 7	−0.9	191.107 2, 135.043 1	CX
67	Glycyrrhizin-7,4'-diglucoside[11]	10.17	$C_{27}H_{32}O_{14}$	$[M-H]^-$	579.172 2	0.4	135.012 8, 255.067 0, 417.118 1	GC
68	Shaftoside/isoshaftoside[14]	13.40	$C_{26}H_{28}O_{14}$	$[M-H]^-$	563.141 2	0.9	353.063 7, 383.074 8, 443.095 8, 473.113 9, 503.119 1	GC
69	Glucoliquiritin[11]	13.94	$C_{27}H_{32}O_{14}$	$[M-H]^-$	579.172 2	0.4	255.066 5, 579.174 4	GC
70	Isoliquiritin apioside[11,14,16]	15.66	$C_{26}H_{30}O_{13}$	$[M-H]^-$	549.161 9	0.9	255.066 9, 135.009 3	GC
71	Liquiritin apioside[a][11,14,16]	16.22	$C_{26}H_{30}O_{13}$	$[M-H]^-$	549.161 3	−0.1	255.066 3, 135.008 0	GC
72	Liquiritin[a][11,14,16,18]	16.53	$C_{21}H_{22}O_9$	$[M-H]^-$	417.119 5	0.9	119.050 2, 135.008 3, 255.065 3, 417.117 6	GC
73	5-Hydroxyliquiritin[11,14]	20.34	$C_{21}H_{22}O_{10}$	$[M-H]^-$	433.113 6	−0.9	271.060 9, 177.018 7, 151.004 3, 119.051 5, 433.114 1	GC
74	Isoliquiritin[11,14-16]	21.42	$C_{21}H_{22}O_9$	$[M-H]^-$	417.118 9	−0.4	119.051 0, 135.008 7, 255.066 1, 417.117 9, 148.017 4	GC
75	Licorice glycoside A[11]	21.61	$C_{36}H_{38}O_{16}$	$[M-H]^-$	725.210 8	2.9	255.065 0, 531.143 8, 549.161 0, 726.214 0, 175.040 3	GC
76	Licorice glycoside B[11]	21.62	$C_{35}H_{36}O_{15}$	$[M-H]^-$	695.199 6	2.0	549.195 9, 531.152 6, 255.065 3, 135.008 6	GC
77	Licorice glycoside E[14]	22.34	$C_{35}H_{35}NO_{14}$	$[M-H]^-$	692.199 6	1.5	255.066 6, 531.153 5, 549.160 7	GC
78	5,7-Dihydroxyflavanone/Pinocembrin/Liquiritigenin[11,14,16]	22.79	$C_{15}H_{12}O_4$	$[M-H]^-$	255.066 1	−0.6	119.050 2, 135.008 0, 255.063 4	GC
79	24-Hydroxy-licorice-saponin E2[11]	23.34	$C_{42}H_{60}O_{17}$	$[M-H]^-$	835.378 8	3.6	351.055 7, 193.036 0	GC
80	Licorice saponine G2[14]	23.77	$C_{42}H_{62}O_{17}$	$[M-H]^-$	837.394 9	4.2	351.056 6, 837.394 5	GC
81	Uralsaponin B[11,14]	24.16	$C_{42}H_{62}O_{16}$	$[M+H]^+$	823.408 8	−2.7	453.334 0, 647.376 6	GC
82	Echinatin[14,18]	24.24	$C_{16}H_{14}O_4$	$[M-H]^-$	269.081 3	−2.2	133.031 1, 161.020 0	GC
83	Glycyrrhizic acid[a][11,14]	24.38	$C_{42}H_{62}O_{16}$	$[M-H]^-$	821.399 6	3.8	351.055 4, 821.399 3	GC
84	Licochalcone B[11]	25.21	$C_{16}H_{14}O_5$	$[M-H]^-$	285.076 4	1.6	285.078 8, 150.037 8	GC
85	Isoliquiritigenin[11,14,16]	25.48	$C_{15}H_{12}O_4$	$[M-H]^-$	255.066 3	−0.6	255.066 3, 135.008 6, 119.050 7	GC
