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Preparative Isolation and Antitumor Activity of Six Ginkgol Monomers from *Ginkgo biloba*

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Research Article

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ABSTRACT

Ginkgols are bioactive compounds from *Ginkgo biloba* L., exhibiting excellent antitumor activity. For research on the structure-activity relationships, four ginkgol monomers (C13:0, C15:1-Δ8, C17:2-Δ9, 12, C15:0) and two ginkgol isomers (C17:1-Δ10, C17:1-Δ12) were isolated successively by preparative HPLC and identified. The results of MTT assay showed that all of the ginkgol monomers exhibited cytotoxicity against HepG2 and HGC cells. Ginkgol monomers with unsaturated side chains performed stronger inhibitory effects than which with saturated side chains. The IC_{50} values of ginkgol C15:1-Δ8, C17:2-Δ9, 12, C17:1-Δ10, C17:1-Δ12 on HepG2 and HGC cells were in sequence: 5.69 and 3.94, 19.95 and 11.09, 19.79 and 10.22, 22.76 and 11.4 μg/mL. Among which, ginkgol C15:1-Δ8 showed the strongest cytotoxicity with the lowest IC_{50} values. As for the two ginkgol C17:1 isomers, double bond at Δ10 performed higher antitumor activities than that at Δ12. The IC_{50} values of ginkgol C17:2 with two double bonds were close to that of ginkgol C17:1 with one double bond.

INTRODUCTION

Ginkgo biloba, which is considered as a "living fossil", has been existing on earth for 200 million years [1]. Extracts of *Ginkgo biloba* leaves (EGb) which contain-bioactive constituents such as terpene trilactones and flavonol glycosides have been used for treating many diseases including Alzheimer's disease, cardiovascular disease, dementia, memory loss, and cerebral ischemia [2]. Nowadays, the EGb is one of the most widely used herbal remedies in Europe and US [3]. Three classes of alkylphenols (ginkgolic acids, cardanols and cardols) have been reported to exist in *Ginkgo*, and occur in different concentrations in commercial extracts due to the different manufacturing processes [4-6]. These alkylphenols which cause contact allergy have been identified as hazardous constituents in the EGb and should be removed by most manufacturers during EGb761 processing [7]. Although the toxicity of *Ginkgo* alkylphenols upon oral administration has never been undoubtedly proven [8-10], most suppliers have limited their content to 5 ppm in extracts [11,12]. However, several therapeutically desired effects have been reported for *Ginkgo* alkylphenols, such as antioxidant activity, antitumor activity [13-15]. These prominent bioactivities lead to interest in the research of alkylphenols with regard to its structure and potential applications as a therapeutic agent in the treatment of cancer and other diseases.

Ginkgolic acids are derivatives of 6-alkylsalicyclic acid with an alkyl side chain varying from 13 to 17 carbons in length and 0, 1 or 2 double bonds. Ginkgolic acids could be converted into ginkgols (3-alkylphenols) by decarboxylation when they were heated [16]. Our previous study showed that ginkgols also exhibited excellent anticancer effects and thermostability, and then ginkgol C13:0, C15:1 and C17:1 was separated and their antiproliferation effects against cancer cells were tested [17]. But ginkgol C17:1 was not a single chemical substance, which exhibited two peaks (1:1) with exactly the same mass spectra in GC-MS analysis [17]. The isomers of ginkgol C17:1 have not been separated and identified, and their bioactivities have not been compared either until now.

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In order to obtain the ginkgol C17:1 monomer, ginkgols were separated by preparative HPLC. The isomers of ginkgol C17:1 and other ginkgol monomers were purified and isolated via fraction collection. The structures of the compounds were identified by means of UV, IR, GC-MS, ^{1}H-NMR and ^{13}C-NMR. Their antitumor activities were compared by MTT assay.

RESULTS AND DISCUSSION

Preparative isolation

According to the isolation procedure depicted in **Figure 1**, the ginkgolic acids were extracted and converted into ginkgols [17]. Then the ginkgols were separated by preparative HPLC [18]. The optimum separation process was achieved on Welch Ultimate AQ-C_{18} HPLC column with the eluent MeOH-H_2O (86:14, v/v) at 24 mL/min. Under the optimum conditions, ginkgols were separated within 68 minutes and appeared five absorption peaks in preparative chromatogram figure **(Figure 2).** In order to obtain single chemical substance, each of the peaks was segmented and collected. From Figure 2, peaks 1-4 were symmetrical, but the fifth peak appeared as a shoulder peak which means it was not a single compound. Thus the fifth peak was divided into seven fractions in sequence: 58.5~59.0, 59.0~60.7, 60.7~62.3, 62.3~63.4, 63.4~64.3, 64.3~66.0 and 66.0~67.5 min. Then all the fractions were analyzed by analytical HPLC, and the fractions displayed a single and symmetrical shape and same retention time were combined. Four compounds (G-1, G-2, G-3 and G-4) were obtained successively from peaks 1-4, G-5 and G-6 were obtained simultaneously from peak 5. The HPLC purity of the six compounds was shown in **Figure 3.**

