

银杏叶单组分双黄酮分离制备及抗氧化活性研究

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摘要:从银杏叶中分离制备高纯度双黄酮对照品,探讨其抗氧化活性强弱。以 60% 乙醇-水提取的银杏叶浸膏为原料,反溶剂沉淀得到含量 80.40% 的双黄酮粗品,再以甲醇-水为流动相,半制备色谱梯度洗脱。结果得到了 4 种符合中药化学对照品要求的高纯度双黄酮,在最佳制备条件下,4 种双黄酮纯度:阿曼托黄素(98.45%)、白果素(98.66%)、银杏黄素异构体(98.87%)、金松双黄酮(99.29%),其产率(mg/kg)分别为:22.5、23.8、192.5、71.8,考察了它们的抗氧化活性,并采用 60% 乙腈和有机酸水溶液对银杏黄素异构体进行了完全分离。建立的制备方法快速简便,所得单组分双黄酮纯度、产量高。本工作为单组分银杏双黄酮新药的研究与开发提供重要的技术支持。

关键词:双黄酮;反溶剂法;中药化学对照品;半制备高效液相色谱;银杏叶

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Isolation of biflavonoids from *Ginkgo biloba* leaves and their antioxidant activity

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Abstract: To isolate and prepare the biflavonoids reference substance with high-purity from *Ginkgo biloba* L., then investigate their antioxidant activity. High-purity biflavonoids were prepared by 60% ethanol-water extract of *Ginkgo biloba* L., through antisolvent precipitation and semi-preparative HPLC. Crude biflavonoids (purity: 80.40%) was obtained by antisolvent precipitation process, then purified by semi-preparative HPLC with the mobile phase comprising of methanol-water in a gradient elution manner. Under the optimal preparation conditions, four biflavonoids were obtained and identified as amentoflavone (98.45%), bilobetin (98.66%), ginkgetin isomers (98.87%) and sciadopitysin (99.29%) with the yield of 22.5, 23.8, 192.5, 71.8 (mg/kg) respectively, which met the requirements of TCM reference standards, and examined their antioxidant activity, then completely separate the ginkgetin isomers by the use of 60% acetonitrile and organic acid aqueous solution. The preparation method is rapid and simple, and the obtained biflavonoids have high purity and high yield. This study provides important technical support for the research and development of one-component ginkgo biflavonoids.

Key words: biflavonoids; antisolvent precipitation; TCM reference substance; semi-preparative HPLC; *Ginkgo biloba* leaves

高浓度的双黄酮具有一定的细胞毒性^[1],在银杏叶(*Ginkgo Biloba* L.)提取物中,双黄酮一直被作为限量物质。但近年来,银杏双黄酮一些特殊药用价值,如抗流感病毒^[2]、抗氧化^[3]、治疗阿尔茨海默症^[4]以及动脉粥样硬化^[5]等作用被陆续发现。

在双黄酮分析及其高纯单组分对照品研究方面,国内起始于 2010 年,孔繁晟^[6]等首先采用硅胶柱色谱和重结晶方法得到 98.5% 的单组分金松双

黄酮。2014 年李冰等^[7]通过硅胶色谱和半制备色谱,分离得到 mg 级纯度 >95% 的金松双黄酮、银杏黄素、异银杏黄素、白果素 4 种单组分双黄酮,但纯度没有达到 >98% 的对照品要求,特别是没有得到单组分阿曼托黄素。因此,国内双黄酮的定量分析一直落后于其药用研究,从而限制了其资源开发。

本工作以银杏叶粗提物为原料,反溶剂沉淀分离得到 80.40% 的多组分双黄酮粗品,再用半制备色谱分离制备得到 >98% 的高纯金松双黄酮、阿曼托黄素、白果素、银杏黄素异构体,系统研究了制备条件,并对银杏黄素异构体进行了分离,为单组分银杏双黄酮新药的研究与开发提供了重要的技术支持。