86	Formononetin[14,18]	25.76	$C_{16}H_{12}O_4$	$[M-H]^-$	267.066 0	−1.0	252.042 5, 223.039 6, 267.065 7, 195.045 0	GC
87	(−)-Vestitol[16]	25.83	$C_{16}H_{16}O_4$	$[M-H]^-$	271.097 3	−1.0	235.044 8, 109.031 1, 271.095 0, 149.062 1, 238.069 9, 121.032 4, 241.061 8, 255.068 8	GC
88	Glycycoumarin[11]	27.37	$C_{21}H_{20}O_6$	$[M-H]^-$	367.118 6	−0.2	309.040 4, 367.118 5, 297.039 9, 284.032 2, 265.051 2, 139.039 7, 201.018 9	GC
89	Genkwanin[11]	27.86	$C_{16}H_{12}O_5$	$[M-H]^-$	283.061 0	−0.8	268.037 0, 239.035 6, 211.036 7	GC
90	Kumatakenin[14]	28.27	$C_{17}H_{14}O_6$	$[M-H]^-$	313.071 8	0.0	283.024 0, 255.029 6, 298.046 6, 313.239 3, 183.045 5, 295.226 9	GC
91	Glycyrin/Licoricone[11,14]	28.66	$C_{22}H_{22}O_6$	$[M-H]^-$	381.134 2	−0.6	351.087 5, 201.019 5, 323.092 6	GC
92	Lupiwighteone[11,18]	29.16	$C_{20}H_{18}O_5$	$[M-H]^-$	337.107 9	−0.7	282.052 7, 281.045 5	GC
93	Neo/Glycyrol/Isoglycyrol[11]	29.24	$C_{21}H_{18}O_6$	$[M-H]^-$	365.102 8	−0.8	307.024 2, 295.024 2, 365.102 0, 282.016 3, 335.056 2	GC
94	Gancaonin A[14]	29.63	$C_{21}H_{20}O_5$	$[M-H]^-$	351.123 4	−1.1	281.045 1, 308.104 3, 336.100 8, 293.082 1, 253.049 3, 307.098 7	GC
95	Glabrol[14]	31.91	$C_{25}H_{28}O_4$	$[M-H]^-$	391.191 2	−0.8	187.114 6, 203.070 8, 391.191 2, 132.058 5	GC
96	Glyinflanin A[14]	34.54	$C_{25}H_{28}O_5$	$[M-H]^-$	407.186 1	−0.8	187.112 3, 133.063 7, 175.075 2	GC
97	Glabric acid[14]	38.10	$C_{30}H_{46}O_5$	$[M+H]^+$	487.346 9	−2.3	487.330 6, 423.324 7, 451.321 9	GC
98	Licorisoflavan A[14]	41.72	$C_{27}H_{34}O_5$	$[M+H]^+$	439.247 7	−0.4	191.104 5, 371.182 2	GC
99	Gancaonin H[11,12,14]	44.90	$C_{25}H_{24}O_6$	$[M-H]^-$	419.150 1	0.2	219.066 0, 419.148 5	GC
100	Vicenin II[11,12,14]	11.35	$C_{27}H_{30}O_{15}$	$[M+H]^+$	595.164 2	−2.6	559.143 9, 511.124 9, 457.112 6, 337.068 9, 325.069 4, 295.060 1	SZR/GC
101	Saponarin[11,12,14]	14.77	$C_{27}H_{30}O_{15}$	$[M-H]^-$	593.151 5	0.5	293.044 7, 413.087 4, 341.064 6	SZR/GC
102	Vitexin[11,14]	15.93	$C_{21}H_{20}O_{10}$	$[M+H]^+$	433.112 6	−0.8	415.0985, 397.082 6, 379.082 6, 337.070 1, 313.068 9, 295.061 1, 283.059 8	SZR/GC
103	Isovitexin[11,14]	16.23	$C_{21}H_{20}O_{10}$	$[M-H]^-$	431.098 1	−0.6	311.055 2, 341.067 9, 323.058 5	SZR/GC

