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Synthesis and antiproliferative activities of polymethoxyflavones aminoalkyl and amino acid derivatives

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Abstract: Twelve novel aminoalkyl derivatives **3a-3f**, **4a-4f** and four novel amino acid derivatives **5a**, **5b**, **6a** and **6b** of polymethoxyflavones **1** and **2** were synthesized through regioselective demethylation, etherification, amination, EDCl-mediated amide condensation and alkaline hydrolysis, using tangeretin and nobiletin as starting materials. Their antiproliferative activities against four different human cancer cell lines (Aspc-1, SUN5, HepG-2 and HCT116) were evaluated by *in vitro* CCK-8 assay. The results show that the majority of the synthetic compounds exhibited moderate to good antiproliferative activity. In particular, the antiproliferative activity of compound **5b** against HepG-2 cells (IC_{50} 0.057 μM) was equal to the positive control drug Staurosporine (IC_{50} 0.0575 μM).

Keywords: polymethoxyflavones, aminoalkylated derivatives, amino acid derivatives, synthesis, antiproliferative activity.

Introduction

Polymethoxyflavonids (PMFs) are a class of natural products, which widely exist in Citrus plants possessing high anticancer activity [1-5], however, their bioavailability is low. The biological interest to develop structural analogs of anticancer agents possessing basic nitrogen atoms seemed highly desirable. It is reported that the introduction of an aminoalkyl side chain leads to a significant increase in biological activity and potency of the parent molecule [6-8]. The amino acid derivatives, such as tricin-amino acid derivatives, demonstrate favorable cell permeability and excellent bioavailability [9]. Quercetin-amino acid derivatives are safe cancer multidrug resistance (MDR) modulators [10,11]. The results of these studies suggested that flavonoid conjugation with aminoalkyl groups or amino acids was the most promising approach to enhance its bioactivity and improve bioavailability.

As part of our screening program dedicated to the search for derivatives of natural flavonoids with anticancer properties[12-15], we herein synthesized two series of PMFs derivatives by attaching aminoakyl or amino acid moiety through a linking spacer to the free hydroxyl groups at the C-5 position. Furthermore, their antiproliferative activities *in vitro* against four different human cancer cell lines including Aspc-1 (human pancreatic cancer), SUN-5 (human gastric cancer), HepG-2 (human hepatocellular carcinoma) and HCT-116 (human colon cancer) were evaluated by CCK-8 (cell counting kit-8) assay using Staurosporine as the positive control drug.

Result and discussion

The synthesis of novel PMF derivatives **3a**-**3f, 4a**-**4f**, **5a, 5b, 6a** and **6b** was performed according to the reaction pathways illustrated in Scheme 1. The inexpensive and easily accessible natural products tangeretin and nobiletin were used as the starting materials. Due to the neighboring-group participation of the C-4 carboxyl oxygen, tangeretin or nobiletin undergo regioselective demethylation of the C-5 methoxy group after treatment with a Lewis acids such as boron trichloride or aluminum chloride to give 5-demethyl tangeretin (**1**) or 5-demethyl nobiletin (**2**), respectively. We obtained compounds **1** and **2** with high yields of 93% and 90% respectively. PMFs **1** or **2** reacted with 1,2-diobromoethane in the presence of anhydrous K_2CO_3 in dry acetone to give compounds **7** or **8**. The two latter reacted with various amines in the presence of K_2CO_3

