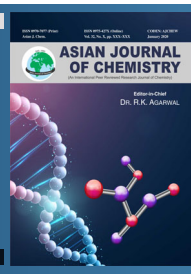
Asian Journal of Chemistry; Vol. 32, No. 3 (2020), 515-518

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2020.22241



Isolation of Kaempferol from Vietnamese *Ginkgo biloba* Leaves by Preparative Column Chromatography

TUONG VAN NGUYEN[1,*] and VINH DINH[2]

[1]Faculty of Pharmacy, Nguyen Tat Thanh Univeristy, Ho Chi Minh City, Vietnam
[2]Faculty of Pharmacy, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam

*Corresponding author: E-mail: ntvan@ntt.edu.vn

Received: 4 June 2019; *Accepted*: 30 September 2019; *Published online*: 31 January 2019; AJC-19751

The aim of this research was to develop a simple procedure to isolate kaempferol from *Ginkgo biloba* leaves extract. Flavonoids present in *Ginkgo biloba* leaves were extracted by 96 % ethanol. These flavonol glycosides which were hydrolyzed in the presence of HCl to convert to aglycones. The flavonol aglycones were extracted by the hydrolysis of solution with ethyl acetate which was evaporated to dryness under reduced pressure to obtain concentrated flavonol aglycones (C2). Flavonol aglycones were then purified by preparative column chromatography with a mobile phase composed of petroleum ether-ethyl acetate to acquire fraction C3. The recrystallization of fraction C3 in acetone realizes to have an amorphous sediment as fraction C4 that was supposed to be a mixture of kaempferol, quercetin and isorhamnetin. Kaempferol in this sediment was purified by column chromatography with a mobile phase composed of $CHCl_3$-MeOH to achieve a fraction denoted as fraction C5. The isolated fraction C5 was assigned by its purity and structure by thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and spectroscopic data analyses.

Keywords: Ginkgo leaves extract, Kaempferol, Silica gel, Column chromatography.

INTRODUCTION

Ginkgo biloba, commonly known as gingko or ginkgo, is among the most oldest plants (a living fossil). *Ginkgo biloba* is the only remaining species of ancient Ginkgoales remaining. *Ginkgo biloba* grows widely in China, spread to Japan, Europe, North America and elsewhere [1]. Crucial constituents contained in this medicinal leaves are the terpene trilactones, flavonoids, alkylphenols, simple phenolic acids, 6-hydroxykynurenic acid, 4-O-methylpyridoxine and polyprenols [2].

Ginkgo flavonoids have been recommended for cerebral-related symtoms such as deterioration functional disturbances, vascular blockage. These flavonoids possess antitumor and anti-inflammatory activities [3]. Crude extracts of ginkgo leaves in concentration of 7.8 µg/mL inhibit activity of both Gram-positive bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) and Gram-negative bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*). Methanol fractions of leaves significantly restrain tumor forming [4,5]. Ginkgo flavonoids are able to diminish the lipid agglomeration in relation to up-regulation of carnitine palmitoyl-transferase 1A (CPT1A), a rate-limiting enzyme, in the β-oxidation of long-chain fatty acids [6]. Totally 13 isolated flavonoids from *Ginkgo biloba* were found to inhibit AChE dose dependently at IC_{50} from 57.8 to 133.1 µg/mL.

The flavonoids found in the leaves of *Ginkgo biloba* could be analyzed qualitatively and quantitatively by RP-HPLC. After being hydrolyzed in acid to aglycones (quercetin, kaempferol and isorhamnetin) and separated by HPLC, original total content of these glycosids is estimated from that of aglycones [2,7]. Kaempferol which is among principal yellow colouring material is crystalline solid. It is soluble in dimethyl sulfoxide, ethanol and ether. The extraction solvents of kaempferol mainly are methanol, ethanol, acetone and water with the assistance of ultrasound, microwaves, ultrasound and microwaves [8]. In relation to advanced technology of extraction, soxhlet extraction technique have been emplyed to extract kaempferol with 80 % methanol as a solvent [9]. Extraction using supercritical fluids (usually CO_2) is an alternative method whose conditions are at lower temperature and higher pressure. This brings about

high extraction performance since substances are not decomposed but requires expensive equipment [8]. The extract is then hydrolyzed with HCl in methanol and partitioned continuouslly with chloroform to eliminate non-polar contaminants and ethyl acetate. The ethyl acetate fractions were collected and separated by column chromatography with 50 % methanol as an eluant [10]. Another way to purify ethyl acetate soluble fraction is further partition with *n*-hexane. The *n*-hexane fraction was then subjected respectively to vaccum liquid chromatography and reverse-phase column chromatography using Sephadex column LH-20 to achieved highly pure kaempferol [11].