2 网络药理学分析

依据实验室的前期研究成果[13]以及文献报道, 在已鉴定的化合物中查找到入血成分 27 个, 如表 2[19-26]所示, 每种成分选取排名靠前的 35 个靶点。以“insomnia”和“sleepless”为关键词, 得到与失眠有关的靶点 5 052 个。成分靶点与疾病靶点取交集得到 156 个关键靶点, 将 27 个入血成分及关键靶点导入 Cytoscape 3.7.2, 构建“化学成分−疾病靶点”的可视化网络, 该网络有 183 个节点, 460 条边, 如图 1 所示。图中绿色菱形代表关键靶点, 粉色圆形代表入血成分。针对关键靶点进行通路富集分析, 筛选 $P < 0.01$ 的通路, 最终富集到 49 条信号通路, 如图 2 所示。选取度值高于 24 的 6 个成分作为复方酸枣仁汤的药效物质对其进行质量控制, 它们依次为新芒果苷 (28)、芒果苷 (27)、洋川芎内酯 I (27)、斯皮诺素 (26)、6'''-阿魏酰斯皮诺素 (25)、阿魏酸 (25)。

Table 2 Degree and source of 27 compounds found in blood

Compound	Degree	Source	Compound	Degree	Source
Jujuboside A[19,20]	21	SZR	Magnoflorine[20]	13	SZR
Jujuboside B[20]	14	SZR	Vicenin II[25]	14	SZR/GC
Spinosin[20]	26	SZR	Isovitexin[25]	15	SZR/GC
6'''-Feruloylspinosin[20]	25	SZR	Timosaponin BII[19, 20]	21	ZM
Coclaurine[20]	14	SZR	Timosaponin BIII[26]	15	ZM
Mangiferin[19]	27	ZM	Isoliquiritin[25]	3	GC
Neomangiferin[21]	28	ZM	Liquiritin apioside[25]	9	GC
Timosaponin AIII[19]	16	ZM	Isoliquiritin apioside[25]	15	GC
Senkyunolide I[22]	14	CX	Licorice glycoside B[25]	2	GC
Senkyunolide A[23]	13	CX	Uralsaponin B[25]	2	GC
Ferulic acid[24]	25	CX	Glycyrin[25]	16	GC
Senkyunolide F[21]	12	CX	Neoglycyrol[25]	11	GC
Glycyrrhizic acid[24]	23	GC	Licorice saponin G2[25]	15	GC
Liquiritin[19]	24	GC			