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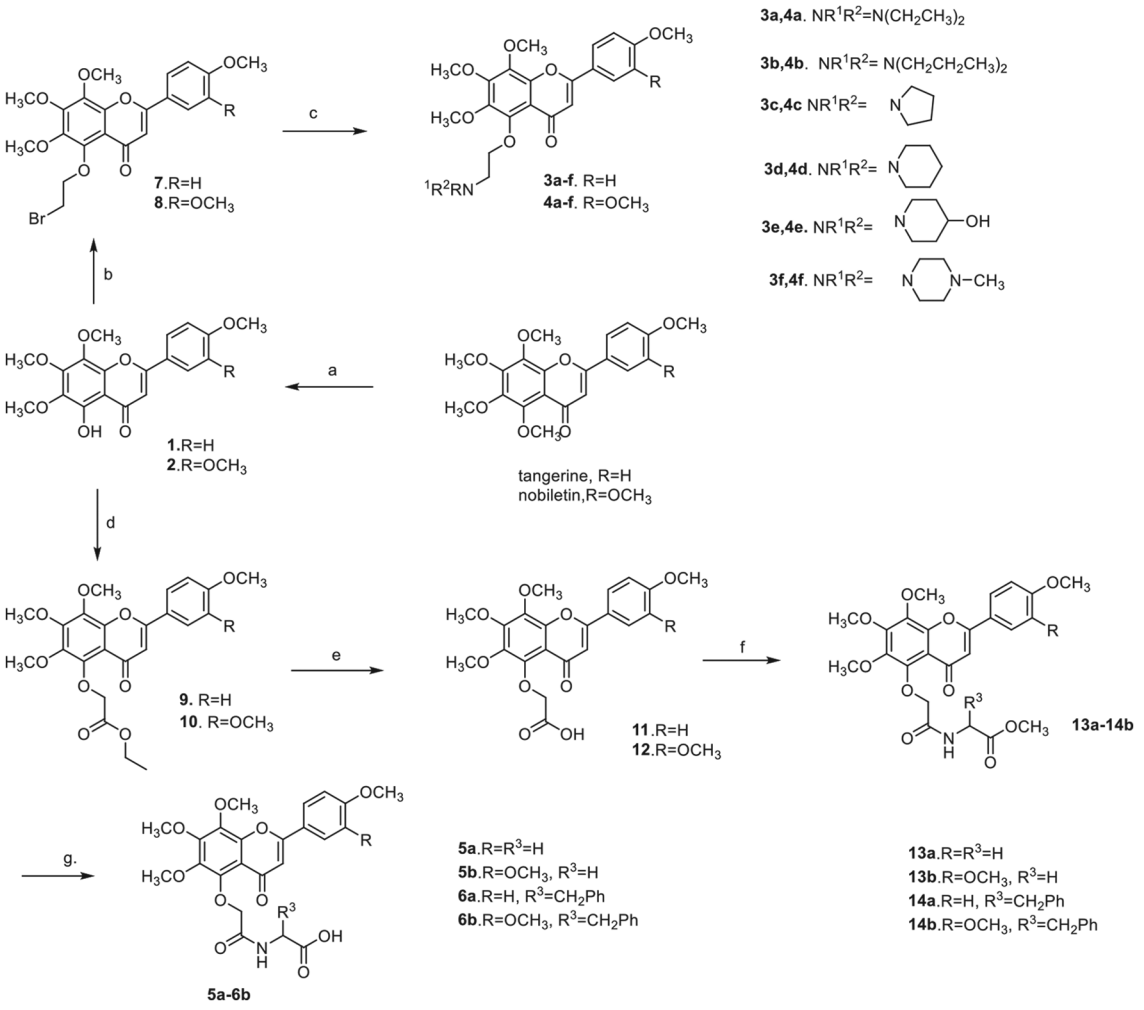

Scheme 1 Synthetic route of aminoalkyl and amino derivatives of polymethoxyflavones
Reagents and conditions: a. $AlCl_3$, $CH_3CO_2C_2H_5$, reflux, then 5% HCl(aq), reflux; b.$BrCH_2CH_2Br$, K_2CO_3, DMF, heat; c. HNR^1R^2, K_2CO_3, CH_3CN, reflux; d. $ClCH_2CO_2C_2H_5$, K_2CO_3, DMF, heat; e. 10% NaOH (aq.), CH_3OH, r.t; f. $H_2NCH(R^3)CO_2CH_3$, EDCl, DMAP, CH_2Cl_2, r.t; g. 10% NaOH (aq), CH_3OH, r.t, then 1 M HCl(aq), r.t.

and KI, to give compounds **3a**-**3f** and **4a**-**4f**. These compounds are extending alkoxyl side chain at the 5-position, and introducing amine hydrogen bond receptor at the end of the side chain. Compounds **9** and **10** were usually prepared from etherification of the 5-OH group by using methyl chloroacetate in the presence of a base. Two key intermediates 5-*O*-carboxyalkylated polymethoxy flavones **11** and **12** were synthesized by basic hydrolysis of **9** and **10** respectively.

Two amino acids, glycine (Gly) and phenylalanine (Phe) were attached to the 5-position of the PMFs ring *via* a non-hydrolysable linker. 5-*O*-carboxyalkylated PMFs **11** or **12** were condensed with glycine methyl ester hydrochloride or phenylalanine methyl ester hydrochloride, respectively, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCl), *N*-hydroxybenzotriazole (HOBt) and 4-dimethylaminopyridine (DMAP) as efficient coupling reagents. The corresponding PMF glycine or phenylyamine derivatives **5a**, **5b**, **6a** and 6**b** were obtained by hydrolysis of **13a**, **13b**, **14a** and **14b**. All the new compounds were purified by recrystallization or chromatography, and their structures were confirmed by means of ^{1}HNMR, ^{13}CNMR, and MS spectra.

The antiproliferative activities of the synthesized polymethoxyflavones **1** and **2** and their aminoalkyl and amino acid derivatives **3a**-**3f**, **4a**-**4f**, **5a, 5b, 6a** and **6b** against four different human cancer cell lines (Aspc-1, SUN-5, HepG-2 and HCT-116) were evaluated by CCK-8 method. Based on this procedure, a dose-response curve was obtained for the four cell lines. Each test compound and the concentration that caused 50% cell growth inhibition (IC_{50}, corresponding to the concentration of compound that inhibition 50% of net cell growth) was determined as described elsewhere.