The one step separation technique to isolate kaempferol from a crude ethanol extract of *Ginkgo biloba* leaves could be high-speed counter-current chromatography method. The separation was carried out by applying a mobile phase constituted of n-hexane-butanol-ethyl acetate-methanol-0.5 % acetic acid (1:0.5:3.5:1:4, v/v) [3].

The polarity of three flavonol aglycones is increased from kaempferol, quercetin and isorhamnetin due to the presence of hydroxyl groups in their structure. Therefore, they could be isolated from each other by preparative column chromatography with normal stationary phase which is suitable for relatively less polar substances. This technique is among the most simple and therefore available for larger scale preparative applications. Solvents for elution is manually or automatically applied. Kaempferol purities were determined by HPLC and structurally identified by IR, MS, ^{1}H and ^{13}C NMR.

EXPERIMENTAL

Plant materials: *Ginkgo biloba* leaves were purchased from local market store of Ho Chi Minh City, Vietnam during March 2018. The sample was identified by the Department of Botany, Pharmacy Faculty, Nguyen Tat Thanh University. After collection, the leaves were dried under 60 ºC for 72 h in an oven with force ventilation and then ground into raw powder.

Methanol and acetonitrile (Merck Darmstadt, Germany) were of HPLC quality. Acetone, ethyl acetate, *n*-hexane, acetic acid were of synthetic quality (Chemsol, HCM city, Vietnam). All the solvents used for HPLC analysis were filtered (0.45 pm) and ultrasonically degassed before use. All the separations were carried out at room temperature (25 ºC). The chromatography column (35 cm × 2 cm) was packed with 25 g of silica gel 60 Merck (0.040-0.063 mm).

Flavonoid extractions and hydrolysis: About 1.5 kg of *Ginkgo biloba* leaves powder were extracted by repeated cold maceration in 2 L of 96 % EtOH at room temperature for one time a day. The same extraction process was repeated for 4 times in the same leave sample, using totally 8 L absolute ethanol. The extract was filtered and concentrated in vacuum at 60 ºC to dryness with a rotary evaporator and denoted as C1. The extract C1 is then distributed into 1 L hot water then added chloroform, shaken and allowed to stand 30 min in order to eliminate non-polar impurities. Each 100 mL of distributed water was shaken with 30 mL $CHCl_3$ until the $CHCl_3$ layer exhibited no colour. The aqueous layer after shaking with $CHCl_3$ was separated and hydrolyzed with 10 % HCl at pH 3-4. The acidic aqueous solution was shaken with EtOAc. Each time of shaking, used 30 mL EtOAc and acidic water layer was collected. This process was repeated until EtOAc layer yellow colour disappeared. The acidic aqueous layer was hydrolyzed by reflux method with 20 % HCl at pH 1.3-1.4 for 4 h and then left for for 1 h. The hydrolyzed solution was fractionally extracted with EtOAc (30 mL) and the EtOAc layer was collected. This layer was concentrated in vacuum evaporator at 40 ºC to dryness and denoted as C2.

Column chromatography: The C2 fraction was collected and then separated using column chromatography (CC) method with silica gel as stationary phase and petroleum ether and EtOAc as mobile phase in the ratio of 1:1. The stationary phase was packed with 25 g of silica gel using a wet method. Subfractions were collected every 15 mL with flow-rate of 2.0 mL per minute. Consequently, total 200 subfractions were obtained (Table-1).

TABLE-1
SUBFRACTIONS OF THE C2

PE-EtOAc	Volume (mL)	Subfractions (15 mL)
100:0	400	Not collected
95:5	300	1-20
90:10	300	21-40
85:5	3000	41-200

Kaempferol in subtractions was verified by TLC (silica gel 60 F_{254}), solvent system $CHCl_3$-MeOH -HCOOH (95:5:1, v/v), monitoring under an UV lamp.. The subfractions from 1 - 200 were collected and concentrated in vacuum evaporator at 40 ºC to dryness and denoted as fraction C3.

Recrystallization in acetone: The fraction C3 was dissoluted in a flask using a minimal amount of acetone. The crude product was eluted out as a pure crystalline solid and leaving mother liquor. Kaempferol with higher purity (C4) was achieved after several times recrystallization. The fraction C4 was verified by the same chomatographic condition and revealed that it consist of three flavonoids.