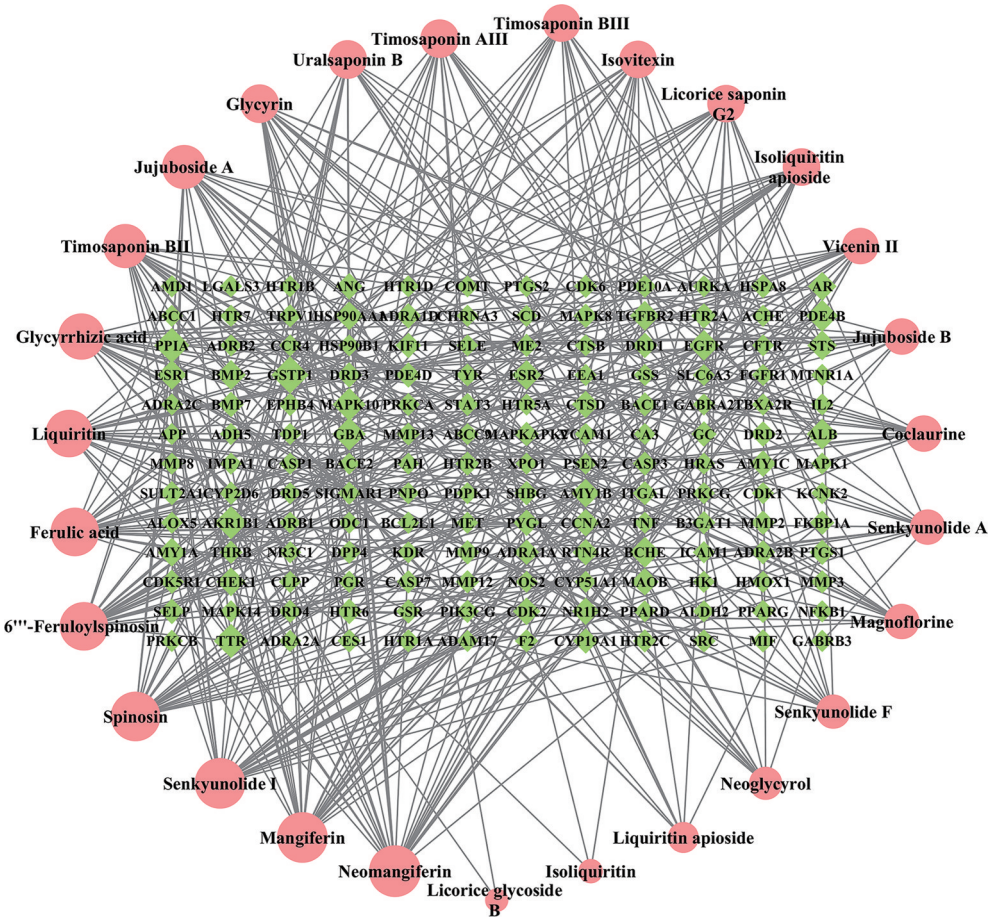

Figure 1 The "component-target" network of Compound Suanzaoren Decoction

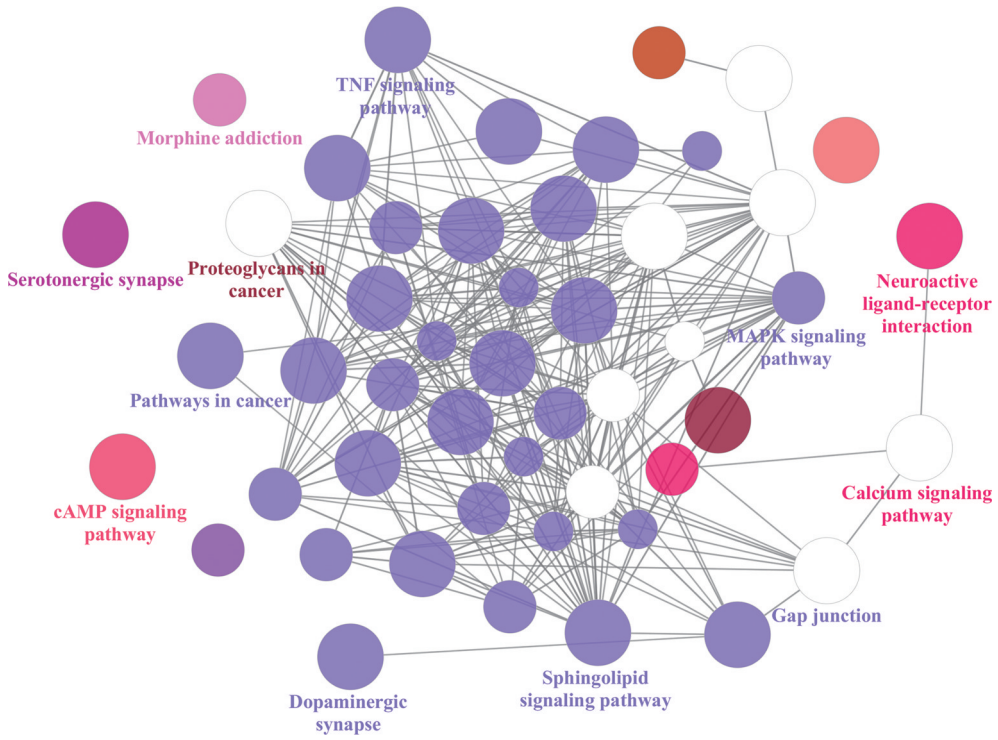

Figure 2　Pathways based on KEGG pathways analysis

3　系统适用性

结果显示按新芒果苷峰计, 理论塔板数 (n) 均大于 4.2×10^4, 相邻色谱峰的分离度 (R) 均大于 1.5, 各色谱峰信噪比 (S/N) 均大于 10, 拖尾因子均介于 0.95～1.05 之间。新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素、6'''-阿魏酰斯皮诺素峰面积的 RSD 分别为 0.8%、0.8%、0.8%、0.6%、0.9%、1.0%, 表明仪器的精密度良好。

4　分析方法验证

4.1　专属性　通过对比混合对照品溶液、供试品溶液、缺味对照溶液, 结果显示, 新芒果苷、芒果苷来自知母, 阿魏酸、洋川芎内酯I来自川芎, 斯皮诺素、6'''-阿魏酰斯皮诺素来自酸枣仁, 该方法专属性良好, 阴性溶液无干扰。典型色谱图见图3。