As shown in Table 1, the majority of the synthetic compounds exhibited moderate to potent antiproliferative

Table 1 Antiproliferative activities [IC_{50}(μM)][a] of synthetic compounds on four human cancer lines

Compound	Aspc-I	SNU5	HepG2	HCT116
1	>50	>50	>50	>50
2	>50	>50	>50	>50
3a	>50	>50	33.48±2.53	26.58±7.67
3b	23.01±3.61	>50	11.51±4.62	12.68±2.59
3c	49.71±2.10	>50	33.08±8.41	29.89±5.11
3d	45.60±3.20	34.97±6.60	26.36±5.34	17.90±3.39
3e	>50	>50	38.21±3.50	>50
3f	>50	>50	32.62±7.34	31.04±1.56
4a	>50	>50	>50	39.03±2.72
4b	43.24±5.62	>50	16.63±1.72	18.24±5.86
4c	>50	>50	>50	>50
4d	42.59±2.43	>50	39.57±2.22	34.62±6.05
4e	>50	>50	>50	>50
4f	>50	>50	>50	>50
5a	>50	>50	>50	>50
5b	>50	>50	0.057±4.9E-05	>50
6a	>50	>50	>50	>50
6b	>50	>50	>50	>50
Staurosporine	0.0052±1.21E-06	0.0208±2.5E-07	0.0575±2.03E-05	0.02±1.0E-06

[a]Data are the mean ± S.D. of three independent experiments performed.

activity. This shows that the introduction of aminoalkyl or amino acid moiety at 5-*O*-position of PMFs **1** or **2** produces the desired effect of increasing the antiproliferation activities. In particular, compound **5b** clearly demonstrated the best results (IC_{50} 0.057 μM) in the HepG-2 cell line studied. In fact, by comparing the structures **2** and **5b**, it can be concluded that the presence of phenylalanine amide moiety on 5-position of 5-demethyl nobiletin was associated with a remarkable increase in the growth inhibitory effect on HepG-2 cells. The benzyl function group between amido and carboxyl structure has more affinity to biological cells, so the biological activity is better than Gly derivative. It is suggested that the importance of this molecular modification is responsible for the activity when compared with the corresponding polymethoxy flavones. The clarification of the structural determinants for the potency would guide the design of novel potent drug molecules for future development.

Conclusion

Twelve novel aminoalkyl derivatives **3a-3f**, **4a-4f** and four novel amino acid derivatives **5a**, **5b**, **6a** and **6b** of polymethoxyflavones were synthesized. Their antiproliferative activities *in vitro* results show that the majority of them exhibited moderate to good antiproliferative activity against four different human cancer cell lines (Aspc-1, SUN5, HepG-2 and HCT116). Among them, PMFs amino acid derivative **5b** against HepG-2 cells (IC_{50} 0.057 μM) was equal to the positive control drug Staurosporine ((IC_{50} 0.0575 μM) deserving further investigation.

Experimental

Melting points were determined *via* an XRC-1 apparatus and are uncorrected. ^{1}HNMR and ^{13}CNMR spectra were recorded on a Bruker-AV 400 spectrometer at 400 and 100MHz, respectively, with TMS as an internal standard in $CDCl_3$ or DMSO-d_6. Mass spectra (MS) and high-resolution mass spectra (HRMS) were determined on a VG Autospec-3000 or Mat 95 XP Spectrometer by the EI or ESI method. Column chromatography was carried out on silica gel 200-300 mesh (Qingdao Ocean Chemical Products of China). Commercially available AR or chemically pure reagents were used, and anhydrous solvents were dried and redistilled using standard experimental procedures.

5-Demethyl tangeretin (**1**) and 5-demethyl nobiletin (**2**) were prepared form tangeretin and noliletin according to previously published procedures [16].

General procedure for synthesis of bromoethanated polymethoxyflavones (7 and 8)

1,2-Dibromoethane (11.5 mmol) was added to a solution of PMFs **1** or **2** (1.4 mmol) and K_2CO_3 (14.5 mmol) in acetone (50 mL), and the mixture was refluxed for 4h. The progress of the reaction was monitored by TLC. After cooling

to room temperature, the mixture was filtered, the solvent was evaporated, and the residue was recrystallized from EtOH to yield compound **7** or **8**.

5-(2-Bromoethoxy)-4',6,7,8-tetramethoxyflavone (7) yellow solid; 76% yield; m.p. 122-124 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, J = 8.3 Hz, 2H, 2'-H and 6'-H), 6.94 (d, J = 8.3 Hz, 2H, 3'-H and 5'-H), 6.49 (s, 1H, 3-H), 4.27 (t, J = 6.5 Hz, 2H, 5-OCH_2), 4.03, 3.95 , 3.88, 3.80 (s/each, 12H, 4OCH_3), 3.70 (t, J = 6.5 Hz, 2H, CH_2Br). ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.24, 162.36, 161.37, 151.41, 147.77, 146.45, 144.19, 138.4, 127.74, 123.66, 114.80, 114.53, 106.56, 74.44, 62.12, 61.69, 55.52, 30.13.