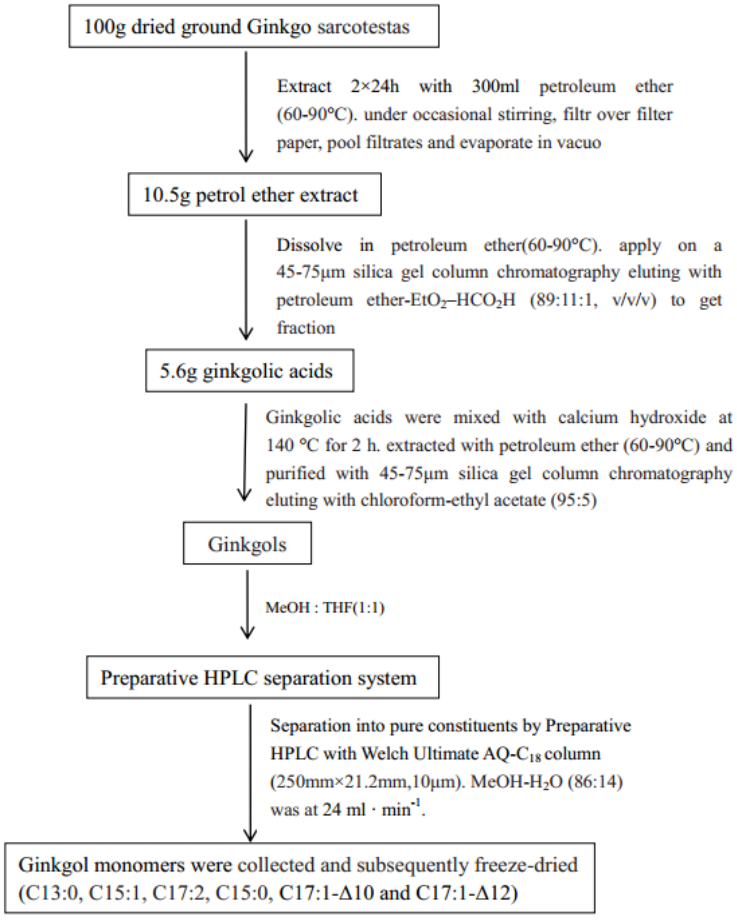

Figure 1. Schematic presentation of the isolation procedure for ginkgols.

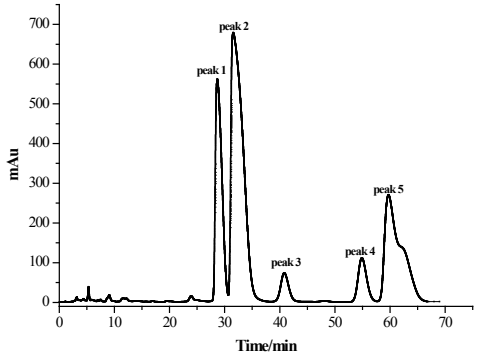

Figure 2. Preparative chromatogram of the ginkgols.

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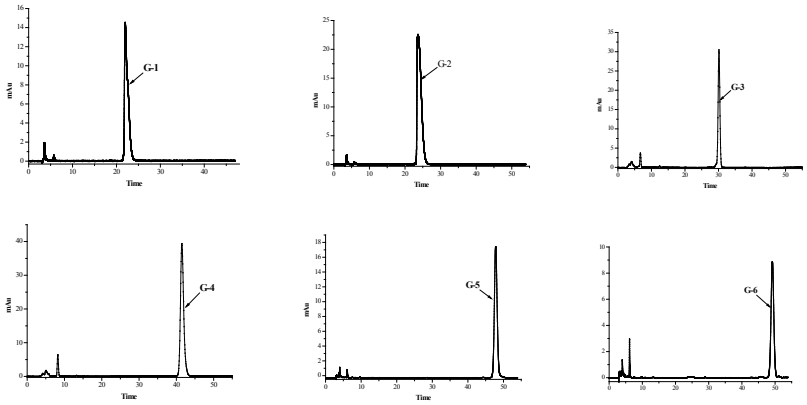

Figure 3. The chromatograms of G-1, G-2, G-3, G-4, G-5 and G-6.