Final purification by column chromatography: The fraction C4 was purified by column chromatography with $CHCl_3$ as a mobile phase at a flow-rate of 2.5 mL per minute. Eluant was not collected until chromatographically pure kaempferol begins to occur (monitored by TLC). The mobile phase was then changed from $CHCl_3$ to MeOH. Elution was performed until kaempferol was completely washed out the column. Evaporation of mobile phase at room temperature obtained a fraction C5. It was preserved from light using a dark membrane.

RESULTS AND DISCUSSION

Purity assessment by TLC: Fraction C5 was analyzed with three different mobile phases with increasing polarity as (1) $CHCl_3$-MeOH-HCOOH (8:2:1,v/v), (2) PE-EtOAc-HCOOH (5:5:1) and (3) $CHCl_3$-EtOAc-HCOOH (5:4:1). The chromatogram of fraction C5 showed only one spot on a developed TLC plate. This indicated that the sample was a single component.

Purity assessment by HPLC: The purity of fraction C5 was also assessed by HPLC using the analytical conditions as detector: PDA2998, λ = 370 nm, column: Luna® 5 µm C18, (250 × 4.6 mm, 5 µm); mobile phase: MeOH-CH_3CN-H_3PO_4 0.05 % (15:25: 60, v/v); volume of injection: 20 µL; Flow-rate: 1 mL/min and column temperature: 25 ºC.

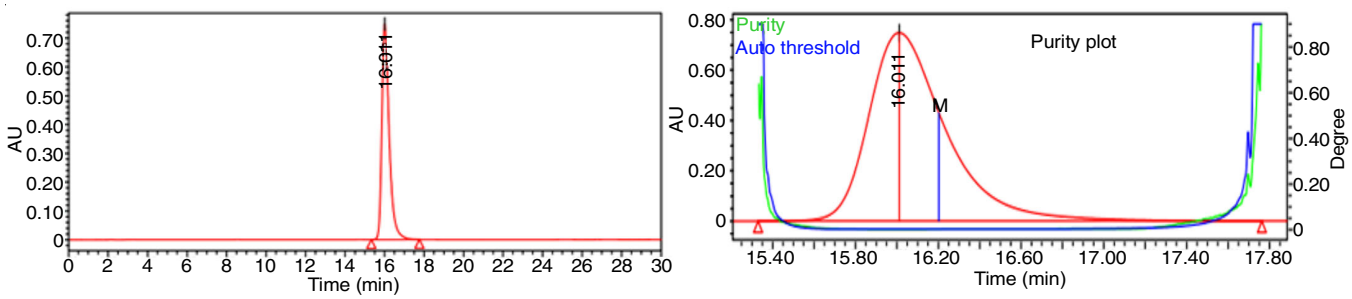


Fig. 1. HPLC chromatograph of fraction C5

Chromatographic purity of fraction C5 was 98 % as according to percentage of peak area over total areas of all peaks in the chromatogram (Fig. 1). In addition, only peak in chomatogram was proved to be not overlaped by any other peaks assessed by threshold value.

FTIR analysis: The infrared spectrum of fraction C5 showed broaded band in the region 3211 cm^{-1} which indicates a hydroxyl group (-OH) and supported by the appearance of absorption at 1090 cm^{-1} is a prime characteristic of C-OH. A strong absorption band formed at 1655 cm^{-1} is due to C=O. Another strong bands at 1613 and 1557 cm^{-1} were assinged to C=C aromatic. Similarly, a band at 818 cm^{-1} was due to -CH bending of an aromatic ring while a weak peak at 1166 cm^{-1} indicates the presence of C-O bond (Fig. 2) [10].

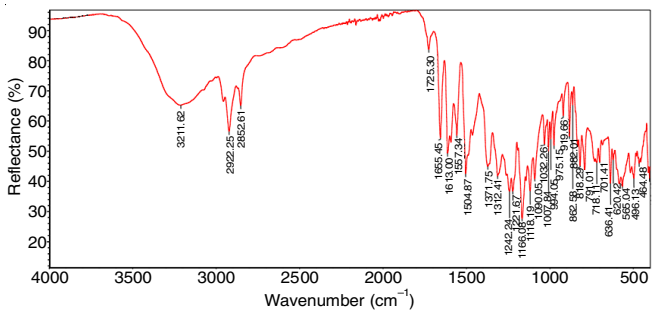


Fig. 2. Infrared spectra of fraction C5

Mass analysis: The mass spectrum in ESI mode of fraction C5 (Fig. 3) showed a quasi-molecular ion with *m/z* 284.8. It indicates that molecular weight of fraction C5 is 286 as same as kaempferol [12].