5-(2-Bromoethoxy)-3',4',6,7,8-pentamethoxyflavone (8) yellow solid; 79% yield; m.p.116-118 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.50 (d, J = 8.4 Hz, 1H, 6'-H), 7.34 (s, 1H, 2'-H), 6.93 (d, J = 8.4 Hz, 1H, 5'-H), 6.53 (s, 1H, 3-H), 4.28 (t, J = 6.5 Hz, 2H, 3-OCH_2), 4.04, 3.96, 3.90 (s/each, 15H, 5OCH_3), 3.71 (t, J = 6.5 Hz, 2H, CH_2Br). ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.27, 161.26, 152.02, 151.48, 149.31, 147.77, 146.52, 144.23, 138.41, 123.89, 119.71, 114.79, 111.25, 108.57, 106.79, 74.47, 62.11, 61.73, 56.05, 30.08.

General procedure for synthesis of aminalkyl-substitued polymethoxyflavone derivatives 3a-3f and 4a-4f

To a suspension of compounds **7** or **8** (0.43 mmol) and K_2CO_3 (7.2 mmol) in acetonitrile (20.0 mL), the amine (14.5 mmol) and a catalytic amount of KI (0.07 mmol) were added. The resulting mixture was refluxed for 6-12h. After filtering, the resulting filtrate was evaporated to dryness under reduced pressure. The residue was suspended in water (10.0 mL) and extracted with dichloromethane (3×30 mL). The combined organic layers were evaporated under reduced pressure, and the residue was recrystallized from EtOH to yield compounds **3a-3f** or **4a-4f.**

5-(2-Diethylaminoethoxy)-4',6,7,8-tetramethoxyflavone (3a) yellow solid; 80% yield; m.p. 110-112 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, J = 7.8 Hz, 2H, 2'-H and 6'-H), 6.95 (d, J = 7.8 Hz, 2H, 3'-H and 5'-H), 6.50 (s, 1H, 3-H), 4.13 (t, J = 5.7 Hz, 2H, 5-OCH_2), 4.03, 3.95, 3.87, 3.81 (s/each, 12H, 4OCH_3), 3.08 (t, J = 5.7 Hz, 2H, CH_2), 2.72 (q, J = 6.8 Hz, 4H, 2NCH_2), 1.06 (t, J = 6.9 Hz, 6H, 2CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.49, 162.37, 161.43, 151.52, 147.74, 146.96, 144.13, 138.22, 127.74, 123.62, 114.6, 106.48, 71.89, 61.98, 61.65, 55.52, 52.20, 47.67, 11.29. ESI- MS: m/z 458 $[M+H]^+$.calc:458.21.

5-(2-Dipropylaminoethoxy)-4',6,7,8-tetramethoxyflavone (3b): yellow solid; 77% yield; m.p. 88-90 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.77 (d, J = 8.1 Hz, 2H, 2'-H and 6'-H), 6.91 (d, J = 8.2 Hz, 2H, 3'-H and 5'-H), 6.48 (s, 1H, 3-H), 4.06 (t, J = 6.7 Hz, 2H, 5-OCH_2), 4.00, 3.93, 3.85, 3.77 (s/each, 12H, 4OCH_3) , 2.95 (t, J = 6.7 Hz, 2H, CH_2), 2.42-2.27 (m, 4H, 2NCH_2), 1.46-1.36 (m, 4H, 2CH_2), 0.78 (t, J = 7.2 Hz, 6H, 2CH_3). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.21, 162.21, 161.02, 151.29, 147.58, 144.05, 137.90, 127.61, 123.72, 114.93, 114.44, 106.52, 73.20, 61.89, 61.58, 56.77, 55.44, 53.71, 20.41, 11.89. ESI-MS: m/z 486$[M+H]^+$. calc:486.24.

5-(2-Pyrrolidinylethoxy)-4',6,7,8-tetramethoxyflavone (3c) yellow solid; 87% yield; m.p. 98-100 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.87 (d, J = 8.0 Hz, 2H, 2'-H and 6'-H), 7.02 (d, J = 8.1 Hz, 2H, 3'-H and 5'-H), 6.57 (s, 1H, 3-H), 4.20 (t, J = 6.0 Hz, 2H, 5-OCH_2), 4.10, 4.03, 3.95, 3.89 (s/each, 12H, 4OCH_3), 3.07 (t, J = 6.0 Hz, 2H, CH_2), 2.72-2.63 (m, 4H, 2NCH_2), 1.83-1.75 (m, 4H, 2CH_2). ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.25, 162.26, 161.12, 151.35, 147.70, 147.37, 144.17, 138.06, 127.68, 123.79, 114.99, 114.49, 106.58, 73.43, 61.94, 61.61, 55.55, 54.38, 23.55. ESI-MS: m/z 478 $[M+Na]^+$. calc:478.19.