Identification of ginkgols

The purity of these compounds was further identified by GC-MS **(Figure 4).** The total ion chromatograms (TICs) of G-1, G-2, G-3, G-4, G-5 and G-6 are shown in **Figures 4A-4F**, all of them showed as a single and symmetrical peak, respectively. The purities of the six compounds were 100%, 98.1%, 100%, 100%, 96.7% and 99%, respectively.

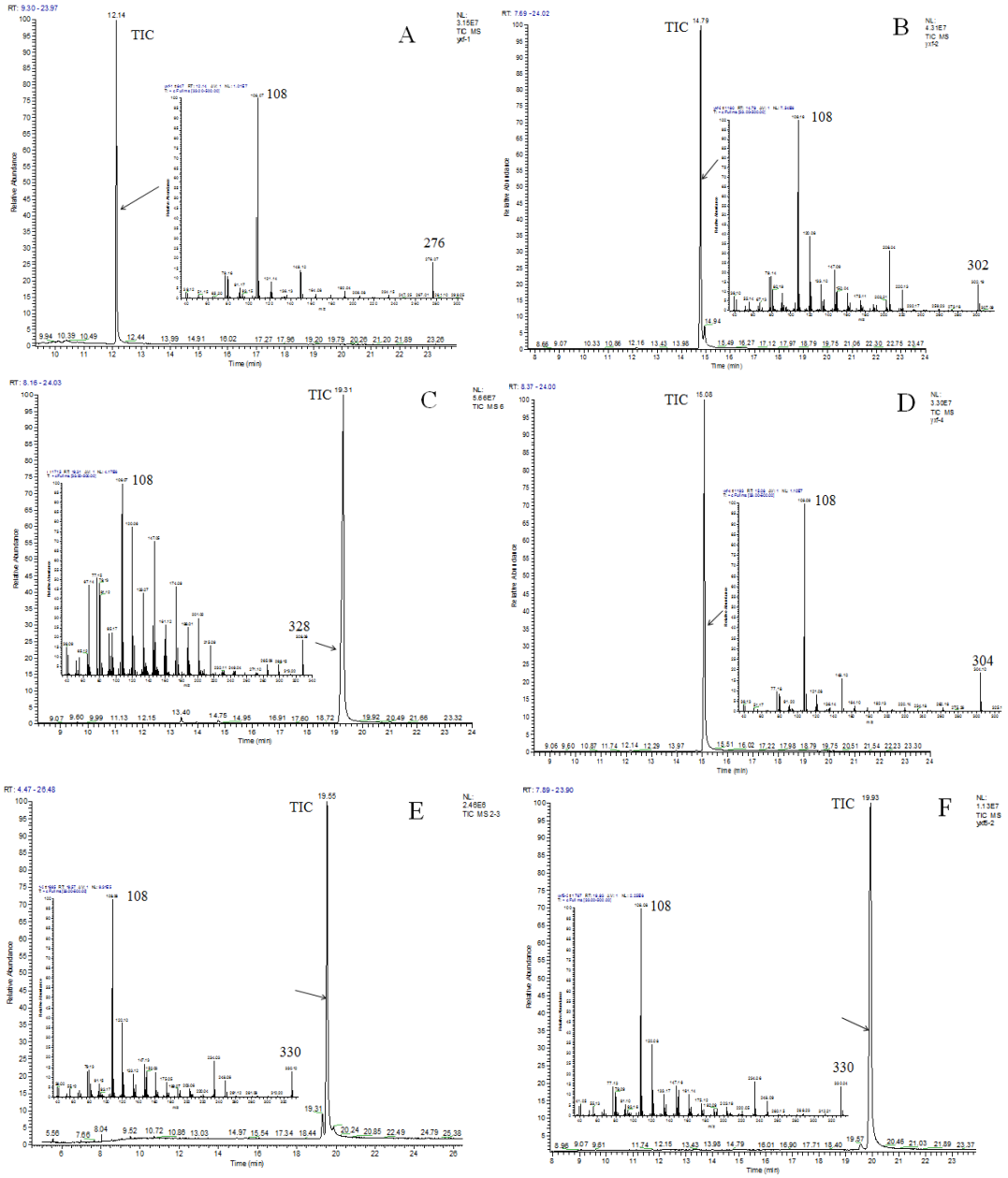

Figure 4. GC-MS chromatogram and EI mass spectra of G-1 (A), G-2 (B), G-3 (C), G-4 (D), G-5 (E) and G-6 (F).