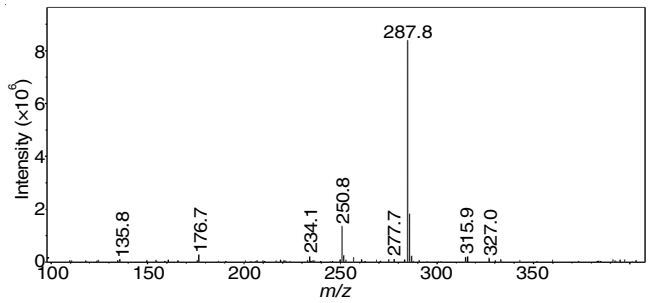


Fig. 3. Mass spectrum of fraction C5

NMR analysis: ^{1}H NMR (500 MHz, DMSO-d_6) spectrum was used to identify the number of proton and its multiciple in the molecular structure of fraction C5. In these data, there are several groups of signals consisting of six protons aromatic (Fig. 4). The emergence of doublet signals at 6.490 ppm (1H, d, J = 1.5 Hz) and 6.229 ppm (1H, d, J = 1.5 Hz) revealed an occurrence of two protons in coupling position of *m*-aromatic ring. The existence of two doublet signals at 8.021 ppm (2H, d, J = 9 Hz) and at 6.943 ppm (2H, d, J = 8.5 Hz) proved that *ortho*-coupling position on C-6′,2′ and C-5′,3′ of B-ring. The emergence of singet signals at δ 10.251 and 12.438 ppm show the position of hydroxyl group. All these data (Table-2) revealed a model of flavonol skeleton.

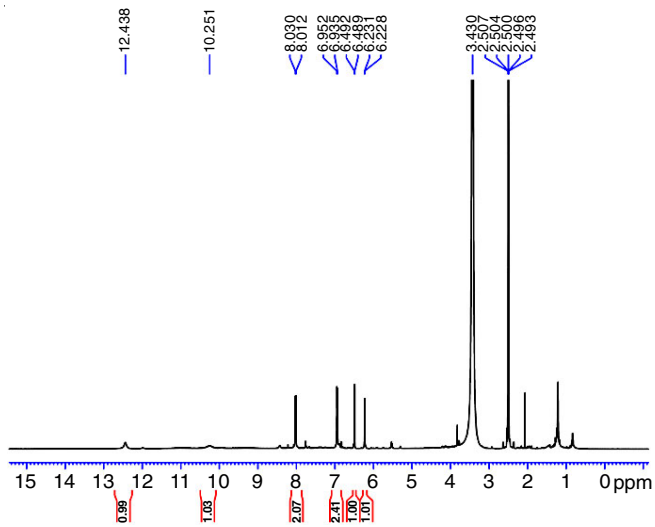


Fig. 4. ^{1}H NMR spectrum of fraction C5 (500 MHz, DMSO)

TABLE-2
INTERPRETATION ^{1}H NMR SPECTRUM OF C5 IN COMPARISON WITH KAEMPFEROL (500 MHz, DMSO)

Proton	110VAN_Kaempferol (ppm)	Kaempferol (ppm) [10]
H-3', H-5'	6.943 (2H; d; 8.5)	6.89 (2H; d; 9)
H-2', H-6'	8.021 (2H; d; 9)	8.07 (2H; d; 9)
H-6	6.229 (1H; d; 1.5)	6.15 (1H; d; 2)
H-8	6.490 (1H; d; 1.5)	6.37 (1H; d; 2)
-OH	10.251 (1H; s)	
-OH	12.438 (1H; s)	

^{13}C NMR (125 MHz, DMSO) spectrum showed that there were 13 separate signals assigned to 15 chemical shifts of 15 carbon atoms at δ_C = 93.58, 98.34, 103.01, 115.53, 121.66, 129.46, 135.72, 146.88, 156.19, 159.33, 164.14, 160.66, 175.97 ppm. All of which were shifted in field of carbons of aromatic ring (Table-3). There was one carbon of a carbonyl group at δ_C = 175.97 ppm. There were also two chemical shifts which have twice abundant at δ_C = 115.53 and 129.46 ppm.