5-(2-Piperidinylethoxy)-4',6,7,8-tetramethoxyflavone (3d) yellow solid; 86% yield; m.p. 106-108 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J = 8.0 Hz, 2H, 2'-H and 6'-H), 6.93 (d, J = 8.0 Hz, 2H, 3'-H and 5'-H), 6.49 (s, 1H, 3-H), 4.10 (t, J = 6.0 Hz, 2H, 5-OCH_2), 4.01, 3.94, 3.86, 3.79 (s/each, 12H, 4OCH_3), 2.82 (t, J = 5.9 Hz, 2H, CH_2), 2.47-2.41 (m, 4H, 2NCH_2), 1.59-1.46 (m, 4H, 2CH_2), 1.36-1.29 (m, 2H, CH_2). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.12, 162.20, 161.05, 151.24, 147.66, 147.27, 144.04, 138.04, 127.59, 123.63, 114.99, 114.42, 106.45, 71.60, 66.95, 61.97, 61.84, 61.56, 58.45, 55.41, 53.78. ESI-MS: m/z 508 $[M+K]^+$. calc:508.21.

5-(2-(4-Hydroxypiperidinyl)ethoxy)-4',6,7,8-tetramethoxyflavone (3e) yellow solid; 77% yield; m.p. 108-110 °C; ^{1}H NMR (400 MHz, $CDCl_3$):δ 7.77 (d, J = 7.7 Hz, 2H, 2'-H and 6'-H), 6.92 (d, J = 7.8 Hz, 2H, 3'-H and 5'-H), 6.48 (s, 1H, 3-H), 4.09 (t, J = 5.2 Hz, 2H, 5-OCH_2), 4.01, 3.93, 3.85 , 3.78 (s/each, 12H, 4OCH_3), 3.65-3.78 (m, 1H, CH), 2.96-2.84 (m, 4H, 2NCH_2), 2.27(t, J = 5.2 Hz, 2H, NCH_2), 1.85-1.76 (m, 2H, CH_2), 1.62-1.53 (m, 2H, CH_2). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.36, 162.25, 161.22, 151.35, 147.69, 147.24, 144.07, 138.01, 127.68, 123.60, 114.84, 114.45, 106.43, 72.12, 67.43, 61.96, 61.62, 57.71, 55.47, 51.31, 34.29, 31.53, 22.61, 14.11. ESI-MS: m/z 508 $[M+Na]^+$. calc:508.20.

5-(2-(1-Methylhexahydropyrazinyl)ethoxy)-4',6,7,8-tetramethoxyflavone (3f) yellow solid; 86% yield; m.p. 104-106 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J = 8.5 Hz, 2H, 2'-H and 6'-H), 6.93 (d, J = 8.5 Hz, 2H, 3'-H and 5'-H), 6.49 (s, 1H, 3-H), 4.09 (t, J = 5.7 Hz, 2H, 5-OCH_2), 4.01, 3.94, 3.86, 3.79 (s/each, 12H, 4OCH_3), 2.84 (t, J = 5.7 Hz, 2H, CH_2), 2.58-2.52 (m, 4H, 2NCH_2), 2.41-2.32 (m, 4H, 2CH_2), 2.20 (s, 3H, CH_3). ^{13}C NMR (101MHz, $CDCl_3$): δ 176.1, 161.22, 160.06, 150.27, 146.69, 146.40, 143.10, 137.02, 126.64, 122.76, 114.04, 113.46, 105.55, 71.08, 60.95, 60.61,

56.92, 54.46, 54.13, 52.32, 45.07. ESI-MS: m/z 507 $[M+Na]^+$. calc:507.22.

5-(2-Diethylaminoethoxy)-3',4',6,7,8-pentamethoxyflavone(4a) yellow solid; 78% yield; m.p. 142-144 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48 (d, J = 8.3 Hz, 1H, 6'-H), 7.33 (s, 1H, 2'-H), 6.91 (d, J = 8.3 Hz, 1H, 5'-H), 6.51 (s, 1H, 3-H), 4.06 (t, J = 6.3 Hz, 2H, 5-OCH_2), 4.02, 3.95, 3.91-3.86 (s/each, 15H, 5OCH_3), 2.98 (t, J = 6.3 Hz, 2H, CH_2), 2.62 (q, J =6.7 Hz, 4H, 2NCH_2), 1.01 (t, J = 6.9 Hz, 6H, 2CH_3). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.28, 160.97, 151.86, 151.40, 149.20, 147.68, 147.42, 144.09, 137.91, 123.90, 119.58, 114.89, 111.18, 108.44, 106.71, 72.87, 61.90, 61.63, 55.98, 52.37, 47.49, 11.75. ESI-MS: m/z 488 $[M+H]^+$. calc:488.22.