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The structures of the six compounds were identified by UV, IR, ^{1}H-NMR, ^{13}C-NMR spectroscopy **(Table 1)** and GC-MS **(Figure 4).** In **Table 1,** the ^{1}H-NMR spectra data of the six compounds showed the existence of a methyl group (δ_H about 0.90 ppm, t, *J* = 6.8 Hz, 3H), a long chain of methylene groups (δ_H about 1.30 ppm, b) and a 1, 3-substituted benzene (δ_H 7.15(t, *J* = 7.6 Hz, 1H), 6.76(d, *J* = 7.6 Hz, 1H), 6.66(m, 2H) ppm). The existence of hydroxyl group was verified by their wide and strong ν_{O-H} vibration at 3330 cm^{-1} of the IR spectrum **(Table 1)** and fragmentation peak at 108 (cresol) on their MS **(Figure 4).** Their UV spectra ($\lambda_{max\ (MeOH)}$ = 275 nm) were identical **(Table 1).** All the data showed that the six compounds were 3-alkyl phenol [19,20]. They were different on the length of an alkyl chain or on the number and position of double bonds in the alkyl chain.

Table 1. Recorded ^{1}H-NMR, ^{13}C-NMR, UV and IR spectral data of isolated compounds.

Compound	Spectrum	Aryl	Side chain
G-1 (Peak 1)	^{1}H[a]	5.35(s,1H),6.67(m,2H),6.76(d,1H),7.15(t,1H)	0.9(t,3H,H13′),1.29(m,20H),1.60(m,2H,H2′),2.56(t,2H,H1′)
	^{13}C[a]	112.5,115.3,120.7,129.3,144.9,155.5	14.1(C13′),22.7(C12′),29.3,29.5,29.7,30.9,31.3,31.9,35.8(C1′)
	UV-(MeOH)	λ_{max} 275 nm	
	IR (cm^{-1})	3353,2925,2855,1593,1458,1265,1154,778,695	
G-2 (Peak 2)	^{1}H	4.88(s,1H),6.66(m,2H),6.77(d,1H),7.15(t,1H)	0.90(t,3H,H15′),1.31(m,16H),1.61(m,2H,H2′),2.02(m,4H),2.57(t.2H, H1′),5.35(m,2H,CH=CH)
	^{13}C	112.5,115.3,120.7,129.3,144.9,155.5	14.1(C15′),22.7(C14′),27.2,27.2,29.0,29.2,29.3,29.4,29.7,31.3,31. 8,35.8(C1′),129.6,130.0
	UV-(MeOH)	λ_{max} 275 nm	
	IR(cm^{-1})	3360,3005,2926,2855,1593,1457,1265,1154,778,694	
G-3 (Peak 3)	^{1}H	4.81(s,1H),6.66(m,2H),6.76(d,1H),7.15(t,1H)	0.90(t,3H,H17′),1.32(m,14H),1.60(m,2H,H2′),2.06(m,4H),2.56(t,2H, H1′),2.78(t,2H,=CH-CH$_2$-CH=),5.37(m,4H,CH=CH)
	^{13}C	112.3,115.3,121.0,129.4,144.9,155.5	14.1(C17′),22.6(C16′),25.7,27.2,29.2,29.3,29.4,29.7,29.7,31.3,31. 6,35.8(C1′),128.0,128.0,130.1,130.2
	UV-(MeOH)	λ_{max} 275 nm	
	IR(cm^{-1})	3338,2925,2856,1684,1581,1456,1406,1268,1151,776,695	
G-4 (Peak 4)	^{1}H	5.38(s,1H),6.68(m,2H),6.77(d,1H),7.15(t,1H)	0.91(t,3H,H15′),1.30(m,24H),1.61(m,2H,H2′),2.57(t,2H,H1′)
	^{13}C	112.5,115.3,120.8,129.3,144.9,155.6	14.1(C15′),22.7(C14′),29.3,29.3,29.5,29.6,29.7,30.9,31.3,31.9,35.8(C1′)
	UV-(MeOH)	λ_{max} 275 nm	
	IR (cm^{-1})	3328,2925,2855,1593,1458,1265,1154,777,695	
G-5 (Peak 5)	^{1}H	4.76(s,1H),6.65(m,2H),6.76(d,1H),7.15(t,1H)	0.89(t,3H,H17′),1.29(m,20H),1.60(m,2H,H2′),2.02(m,4H),2.56(t.2H, H1′),5.36(m,2H,CH=CH)
	^{13}C	112.4,115.3,120.9,129.3,144.9,155.4	14.1(C17′),22.7(C16′),27.2,27.2,29.0,29.3,29.5,29.5,29.7,29.7,29. 8,31.3,31.8,35.8(C1′),129.9,129.9
	UV-(MeOH)	λ_{max} 275 nm	
	IR (cm^{-1})	3307,2925,2855,1710,1596,1456,1266,1153,738,697	
G-6 (Peak 5)	^{1}H	4.74(s,1H),6.65(m,2H),6.76(d,1H),7.15(t,1H)	0.91(t,3H,H17′),1.31(m,20H),1.60(m,2H,H2′),2.02(m,4H),2.56(t.2H, H1′),5.36(m,2H,CH=CH)
	^{13}C	112.5,115.3,120.9,129.3,145.0,155.4	14.0(C17′),22.3(C16′),26.9,27.2,29.3,29.5,29.5,29.6,29.6,29.6,29. 7,29.8,31.3,32.0,35.8(C1′),129.8,129.9
	UV-(MeOH)	λ_{max} 275 nm	
	IR (cm^{-1})	3306,2925,2855,1594,1458,1266,1154,940,778,696	