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1 仪器与材料

半制备液相色谱仪 LC3000 北京创新恒通科技有限公司; Daisogel C₁₈ (50 mm × 250 mm) 色谱柱; 高效液相色谱仪 Agilent LC-1260, Elite Hypersil ODS2 (200 mm × 4.0 mm, 5 μm) 色谱柱; 超导傅里叶核磁共振波谱仪 AscendTM 600 MHz 瑞士 Bruker 公司; 二维液相色谱-离子阱质谱联用仪 Agilent 1100LC/MSD Trap; 紫外分光光度计 N4 上海仪电电子股份有限公司; 甲醇、乙腈为色谱纯; 水为双蒸水; DPPH、ABTS、K₂S₂O₈ 均购自阿拉丁; 其他试剂均为分析纯; 原料银杏叶采自本校南湖校区。

2 分离方法与结果

2.1 双黄酮粗品分离与提取

60% 乙醇提取的银杏叶浸膏, 用 5 倍水溶散后, 加入等体积 1:1 乙酸乙酯-正丁醇, 搅拌提取 3 次。合并有机相, 旋蒸浓缩, 再用水分散, 加入石油醚搅拌沉淀得到双黄酮粗提物。

取 5 g 双黄酮粗提物溶于 40 mL 乙酸乙酯, 分 3 次加入 80 mL 石油醚, 边加边搅拌, 析出沉淀, 离心分取, 沉淀再溶于 50 mL 甲醇, 加水再次沉淀。反复多次, 得到含量为 80.40% 双黄酮粗品。图 1 为三次加水沉淀后的 HPLC 图谱。

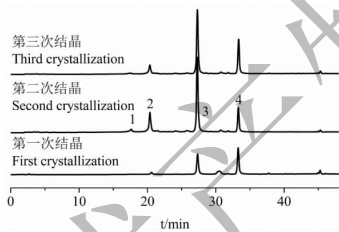


图 1 三次加水沉淀后双黄酮粗品 HPLC 谱图

Fig. 1 HPLC chromatograms of crude biflavonoids

after three times of water precipitation

注: 化合物 1~4 分别为: 阿曼托黄素、白果素、银杏黄素异构体、金松双黄酮。

Note: Compounds 1-4: amentoflavone, bilobetin, ginkgetin, isomers, sciadopitysin.

2.2 单组分双黄酮色谱制备

称取 1.00 g 双黄酮粗品, 用 80% 甲醇约 100 mL 至固体恰好溶解, 离心分离, 微孔滤膜过滤后进半制备色谱, 洗脱分离, 流速 30 mL/min, 柱温 35 °C, 检测波长 330 nm。

收集目标液, 采用红外、核磁氢谱、质谱和分析

型高效液相色谱对其结构及纯度进行检测。

2.2.1 洗脱梯度考察

考察流动相组成和多个洗脱梯度对分离效果影响。结果表明, 制备条件为甲醇(A)-有机酸水溶液(B), 0~2 min, 76% A; 2~45 min, 76%~100% A; 45~50 min, 100% A, 可完全分离阿曼托黄素、白果素、银杏黄素异构体和金松双黄酮(分别记为目标化合物 1、2、3、4), 其保留时间分别为 14.1 min、18.5 min、28.2 min 和 41.1 min。此外, 实验证明, 流动相中加入 0.005 mol/L 三氟乙酸以及 0.005 mol/L 乙酸可规整峰型。

2.2.2 二次制备

将一次制备所得的阿曼托黄素(化合物 1)和白果素(化合物 2)用适量甲醇复溶, 调节洗脱梯度为: 甲醇(A)-有机酸水溶液(B), 0~2 min, 50%~80% A; 2~45 min, 80%~100% A; 45~50 min, 100% A。两次制备所得 4 种单组分双黄酮的 HPLC 谱图如图 2。

4 种单组分双黄酮阿曼托黄素、白果素、银杏黄素异构体、金松双黄酮产率(mg/kg)经计算分别为: 22.5、23.8、192.5、71.8。

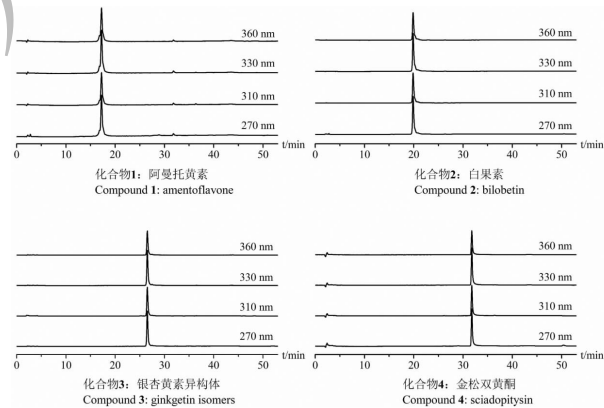


图 2 4 种高纯单组分双黄酮的 HPLC 谱图

Fig. 2 HPLC chromatograms of four high-purity one-component biflavonoids

3 定性及定量分析

3.1 结构鉴定

对得到的 4 个化合物进行了熔点、红外、紫外、质谱、¹H NMR、¹³C NMR 等检测, 结果如下:

化合物 1 黄色粉末, mp. 268~269 °C, 易溶于 DMSO、甲醇、乙酸乙酯、丙酮。UV (MeOH) λ_{Max} 266, 337 nm; IR (KBr) ν_{max} 3 453 (OH), 1 653 (共轭 C=O), 1 498 (芳 C), 1 240, 827 cm⁻¹; Positive

ESI-MS: m/z 539.1 $[M + H]^+$ (Calcd for $C_{30}H_{18}O_{10}$); 1H NMR (500 MHz, DMSO- d_6) δ 13.11 (s, 1H, OH-5''), 12.98 (s, 1H, OH-5), 10.86 (s, 2H, OH-7, 7''), 10.31 (s, 2H, OH-4', 4''), 8.03 (d, $J = 2.5$ Hz, 1H, H-2'), 8.0 (dd, $J = 8.8, 2.5$ Hz, 1H, H-6'), 7.58 (d, $J = 8.5$ Hz, 2H, H-2''', 6'''), 7.17 (d, $J = 8.5$ Hz, 1H, H-5'), 6.85 (s, 1H, H-3), 6.81 (s, 1H, H-3''), 6.73 (d, $J = 8.4$ Hz, 2H, H-3''', 5'''), 6.48 (d, $J = 2.1$ Hz, 1H, H-8), 6.42 (s, 1H, H-6''), 6.20 (d, $J = 2.1$ Hz, 1H, H-6); ^{13}C NMR (150 MHz, DMSO- d_6) δ 182.1 (C-4, 4'), 166.4 (C-7), 163.6 (C-2, 2'), 163.5 (C-7''), 161.8 (C-5), 160.7 (C-5''), 160.5 (C-2'), 158.8 (C-9), 157.7 (C-4'''), 156.3 (C-9''), 132.6 (C-4'), 129.2 (C-2''', 6'''), 128.1 (C-6'), 123.4 (C-5'), 122.9 (C-1'''), 120.8 (C-3'), 117.6 (C-1'), 115.8 (C-3''', 5'''), 104.5 (C-3, 3'), 104.4 (C-10), 104.1 (C-8''), 104.0 (C-10''), 98.8 (C-6''), 98.3 (C-6), 94.0 (C-8)。以上数据与文献^[9,10]基本一致,故确定化合物**1**为阿曼托黄素(amentoflavone)。

化合物2 黄色晶体(CH_2Cl_2 - CH_3OH), mp. 233 ~ 235 °C, 易溶于甲醇、乙酸乙酯、丙酮。UV (MeOH) λ_{Max} 267, 335 nm; IR (KBr) ν_{max} 3 498 (OH), 1 654 (共扼 C = O), 1 496 (芳 C), 1 243, 836 cm^{-1} ; Negative ESI-MS: m/z 551.0 $[M-H]^-$ (Calcd for $C_{31}H_{20}O_{10}$); 1H NMR (500 MHz, DMSO- d_6) δ 13.11 (s, 1H, OH-5''), 12.94 (s, 1H, OH-5), 10.85 (s, 1H, OH-7''), 10.83 (s, 1H, OH-7), 10.29 (s, 1H, OH-4'''), 8.20 (dd, $J = 8.8, 2.5$ Hz, 1H, H-6'), 8.08 (d, $J = 2.4$ Hz, 1H, H-2'), 7.52 (d, $J = 8.9$ Hz, 2H, H-2''', 6'''), 7.36 (d, $J = 8.9$ Hz, 1H, H-5'), 6.95 (s, 1H, H-3), 6.82 (s, 1H, H-3''), 6.72 (d, $J = 8.9$ Hz, 2H, H-3''', 5'''), 6.50 (d, $J = 2.1$ Hz, 1H, H-8), 6.41 (s, 1H, H-6''), 6.21 (d, $J = 2.1$ Hz, 1H, H-6), 3.79 (s, 3H, OCH₃-4'); ^{13}C NMR (150 MHz, DMSO- d_6) δ 182.1 (C-4, 4''), 166.4 (C-7), 163.6 (C-2, 2''), 163.5 (C-7''), 161.8 (C-5), 161.7 (C-2'), 160.7 (C-5''), 158.8 (C-9), 157.7 (C-4'''), 156.3 (C-9''), 132.2 (C-4'), 129.2 (C-2''', 6'''), 127.7 (C-6'), 123.1 (C-5'), 122.9 (C-1'''), 121.9 (C-3'), 115.8 (C-3''', 5'''), 111.0 (C-1'), 104.5 (C-3, 3''), 104.4 (C-10), 104.1 (C-8''), 104.0 (C-10''), 98.8 (C-6''), 98.3 (C-6),