5-(2-(Dipropylaminoethoxy)-3',4',6,7,8-pentamethoxyflavone (4b) yellow solid; 75% yield; m.p. 160-162 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (d, J = 8.2 Hz, 1H, 6'-H), 7.31 (s, 1H, 2'-H), 6.90 (d, J = 8.2 Hz, 1H, 5'-H), 6.50 (s, 1H, 3-H), 4.02 (t, J = 6.1 Hz, 2H, 5-OCH_2), 4.02, 3.94, 3.86 (s/each, 15H, 5OCH_3), 2.94 (t, J = 6.1 Hz, 2H, CH_2), 2.41-2.32 (m, 4H, 2NCH_2), 1.40-1.34 (m, 4H, 2CH_2), 0.78 (t, J = 6.9 Hz, 6H, 2CH_3). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.16, 160.82, 151.78, 151.31, 149.15, 147.64, 147.54, 144.04, 137.79, 123.90, 119.51, 114.90, 111.13, 108.36, 106.70, 73.22, 61.89, 61.80, 61.59, 56.74, 56.00, 55.84, 53.64, 20.39, 11.89. ESI-MS: m/z 516 $[M+H]^+$. calc: 516.25.

5-(2-(Pyrrolidinylethoxy)-3',4',6,7,8-pentamethoxyflavone (4c) yellow solid; 89% yield; m.p. 124-126 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, J = 8.3 Hz, 1H, 6'-H), 7.32 (s, 1H, 2'-H), 6.91 (d, J = 8.4 Hz, 1H, 5'-H), 6.50 (s, 1H, 3-H), 4.13 (t, J = 5.4 Hz, 2H, 5-OCH_2), 4.02, 3.95, 3.88 (s/each, 15H, 5OCH_3), 3.01 (t, J = 5.4 Hz, 2H, CH_2), 2.67-2.59 (m, 4H, 2NCH_2), 1.76-1.71 (m, 4H, 2CH_2). ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.24, 160.97, 151.87, 151.39, 149.21, 147.33, 144.15, 137.97, 123.91, 119.58, 114.89, 111.19, 108.47, 106.71, 78.98, 73.30, 67.76 , 64.67, 54.35, 23.51. ESI-MS: m/z 508 $[M+Na]^+$. calc:508.20.

5-(2-(Piperidinylethoxy)-3',4',6,7,8-pentamethoxyflavone (4d) yellow solid; 88% yield; m.p. 148-150 °C;^{1}H NMR (400 MHz, $CDCl_3$): δ 7.49 (d, J = 7.3 Hz, 1H, 6'-H), 7.33 (s, 1H, 2'-H), 6.92 (d, J = 8.1 Hz, 1H, 5'-H), 6.51 (s, 1H, 3-H), 4.15 (t, J = 5.1 Hz, 2H, 5-OCH_2), 4.02,3.95, 3.91,3.86 (s/each, 15H, 5OCH_3), 2.95 (t, J = 5.1 Hz, 2H, CH_2), 2.64-2.58 (m, 4H, 2NCH_2), 1.60-1.53 (m, 4H, 2CH_2), 1.42-1.38 (m, 2H, CH_2). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.37, 161.13, 151.93, 151.47, 149.23, 147.70, 147.19, 144.11, 138.04, 123.86, 119.65, 114.81, 111.20, 108.48, 106.71, 71.75, 61.94, 61.65, 58.36, 56.01, 54.56, 25.47, 23.89. ESI-MS: m/z 500 $[M+H]^+$. calc:500.22.

5-(2-(4-Hydroxypiperidinyl) ethoxy)-3',4',6,7,8-pentamethoxyflavone (4e) yellow solid; 74% yield; m.p. 195-197 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47 (d, J = 8.2 Hz, 1H, 6'-H), 7.32 (s, 1H, 2'-H), 6.90 (d, J = 8.4 Hz, 1H, 5'-H), 6.50 (s, 1H, 3-H), 4.09 (t, J = 5.3 Hz, 2H, 5-OCH_2), 4.02, 3.94, 3.87 (s/each, 15H, 5OCH_3), 3.65-3.61 (m, 1H, CH), 2.90-2.83 (m, 4H, 2NCH_2), 2.27 (t, J = 5.3 Hz, 2H, NCH_2), 1.85-1.76 (m, 2H, CH_2), 1.57-1.51 (m, 2H, CH_2). ^{13}C NMR (101MHz, $CDCl_3$): δ 177.31, 161.07, 151.89, 151.40, 149.20, 147.67, 147.29, 144.10, 137.95, 123.84, 119.63, 114.84, 111.20, 108.48, 106.65, 72.14, 67.38, 61.91, 61.62, 57.73, 55.98, 51.28, 34.30. ESI-MS: m/z 516 $[M+H]^+$. calc:516.22.