[a]NMR values in ppm relative to TM

The length and the degree of unsaturation of the alkyl chains were deduced by the data of ^{1}H-NMR, ^{13}C-NMR and molecular ion peak in the MS. In **Figures 4A-4F**, the molecular ion peaks of G-1, G-2, G-3, G-4, G-5 and G-6 (m/z 276, 302, 328, 304, 330 and 330) corresponded to ginkgols with the alkyl chains of C13:0, C15:1, C17:2, C15:0, C17:1 and C17:1, respectively. G-5 and G-6 were analyzed with the same mass spectra. It was assumed that the two compounds correspond with isomers with the double bond at different positions or as cis-trans isomerism.

In order to confirm the exact positions of the double bonds of G-2 (C15:1), G-5 (C17:1) and G-6 (C17:1). The double bonds of them were oxidatively decomposed with $KMnO_4$ and $NaIO_4$, the products were methylated with $(CH_3OH)_2BF_3$ and their methyl esters products were analyzed by GC-MS **(Figure 5).** The position of the double bonds in the alkyl chain was deduced from the molecular ion peak of the methyl esters of oxidation products [20]. For example, the NMR signal of G-5 at δ_H 5.36 (2H), δ_C 129.9 (2C) ppm showed a double bond in the alkyl chain. The position of the double bond was determined to be Δ10-C17:1 from the molecular ion peak at m/z 278 in the MS of the methyl esters product **(Figure 5B).** The alkenyl carbons were observed at δ_C 27.2 ppm, thus the double bond was concluded to be *cis* [21]. Therefore, G-5 was identified as 3-[(10Z)-heptadecenyl] phenol. The double bonds of G-2 and G-6 were determined to be Δ8-C15:1 and Δ12-C17:1 according to the molecular ion peaks (m/z 250 and 306) in the MS **(Figures 5A and 5C).** Hence G-2 was identified as 3-[(8Z)-pentadecenyl] phenol and G-6 was identified as 3-[(12Z)-heptadecenyl] phenol [19-22].