4.2　线性与范围　以各对照品质量浓度 (μg·mL^{-1}) 为横坐标 (x), 以峰面积 A 为纵坐标 (y), 绘制标准曲线, 计算回归方程。新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素、6'''-阿魏酰斯皮诺素在考察范围内线性关系良好, 相关系数均大于 0.999 0, 结果见表3。

4.3　检测限与定量限　结果显示, 新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素和6'''-阿魏酰斯皮诺素的检测限质量浓度分别为 0.201 5、0.504 5、0.372 2、0.191 9、0.284 7 和 0.188 9 μg·mL^{-1}, 各成分定量限质量浓度分别为 0.604 6、0.756 8、0.744 5、0.383 8、0.569 4 和 0.377 9 μg·mL^{-1}, 表明方法灵敏度良好。

4.4　稳定性　新芒果苷、芒果苷、阿魏酸、洋川芎内酯

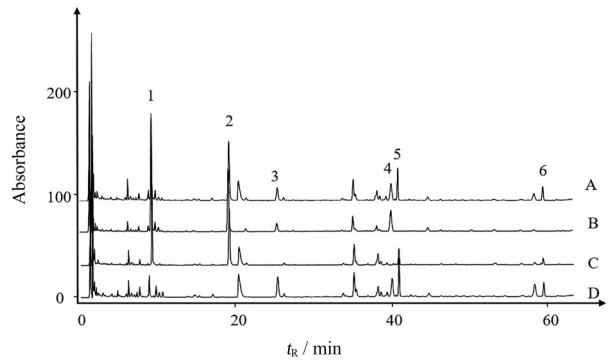

Figure 3　UHPLC chromatogram of test solution of Compound Suanzaoren Decoction (A); UHPLC chromatogram of negative solution: negative control sample of Compound Suanzaoren Decoction without Ziziphi Spinosae semen (B); negative control sample of Compound Suanzaoren Decoction without Chuanxiong Rhizoma (C); negative control sample of Compound Suanzaoren Decoction without Anemarrhenae Rhizoma (D). 1: Neomangiferin; 2: Mangiferin; 3: Ferulic acid; 4: Senkyunolide I; 5: Spinosin; 6: 6'''-Feruloylspinosin

Table 3　Results of linearity test

Analyte	Linearity		
	Range/μg·mL^{-1}	Equation	r
Neomangiferin	19.35–386.9	$y = 3.077\ 9x - 2.852\ 2$	0.999 8
Mangiferin	13.59–271.9	$y = 4.556\ 6x - 7.191\ 8$	0.999 8
Ferulic acid	2.216–44.32	$y = 8.265x - 2.566\ 7$	0.999 8
Senkyunolide I	1.437–28.74	$y = 18.867x - 2.224\ 3$	0.999 8
Spinosin	4.657–93.15	$y = 5.985x - 0.093\ 2$	0.999 9
6'''-Feruloylspinosin	1.694–33.87	$y = 7.618\ 4x - 0.753\ 1$	0.999 8

I、斯皮诺素和6'''-阿魏酰斯皮诺素峰面积的RSD分别为1.6%、1.4%、2.0%、1.6%、1.4%和1.6%, 各成分在24 h内稳定 (RSD < 2.0%)。

4.5 精密度 重复性实验中新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素和6'''-阿魏酰斯皮诺素含量的RSD分别为2.1%、1.2%、1.1%、0.8%、1.1%、1.3%, 表明方法重复性良好 (RSD < 3.0%)。中间精密度实验中各成分含量的RSD分别为0.6%、0.8%、1.0%、0.5%、0.7%和1.2%, 表明方法中间精密度良好 (RSD < 3.0%)。

4.6 准确度 测得新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素和6'''-阿魏酰斯皮诺素的平均回收率在104.2%～108.9%, RSD均不高于2.1%, 表明方法准确度良好。

4.7 耐用性 各色谱条件下, 新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素和6'''-阿魏酰斯皮诺素含量的RSD均不超过2.5%, 表明方法耐用性良好。