5-(2-(1-Methylhexahydropyrazinyl)ethoxy)-3',4',6,7,8-pentamethoxyflavone (4f) yellow solid; 85% yield; m.p. 138-140 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48 (d, J = 8.4 Hz, 1H, 6'-H), 7.32 (s, 1H, 2'-H), 6.91 (d, J = 8.4 Hz, 1H, 5'-H), 6.50 (s, 1H, 3-H), 4.09 (t, J = 5.3 Hz, 2H, 5-OCH_2), 4.02, 3.95, 3.88 (s/each, 15H, 5OCH_3), 2.83 (t, J = 5.3 Hz, 2H, CH_2), 2.57-2.49 (m, 4H, 2NCH_2), 2.41-2.34 (m, 4H, 2CH_2), 2.20 (s, 3H, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.16, 160.90, 151.84, 151.31, 149.20, 147.66, 147.42, 144.11, 137.93, 123.93, 119.55, 115.00, 111.17, 108.44, 106.73, 72.04, 61.91, 61.62, 57.92, 55.96, 55.12, 53.32, 46.07. ESI-MS: m/z 537 $[M+Na]^+$. calc: 537.23.

General procedure for synthesis of carboxylic acid derivatives of polymethoxyflavonoids 11 and 12

A mixture of **1** or **2** (1.3 mmol) and potassium carbonate (1.6 mmol) were dissolved in 40 ml DMF and added into a 100 ml three-necked round-bottomed flask. The reaction mixture was heated to 40 °C, then ethyl chloroacetate (9.07 mmol) was added dropwise into the mixture. The reaction mixture was stirred for 6h at 40 °C and then cooled to room temperature. A total of 500 ml of cold water was added to the mixture. The solid that separated was filtered, washed with water and dried to obtain 0.48 g of compound **9** or **10**.

A mixture of **9** or **10** (1.13 mmol) was dissolved in 40 ml methanol and added into a 100 ml three-necked round-bottomed flask equipped and stirred at room temperature. 10 mL of 10% NaOH aqueous solution was added dropwise into the mixture. The reaction mixture was stirred for 2h at room temperature and then was concentrated under reduced pressure to obtain a white liquid. The pH of the mixture was adjusted to be between 2 and 3 using 6 mol/L hydrochloric acid, The mixture was extracted with dichloromethane, the organic layer was dried over anhydrous magnesium sulfate, and concentrated under reduced pressure to obtain white powder, which was recrystallized with ethanol and filtered to obtain 0.38 g compound **11** or **12**.

5-(*O*-Ethoxyacetyl)-4',6,7,8-tetramethoxyflavone (9) yellow solid; 80% yield; m.p.110-112 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J = 8.0 Hz, 2H, 2'-H and 6'-H), 6.94 (d, J = 8.0 Hz, 2H, 3'-H and 5'-H), 6.50 (s, 1H, 3-H), 4.61 (s, 2H, 3-OCH_2), 4.22 (q, J = 6.9 Hz, 2H, CH_2), 4.02, 3.95, 3.89,

3.80 (4s, 12H, 4OCH$_3$), 1.25 (t, J = 7.0 Hz, 3H, CH$_3$) ; ^{13}C NMR (101MHz, CDCl$_3$): δ175.99, 168.00, 161.29, 160.27, 150.28, 146.58, 145.02, 142.87, 137.51, 126.68, 122.63, 113.71, 113.48, 105.53, 70.08, 61.11, 61.03, 60.63, 59.95, 54.47, 13.21.

5-(*O*-Ethoxyacetyl)-3',4',6,7,8-pentamethoxyflavone (10) yellow solid; 78% yield; m.p.112-114 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.47 (d, J = 8.4 Hz, 1H, 6'-H), 7.31 (s, 1H, 2'-H), 6.91 (d, J = 8.4 Hz, 1H, 5'-H), 6.51 (s, 1H, 3-H), 4.61 (s, 2H, 5-OCH$_2$), 4.22 (q, J = 6.9 Hz, 2H, CH$_2$), 4.02, 3.95 (2s, 6H, 2OCH$_3$), 3.89(s, 6H, 2OCH$_3$), 3.87 (s, H, OCH$_3$), 1.24 (t, J = 7.0 Hz, 3H, CH$_3$); ^{13}C NMR (101MHz, CDCl$_3$): δ 175.95, 167.97, 160.11, 150.93, 150.33, 148.23, 146.56, 145.07, 142.88, 137.44, 122.83, 118.62, 113.69, 110.21, 107.49, 105.72, 70.09, 61.10, 60.95, 60.64, 59.95, 55.06, 54.92, 13.21.

5-(*O*-Carboxymethyl)-4',6,7,8-tetramethoxyflavone (11) yellow solid; 76% yield; m.p.138-140 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ 13.93 (s, 1H, COOH), 7.83 (d, J = 8.1 Hz, 2H, 2'-H and 6'-H), 6.98 (d, J = 8.2 Hz, 2H, 3'-H and 5'-H), 6.63 (s, 1H, 3-H), 4.75 (s, 2H, 5-OCH$_2$), 4.07, 3.97 (2s, 6H, 2OCH$_3$), 3.83 (s, 6H, 2OCH$_3$); ^{13}C NMR (101 MHz, CDCl$_3$): δ 177.75, 169.79, 162.07, 161.92, 151.38, 146.65, 145.38, 141.69, 137.27, 127.07, 121.91, 113.72, 111.09, 105.04, 71.23, 61.21, 60.72, 60.36, 54.57. ESI-MS: m/z 455[M+K]$^+$. calc: 455.11.