94.0 (C-8), 56.1 (OCH₃-2')。以上数据与文献^[9,11]一致,故确定化合物**2**为白果素(bilobetin)。

化合物3 黄色晶体(CH_2Cl_2 - CH_3OH), mp. 24 ~ 246 °C, 易溶于乙酸乙酯、丙酮。UV (MeOH) λ_{Max} 266, 330 nm; IR (KBr) ν_{max} 3 434 (OH), 1 658 (共扼 C = O), 1 500 (芳 C), 1 249, 836 cm^{-1} ; Negative ESI-MS: m/z 564.9 $[M-H]^-$ (Calcd for $C_{32}H_{22}O_{10}$); 由 1H NMR 谱图推出化合物**3**为银杏黄素(ginkgetin)与异银杏黄素(isoginkgetin)的混合物,将其进一步分离后得到银杏黄素 1H NMR (500 MHz, DMSO- d_6) δ 13.10 (s, 1H, OH-5''), 12.93 (s, 1H, OH-5), 10.83 (s, 1H, OH-7''), 10.29 (s, 1H, OH-4'''), 8.24 (d, $J = 9.1$ Hz, 1H, H-1'), 8.11 (d, $J = 2.4$ Hz, 1H, H-5'), 7.51 (d, $J = 8.5$ Hz, 2H, H-2''', 6'''), 7.38 (d, $J = 8.8$ Hz, 1H, H-2'), 7.03 (s, 1H, H-8), 6.83 (d, $J = 2.8$ Hz, 2H, H-3, 3''), 6.72 (d, $J = 8.6$ Hz, 2H, H-3''', 5'''), 6.43 ~ 6.36 (m, 2H, H-6, 6''), 3.84 (s, 3H, OCH₃-7), 3.80 (s, 3H, OCH₃-4'); ^{13}C NMR (150 MHz, DMSO- d_6) δ 182.1 (C-4, 4''), 167.3 (C-7), 163.6 (C-2, 2''), 163.5 (C-7''), 161.7 (C-2'), 161.4 (C-5), 160.7 (C-5''), 158.4 (C-9), 157.7 (C-4'''), 156.3 (C-9''), 132.2 (C-4'), 129.2 (C-2''', 6'''), 127.7 (C-6'), 123.1 (C-5'), 122.9 (C-1'''), 121.9 (C-3'), 115.8 (C-3''', 5'''), 111.0 (C-1'), 104.5 (C-3, 3''), 104.1 (C-10, 8''), 104.0 (C-10''), 98.8 (C-6''), 98.0 (C-6), 92.8 (C-8), 56.1 (OCH₃-2'), 55.8 (OCH₃-7); 异银杏黄素 1H NMR (500 MHz, DMSO- d_6) δ 13.07 (s, 1H, OH-5''), 12.94 (s, 1H, OH-5), 10.85 (d, $J = 8.2$ Hz, 2H, OH-7, 7''), 8.21 (dd, $J = 8.8, 2.4$ Hz, 1H, H-1'), 8.07 (d, $J = 2.3$ Hz, 1H, H-5'), 7.63 (d, $J = 8.8$ Hz, 2H, H-2''', 5'''), 7.37 (d, $J = 8.9$ Hz, 1H, H-2'), 6.94 (dd, $J = 9.3, 4.9$ Hz, 4H, H-3, 3'', 3''', 5'''), 6.49 (d, $J = 2.2$ Hz, 1H, H-6''), 6.42 (s, 1H, H-8), 6.20 (d, $J = 2.1$ Hz, 1H, H-6), 3.78 (d, $J = 16.3$ Hz, 6H, OCH₃-4', 4''); ^{13}C NMR (150 MHz, DMSO- d_6) δ 182.1 (C-4, 4''), 166.4 (C-7), 163.6 (C-2, 2''), 163.5 (C-7''), 161.8 (C-5), 161.7 (C-2'), 160.7 (C-5''), 159.8 (C-4'''), 158.8 (C-9), 156.3 (C-9''), 132.2 (C-4'), 128.8 (C-2''', 6'''), 127.7 (C-6'), 123.1 (C-5'), 122.6 (C-1'''), 121.9 (C-3'), 114.2 (C-3''', 5'''), 111.0 (C-1'), 104.5 (C-