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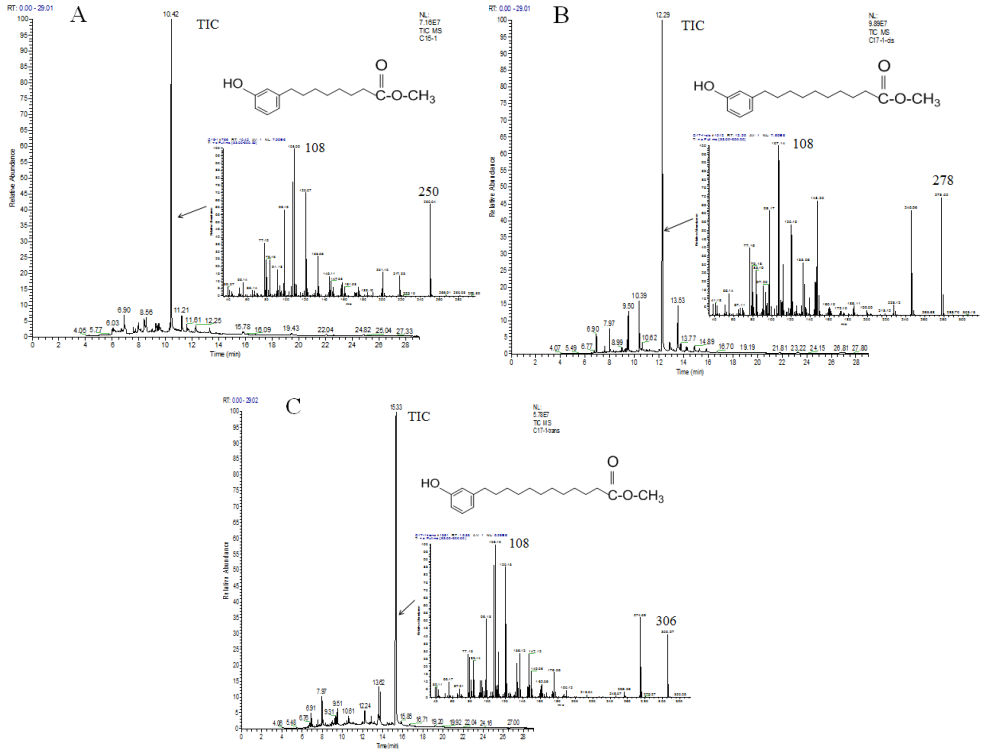

Figure 5. GC-MS chromatogram and EI mass spectra of the methyl esters of G-2 (A), G-5 (B) and G-6 (C).

The spectra data of G-1, G-3 and G-4 were consistent with the previous reports [19-22]. These compounds were identified as 3-tridecyl phenol, 3-[(9Z, 12Z)-heptadecadieny] phenol and 3-pentadecyl phenol, respectively. And their structural formulas were listed in **Figure 6.**

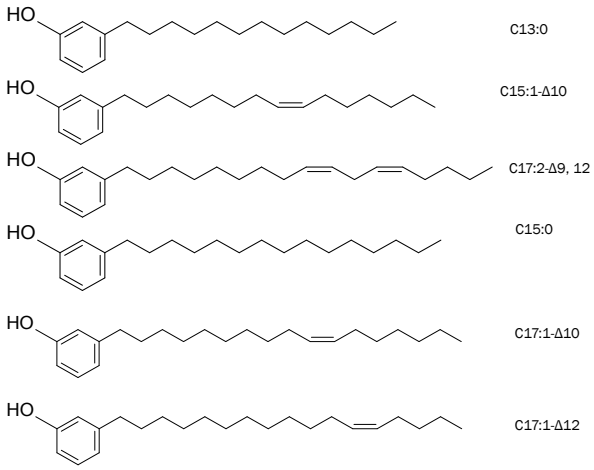

Figure 6. Structures of the six ginkgol monomers.

Among the six compounds, 3-tridecyl phenol and 3-pentadecyl phenol were white powder, 3-[(8Z)-pentadecenyl] phenol, 3-[(9Z, 12Z)-heptadecadieny] phenol, and 3-[(12Z)-heptadecenyl] phenol were light yellow oil.

Antitumor activity of ginkgol monomers

The inhibition effects of these six ginkgol monomers on cancerous HepG2, HGC and SW480 cells were tested by MTT method. All of the tested cells were treated with the six monomers at the concentration of 5, 10, 20, 30 and 40 μg/mL for 48 h, respectively. Results showed that all the ginkgol monomers had significant growth-inhibition effects against HepG2 and HGC cells, but exhibited a growth stimulating effect on SW480 cells with the negative inhibition rates **(Figure 7).** It was important to note that different cancer cells vary in their sensitivity to bioactive compounds. The IC_{50} values of the six monomers (C13:0, C15:1-Δ8, C17:2-Δ9, 12, C15:0, C17:1-Δ10, C17:1-Δ12) against HepG2 and HGC cells were in sequence **(Table 2)**: 43.28 and 37.98 μg/mL, 5.69 and 3.97 μg/mL, 19.95 and 11.09 μg/mL, 38.94 and 29.73 μg/mL, 19.79 and 10.22 μg/mL, 22.76 and 11.4 μg/mL. The HGC cell was more sensitive to all the ginkgol monomers with lower IC_{50} values than HepG2 cell. It seemed that ginkgols with unsaturated alkyl side chains showed higher toxicity against HepG2 and HGC cells than those with saturated side chains. In the four ginkgol monomers with unsaturated side chains, ginkgol C15:1 showed the highest antitumor activity. Similar work was reported by Lee et al. [23] who reported that cardanol C15:1 showed more potent inhibition activities than cardanol C17:1 on A549,