TABLE-3
INTERPRETATION ^{13}C NMR SPECTRUM OF C5 IN COMPARISON WITH KAEMPFEROL (125 MHz, DMSO)

Position of C	C5 (ppm)	Kaempferol [13] (ppm)	Position of C	C5 (ppm)	Kaempferol [13] (ppm)
C-2	146.88	146.1 (s)	C-10	103.01	102.9 (s)
C-3	135.72	135.5 (s)	C-1'	121.66	121.6 (s)
C-4	175.97	175.7 (s)	C-2'	129.46	129.3 (s)
C-5	156.19	156.0 (s)	C-3'	115.53	115.3 (d)
C-6	98.34	98.2 (d)	C-4'	159.33	159.0 (s)
C-7	164.14	163.8 (s)	C-5'	115.53	115.3 (d)
C-8	93.58	93.4 (d)	C-6'	129.46	129.3 (d)
C-9	160.66	160.5 (s)	–	–	–

Based on spectroscopic data analysis, it can be concluded that the fraction C5 was found to be kaempferol (Fig. 5) having molecular formula $C_{15}H_{10}O_6$.

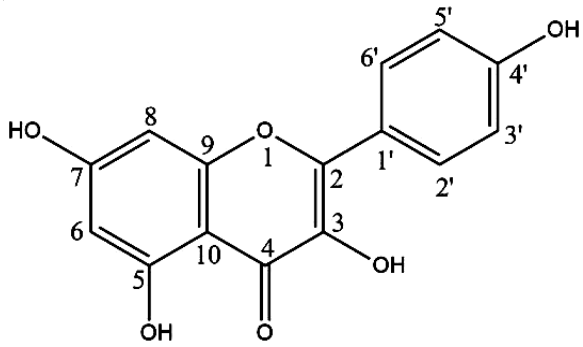


Fig. 5. Structure of kaempferol

Conclusion

The isolation of flavonol glycoside (kaempferol) from *Ginkgo biloba* leaves extract using simple column chromatography was reported [14]. Flavonoids were first hydrolyzed from *Ginkgo biloba* leaves extract and then by converting them into aglycones. The resulting three aglycones were purified by column chromatography with normal phase silica gel. Isolated kaempferol confirmed by spectral (IR and NMR) and mass analysis. Isolated kaempferol showed a molecular ion peak at *m/z* 286 having molecular formula $C_{15}H_{10}O_6$.

ACKNOWLEDGEMENTS

This research was funded by Nguyen Tat Thanh Foundation for Science and Technology Development under grant number 2018.01.37.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. L. Hsieh, *The Forestry Chronicle*, **68**, 612 (1992); https://doi.org/10.5558/tfc68612-5
2. T.A. van Beek, *J. Chromatogr. A*, **967**, 21(2002); https://doi.org/10.1016/S0021-9673(02)00172-3
3. Q. Zhang, L.-J. Chen, H.-Y. Ye, L. Gao, W. Hou, M. Tang, G. Yang, Z. Zhong, Y. Yuan and A. Peng, *J. Sep. Sci*., **30**, 2153 (2007); https://doi.org/10.1002/jssc.200600544
4. S.C. Sati and S. Joshi, *The Scientific World J.*, **11**, 2241 (2011); https://doi.org/10.1100/2011/545421
5. T. Boonkaew and N.D. Camper, *Phytomedicine*, **12**, 318 (2005); https://doi.org/10.1016/j.phymed.2003.06.008
6. T. Wei, F.F. Xiong, S.D. Wang, K. Wang, Y.-Y. Zhang and Q.-H. Zhang, *J. Biomed. Sci.*, 21 (2014); https://doi.org/10.1186/s12929-014-0087-x
7. A. Hasler and O. Sticher, *J. Chromatogr. A*, **605**, 41(1992); https://doi.org/10.1016/0021-9673(92)85026-P
8. S.C. Ortegal andJ. Alberto, *Food Technol. Biotechnol*., **56**, 480 (2018); https://doi.org/10.17113/ftb.56.04.18.5870
9. M.P. Nandan and V. Meena, *Int. J. Appl. Sci. Biotechnol*., **3**, 588 (2015); https://doi.org/10.3126/ijasbt.v3i4.13360
10. Diniatik, S. Pramono and S. Riyanto, *Asian J. Pharm. Clin. Res*., **10**, 322 (2017); https://doi.org/10.22159/ajpcr.2017.v10i5.15970
11. A. Wahab, Tahira, S. Begum, I. Mahmood, T. Mahmood, A. Ahmad and N. Fayyaz, *FUUAST J. Biol.*, **4**, 1 (2014).
12. F. Cuyckens and M. Claeys, *J. Mass Spectrosc*., **39**, 1 (2004); https://doi.org/10.1002/jms.585
13. V.M. Ivanov, Analysis of NMR spectra, In: A Handbook on Analytical Chemistry, Chap. 7, Chemical Industry Press, Pekin (2006).
14. Y. Wei, Q. Xie, D. Fisher and I.A. Sutherland, *J. Chromatogr. A*, **36**, 6206 (2011); https://doi.org/10.1016/j.chroma.2011.01.058