3,3''), 104.4 (C-10), 104.1 (C-8''), 104.0 (C-10'''), 98.8 (C-6''), 98.3 (C-6), 94.0 (C-8), 56.1 (OCH₃-2'), 55.8 (OCH₃-4'''). 以上数据与文献^[9,12]基本一致,故确定化合物**3**为银杏黄素异构体(ginkgetin isomers)。

化合物 4 淡黄色晶体 (CH₂Cl₂, CH₃OH), mp. 298 ~ 300 °C, 易溶于 DMF、丙酮。UV (MeOH) λ_{Max} 268, 330 nm; IR (KBr) ν_{max} 3 423 (OH), 1 652 (共扼 C = O), 1 504 (芳 C), 1 245, 827 cm⁻¹; Negative ESI-MS: *m/z* 578.8 [M-H]⁻ (Calcd for C₃₃H₂₄O₁₀); ¹H NMR (500 MHz, DMSO-*d*₆) δ 13.07 (s, 1H, OH-5''), 12.93 (s, 1H, OH-5), 10.86 (s, 1H, OH-7''), 8.25 (dd, *J* = 8.8, 2.5 Hz, 1H, H-6'), 8.10 (d, *J* = 2.4 Hz, 1H, H-2'), 7.62 (d, *J* = 9.0 Hz, 2H, H-2''', 6'''), 7.38 (d, *J* = 8.9 Hz, 1H, H-5'), 7.03 (s, 1H, H-3), 6.95 (d, *J* = 9.0 Hz, 2H, H-3''', 5'''), 6.93 (s, 1H, H-3''), 6.82 (d, *J* = 2.3 Hz, 1H, H-8), 6.43 (s, 1H, H-6''), 6.38 (d, *J* = 2.3 Hz, 1H, H-6), 3.91-3.70 (m, 9H, OCH₃-7, 4', 4'''); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 182.1 (C-4, 4''), 167.3 (C-7), 163.6 (C-2, 2''), 163.5 (C-7''),

161.7 (C-2'), 161.4 (C-5), 160.7 (C-5'''), 159.8 (C-4'''), 158.4 (C-9), 156.3 (C-9''), 132.2 (C-4'), 128.8 (C-2''', 6'''), 127.7 (C-6'), 123.1 (C-5'), 122.6 (C-1'''), 121.9 (C-3'), 114.2 (C-3''', 5'''), 111.0 (C-1'), 104.5 (C-3, 3''), 104.1 (C-10, 8''), 104.0 (C-10'''), 98.8 (C-6''), 98.0 (C-6), 92.8 (C-8), 56.1 (OCH₃-2'), 55.8 (OCH₃-7, 4'''). 以上数据与文献^[9,13]一致,故确定化合物**4**为金松双黄酮(Sciadopitysin)。

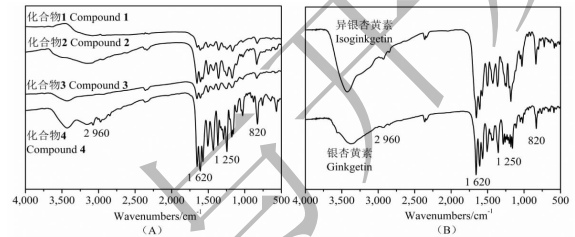


图3 单组分双黄酮的红外光谱图

Fig.3 Infrared spectra of one-component bioflavonoids

注:阿曼托黄素、白果素、银杏黄素异构体、金松双黄酮(A) 银杏黄素及异银杏黄素(B)。

Note: Amentoflavone, Bilobetin, Ginkgetin isomers, Sciadopitysin (A) Ginkgetin and Isoginkgetin (B).