MCF-7 cells. Ginkgol C17:1-Δ10 performed higher activities than ginkgol C17:1-Δ12 on HGC and HepG2 cell. However, ginkgol C17:2-Δ9, 12 with two double bonds did not exhibit stronger cytotoxicity than ginkgol C17:1 with only one double bond. Besides, our previous study showed that ginkgol C17:1 mixture consisting of two isomers showed stronger inhibition effects than ginkgol C15:1 monomer on A549, U251 and SMMC7721 cells [17]. It was assumed that: ginkgol isomers C17:1-Δ10 and C17:1-Δ12 had synergistic effect in the ginkgol C17:1 mixture.

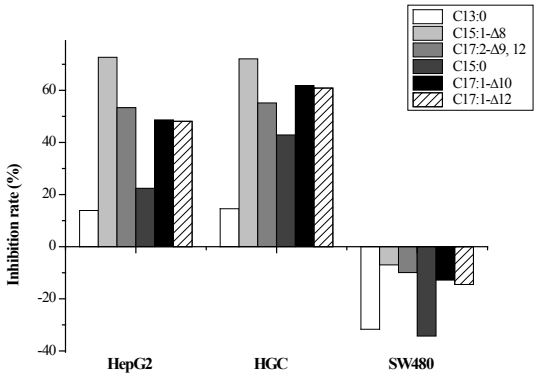

Figure 7. Inhibitive effects of ginkgol C13:0, C15:1-Δ8, C17:2-Δ9, 12, C15:0, C17:1-Δ10 and C17:1-Δ12 on HepG2, HGC and SW480 cell lines at concentration of 20 μg/mL.

Table 2. Inhibition effects of the ginkgol monomers on the HepG2 and HGC cells.

Cell lines	48h-IC_{50} (μg/mL)					
	C13:0	C15:1-Δ8	C17:2-Δ9, 12	C15:0	C17:1-Δ10	C17:1-Δ12
HepG2	43.28 ± 0.83	5.69 ± 0.78	19.95 ± 0.95	38.94 ± 0.79	19.79 ± 0.99	22.76 ± 0.99
HGC	37.98 ± 0.89	3.97 ± 0.88	11.09 ± 0.90	29.73 ± 0.98	10.22 ± 0.34	11.4 ± 0.48

EXPERIMENTAL SECTION

Materials and chemicals

Sunlight-dried *Ginkgo biloba* L. sarcotestas, collected in the Jiangsu province, China in October, 2012, were identified by the Pharmacognosy Laboratory, School of Pharmaceuticals. Voucher specimens were deposited in the Pharmacognosy Laboratory, School of Pharmaceutical, Jiangsu University, China.

Methanol (HPLC grade) was purchased from China National Medicines Co., Ltd. Wahaha purified water (Hangzhou Wahaha Group Co., Ltd.) was used in the HPLC experiments. The other chemicals used in this study were all analytical grade unless specified. The DMEM and fetal bovine serum were purchased from Gibco. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and $(CH_3OH)_2BF_3$-14% (batch number, 101624954) were purchased from Sigma.

General analysis

UV spectra were obtained on a UV-2450 spectrometer (Shimadzu, Japan) using methanol as the solvent. FT-IR spectra were recorded on a Nicolet Nexus 470 FT-IR spectrometer (Thermo Electron Corp., USA) using dichloromethane as the diluting agent.

An Agilent 1260 HPLC system consisting of a G1311A pump and a G1314b VWD detector (Agilent Corp., USA) was used to perform all experiments. Agilent Chemstation software B.02 was used for data collection and processing. The analysis was performed on a SHIMADZU Shimp-pack VP-ODSC18 column (250 mm × 4.6 mm, 5 μm). The mobile phase used was methanol and water (90:10, v/v) at a flow of 1.0 mL/min. The ginkgols in the eluent were detected at 275 nm.

GC-MS analysis was performed using an ITQ1100 Selective Detector coupled to a TRACE GC Ultra (Thermo Scientific, USA) fitted with a TR-5MS capillary column. Helium was used as the carrier gas with a linear pressure of 0.38 MPa. Sample aliquots of 1μL were injected. The oven temperature program was as follows: initial temperature at 100°C for 2 min and then ramped by 20°C/min to 240°C, ending in 20 min at 240°C. The injector temperature was maintained at 250°C. EI spectra were acquired between 25 and 550 Da.