5 含量测定结果

测得10批复方酸枣仁汤中新芒果苷、芒果苷、阿魏酸、洋川芎内酯I、斯皮诺素和6'''-阿魏酰斯皮诺素的含量, 结果如表4所示。

讨论

实验研究表明, 失眠与体内神经递质如5-HT (5-羟色胺)、NO (一氧化碳)、GABA (γ-氨基丁酸) 等密切相关[27]。5-HT是引发睡眠的重要神经递质, 中缝核内的5-HT能神经元分泌的5-HT与快眼动睡眠时间成正比[28]。研究发现, 长期使用地西泮治疗失眠, 能够使神经活性配体-受体相互作用信号通路显著变化, 证明该通路和失眠存在很强相关性[29]。本研究网络药理学中通路富集分析结果最显著的是5-羟色胺能突触通路, 其次是神经活性配体-受体相互作用通路, 这与文献报道结果吻合。除此之外, 富集得到的通路还包含TNF信号通路、多巴胺能信号通路、cAMP信号通路、钙信号通路等。因而推断复方酸枣仁汤治疗失眠可能是多通路协同作用。

网络药理学中"成分−靶点−通路"相互作用不但可以预测中药复方制剂治疗疾病的机制, 还能够辅助中药复方制剂中有效成分的筛选[30-32]。有研究表明, 6'''-阿魏酰斯皮诺素能够不同程度地改变大鼠的自主活动、入睡数量和睡眠时间[33], 斯皮诺素能够作用于5-HT1A, 增强戊巴比妥钠诱导的小鼠的翻正反射丧失[34]。阿魏酸能够提高脑内5-HT的浓度[35], 影响大脑活动, 诱导睡眠。通过"化学成分−疾病靶点"网络, 推测这些化合物促进睡眠作用的发挥可能是通过作用于MAOB、PTGS1、PTGS2等靶点参与5-羟色胺能通路。大量研究发现, 细胞因子能够调节睡眠[36]。长期失眠的患者由于细胞内的炎症反应, 体内细胞因子发生明显变化, 例如IL-6、TNF-α等明显增加, 导致睡眠质量下降[37], 而芒果苷、新芒果苷、洋川芎内酯I可以通过调节MAPK8、CASP3、MMP3、PIK3CG等靶点参与TNF信号通路, 发挥抗炎作用[38-40], 从而改善睡眠质量。这体现了中药复方"多成分、多靶点、多通路"的作用特点, 同时也为复方酸枣仁汤药效物质的选择提供了依据。

《中华人民共和国药典》2020年版[41]并未收载复方酸枣仁汤相关制剂, 只对单味饮片含量测定指标进行了规定, 酸枣仁、知母、川芎饮片中非皂苷类成分含量测定的指标分别为斯皮诺素、芒果苷和阿魏酸, 本研究依据"化学成分−疾病靶点"网络分析结果, 从质量控制指标的选择原则和实际操作出发, 选取了酸枣仁中的斯皮诺素和6'''-阿魏酰斯皮诺素, 知母中的新芒果苷和芒果苷, 川芎中的阿魏酸和洋川芎内酯I这6种度值大于24的药效物质对其进行含量测定。所建立的UHPLC法能够高效、简单、快速地完成对10批复方酸枣仁汤中6种药效物质的含量测定, 为基于药效的复方酸枣仁汤的质量评价提供了依据。

Table 4 The content results of 6 compounds in ten batches

No.	Content/%					
	Neomangiferin	Mangiferin	Ferulic acid	Senkyunolide I/×10^{-3}	Spinosin	6'''-Feruloylspinosin/×10^{-3}
S1	0.012 19	0.138 5	0.074 95	6.655	0.016 73	4.926
S2	0.030 19	0.113 2	0.061 30	9.08	0.015 49	5.009
S3	0.113 5	0.078 98	0.042 76	9.51	0.015 27	5.263
S4	0.010 24	0.053 61	0.012 48	6.303	0.015 60	5.296
S5	0.109 2	0.078 32	0.042 40	8.17	0.015 33	5.223
S6	0.026 77	0.143 6	0.077 76	12.49	0.015 25	5.111
S7	0.103 2	0.087 5	0.047 39	5.907	0.016 12	5.792
S8	0.093 2	0.098 8	0.053 46	7.445	0.017 46	4.859
S9	0.024 54	0.144 5	0.078 20	13.60	0.016 16	6.330
S10	0.101 6	0.084 4	0.045 66	11.92	0.020 07	6.766
Mean ± SD	0.062 46 ± 0.040 4	0.102 1 ± 0.028 5	0.053 64 ± 0.018 44	9.11 ± 2.476	0.016 35 ± 0.001 349	5.458 ± 0.557 3

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