表1 两次色谱制备得到的单组分双黄酮纯度定值

Table 1 Purity assessment of one-component biflavonoids after two chromatographic preparation

对照品 Reference substance	色谱纯度 Chromatographic purit (%)	水分 Water conten (%)	无机杂质 Inorganic impurities (%)	定值 Constant value (%)
阿曼托黄素 Amentoflavone	98.45	0.22	0.07	98.16
白果素 Bilobetin	98.66	0.24	0.08	98.34
银杏黄素异构体 Ginkgetin isomers	98.87	0.27	0.10	98.50
金松双黄酮 Sciadopitysin	99.29	0.12	0.06	99.11

3.2 HPLC-DAD 测定纯度

根据中国计量科学研究院关于国家标准物质纯度测定的要求^[14],对双黄酮制备品以面积归一法计算纯度,测定4种双黄酮色谱纯度。

采用硅胶薄层色谱法对单组分双黄酮定性分析,观察发现4种单组分双黄酮均为单一黄绿色斑点。以质量平衡法测定单组分双黄酮纯度,结果如表1所示。

4 抗氧化活性和异构体分离

4.1 抗氧化活性测定

参照文献^[8]测定桑椹根化学成分的抗氧化活性方法,分别在517 nm及734 nm处测定4种双黄

酮样品清除 DPPH、ABTS⁺ 自由基的清除率及半数抑制率 IC₅₀值。每份样品平行操作测定3次,取平均值,得到4种双黄酮的抗氧化活性结果见表2。结果显示4种双黄酮具有明显的抗氧化活性,与文献报道一致。

4.2 银杏黄素异构体进一步分离

关于银杏黄素异构体的进一步分离,我们尝试采用乙腈-水体系的方法^[7],将甲醇-水分离体系得到的银杏黄素异构体(化合物**3**)用适量丙酮复溶,以60%乙腈和有机酸水溶液洗脱分离,所用有机酸为0.005 mol/L 三氟乙酸-0.005 mol/L 乙酸混合物,可实现银杏黄素和异银杏黄素的完全分离,其纯度

定值分别为 98.56%、95.70%，所得 HPLC 谱图如图 4。

表 2 待测化合物清除 DPPH 自由基和 ABTS⁺ 自由基的 IC₅₀ 值

Table 2 IC₅₀ values of DPPH and ABTS⁺ radical scavenging of the isolated compounds

化合物 Compound	DPPH IC ₅₀ (mg/mL)	ABTS ⁺ IC ₅₀ (mg/mL)
阿曼托黄素 Amentoflavone	0.13	0.02
白果素 Bilobetin	0.45	0.32
银杏黄素异构体 Ginkgetin isomers	0.64	0.41
金松双黄酮 Sciadopitysin	1.14	0.53

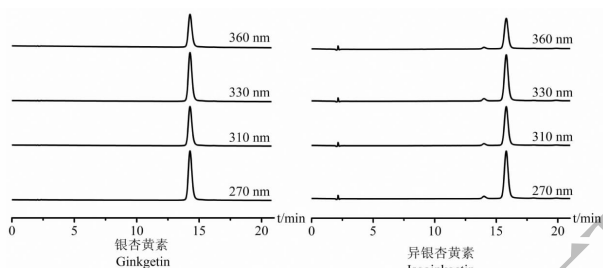


图 4 银杏黄素及异银杏黄素的 HPLC 谱图

Fig. 4 HPLC chromatograms of ginkgetin and isoginkgetin

5 结论

本实验以 60% 乙醇提取的银杏叶浸膏为原料，水-石油醚多次反溶剂沉淀分离得到纯度为 80.40% 的双黄酮粗品。再以甲醇-水为流动相，用制备色谱梯度洗脱分离得到 4 种符合中药化学对照品要求的单组分双黄酮，并采用 60% 乙腈和有机酸水溶液对银杏黄素异构体进行了完全分离，得到了 5 种纯度均大于 95% 的双黄酮，为双黄酮标样的生产提供思路，通过 DPPH 和 ABTS⁺ 体外自由基清除实验，此次分离得到的五种双黄酮均具有一定抗氧化活性，也为双黄酮的开发利用提供了科学依据。

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