NMR spectra were collected on a Bruker DRX500 NMR spectrometer in $CDCl_3$ operating at 400 MHz for ^1H analysis and at 100 MHz for ^{13}C analysis, using tetramethylsilane (TMS) as an internal standard.

Preparation and isolation of ginkgol monomers

Preparative isolations of ginkgolic acids from Ginkgo leaves or sarcotestas have been described by many researchers [16-18,22]. It was further decided to roughly follow the procedure described by Yang et al. [17]. Decarboxylation of the ginkgolic acids was conducted as described by Paramashivappa et al. [16]. The entire isolation and purification procedure was schematically presented in **Figure 1.** In brief, dried *Ginkgo biloba* sarcotesta was extracted to give petroleum ether extracts, the petroleum ether extract was separated by silica gel column chromatography eluting with petroleum ether-EtO_2-HCO_2H (89:11:1, v/v/v) to get ginkgolic acids fraction. The ginkgolic acids

were mixed with calcium hydroxide at 140 °C for 2 h. The mixture was extracted with petroleum ether (60-90 °C) and concentrated to give brown oil. The brown oil was further purified to yield ginkgols.

Separations of ginkgol monomers were performed with two WK500LC-500P HPLC pumps in combination with a SPD-500 UV detector and a Marathon XT manual injector (Xu Yu Technology Co., Ltd. Hangzhou, China) equipped with a 1 mL loop. The separation took place on Welch Ultimate AQ-C_{18} HPLC column (250 mm × 21.2 mm, 10 μm) and elution by mobile phase consisting with MeOH-H_2O (86:14, v/v) with the flow rate at 24.0 mL/min, the ginkgol monomers were monitored by UV detector at 280 nm.

Cleavage of olefinic chains of ginkols by $KMnO_4$ and $NaIO_4$

The positions of double bonds were measured by oxidative degradation [20]. Ginkgol monomer (1 mg) was dissolved in 1 mL of ter-BuOH, and then 1 mL of 5 mM $KMnO_4$/20 mM $NaIO_4$ and 1 mL of 4 mM Na_2CO_3 were added. The reaction mixture was shaken for 1 h at 27°C. The product was methylated with $(CH_3OH)_2BF_3$-14% and the methyl esters product was identified by GC-MS (column, TR-5MS). The position of the last double bond in the chain was deduced from the methyl esters product.

Cancer cell lines and culture

The cancer cell lines of HepG2 (hepatocellular carcinoma), HGC (gastric cancer) and SW480 (colon cancer) were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were adapted in a DMEM medium containing 10% fetal bovine serum, 100 unit/mL penicillin and 125 mg/L streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Antitumor activity assay

The antitumor activity in vitro of ginkgols was assayed by MTT method. 5 × 10^4/mL cell suspension was made with HepG2, HGC and SW480 cells in logarithmic growth phase, respectively. Then the cells suspension was inoculated into a 96-well culture plate with 100 μL in each well for 24 h. 100 μL of the six ginkgols were respectively added. At the same time, control well was set, and there were 6 parallel holes in each group. After another 24 h culture, 20 μL MTT solution (5 mg/mL) was added to each well and incubated for another 4 h. Then the growth medium was removed, and 100 μL DMSO was added into each well. The culture plate was fully oscillated. After the reaction stopped, absorbance value at 570 nm was detected by enzyme-labeling instrument (Bio-Rad, USA). The inhibition rate of cell growth was calculated according to the following formula:

Statistical analysis

The data were presented as the means ± SD. A statistical analysis was evaluated with Student's test by the SPSS 16.0 software.

CONCLUSION

A straightforward one-step preparative separation procedure by preparative RP-HPLC was proposed and six ginkgol monomers were isolated from ginkgol homologues and identified. Two ginkgol isomers C17:1-Δ10 and C17:1-Δ12 were first separated and identified from the ginkgol C17:1 mixture. Results of antitumor activities showed that ginkgol monomers with unsaturated side chains performed higher activities than those with saturated side chains. Ginkgol C15:1 had the strongest inhibitory effect in all monomers. For the ginkgol C17:1 isomers, double bond at Δ10 performed higher antitumor activities than that at Δ12. Besides, ginkgol C17:2 showed similar antitumor activities with ginkgol C17:1.

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