5-(*O*-Carboxymethyl)-3',4',6,7,8-pentamethoxyflavone (12) yellow solid; 76% yield; m.p. 212-214 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ 7.53 (d, J = 8.4 Hz, 1H, 6'-H), 7.35 (s, 1H, 2'-H), 6.95 (d, J = 8.5 Hz, 1H, 5'-H), 6.66 (s, 1H, 3-H), 4.76 (s, 2H, 5-OCH$_2$), 4.07, 3.97 (2s, 6H, 2OCH$_3$), 3.91 (s, 6H, 2OCH$_3$), 3.83 (s, H, OCH$_3$); ^{13}C NMR (101 MHz, CDCl$_3$): δ 178.78, 170.80, 162.98, 152.67, 152.47, 149.48, 147.68, 146.44, 142.76, 138.24, 123.12, 120.20, 112.12, 111.36, 108.67, 106.29, 72.26, 62.16, 61.76, 61.41, 56.18, 56.03. ESI-MS: m/z 485[M+K]$^+$. calc:485.12.

General procedure for synthesis of amino acid ester derivatives of polymethoxyflavonoids 13a, 13b, 14a and 14b

A mixture of **11** or **12** (0.48 mmol), EDCl (0.72 mmol), 4-*N*,*N*-dimethylaminopyridine (DMAP, 0.96 mmol) and glycine methyl ester hydrochloride or *L*-phenylalanine methyl ester hydrochloride (0.58 mmol) were dissolved in 20 mL dichloromethane, and the reaction was carried out at room temperature. The progress of the reaction was followed by TLC. After the reaction was completed, the solvent was removed, and the residue was poured into 50 mL of HCl (1 mol/L), and extracted with CH_2Cl_2 (3×20 mL). The organic phases were combined and dried over anhydrous Na_2SO_4. After filtration, the solvent was evaporated to give a crude material. Purification by silica gel column chromatography [*V* (petroleum ether): *V* (ethyl acetate) = 2:1] afforded compounds **13a, 13b, 14a** and **14b**.

5-(*O*-Acetylglycine methyl ester)-4',6,7,8-tetramethoxyflavone (13a) yellow solid; 90% yield; m.p.164-166 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ 10.02 (s, 1H, CONH), 7.81 (d, J = 8.2 Hz, 2H, 2′-H and 6′-H), 6.96 (d, J = 8.3 Hz, 2H, 3′-H and 5′-H), 6.56 (s, 1H, 3-H), 4.74 (s, 2H, 5-OCH$_2$), 4.13 (d, J = 5.8 Hz, 2H, CH$_2$CO), 4.05, 3.95, 3.82, 3.81 (4s, 12H, 4OCH$_3$), 3.68 (s, 3H, CH$_3$). ^{13}C NMR (101MHz, CDCl$_3$): δ 178.12, 170.22, 170.07, 162.60, 161.88, 152.05, 147.90, 146.84, 142.29, 137.79, 127.85, 123.37, 114.64, 112.93, 106.54, 73.65, 62.15, 61.68, 61.45, 55.55, 52.15, 40.81.

5-(*O*-Acetylglycine methyl ester)-3',4',6,7,8-pentamethoxyflavone (13b) yellow solid; 87% yield; m.p.140-142 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ 10.02 (s, 1H, CONH), 7.48 (d, J = 8.4 Hz, 1H, 6'-H), 7.31 (s, 1H, 2'-H), 6.91 (d, J = 8.5 Hz, 1H, 5'-H), 6.55 (s, 1H, 3-H), 4.72 (s, 2H, 5-OCH$_2$), 4.11 (d, J = 5.7 Hz, 2H, CH$_2$CO), 4.04, 3.94 (2s, 6H, 2OCH$_3$), 3.87 (s, 6H, 2OCH$_3$), 3.80 (s, 3H, OCH$_3$), 3.67 (s, 3H, CH$_3$). ^{13}C NMR (101MHz, CDCl$_3$): δ 177.04, 169.19, 169.06, 160.69, 151.25, 151.05, 148.32, 146.83, 145.75, 141.25, 136.67, 122.41, 118.81, 111.80, 110.30, 107.52, 105.60, 72.58, 61.03, 60.65, 60.41, 55.08, 54.92, 51.12, 39.76.

5-(*O*-Acetylphenylalanine methyl)-4',6,7,8-tetramethoxyflavone (14a) yellow solid; 87% yield; m.p.104-106 °C; ^{1}H NMR (400 MHz, CDCl$_3$): δ 9.94 (d, J = 7.8 Hz, 1H, CONH), 7.68 (d, J = 8.6 Hz, 2H, 2'-H and 6'-H), 7.17-6.97 (m, 5H, C$_6$H$_5$), 6.80 (d, J = 8.6 Hz, 2H, 3'-H and 5'-H), 6.46 (s, 1H, 3-H), 4.76-4.70 (m, 1H, CH), 4.56 (s, 2H, 5-OCH$_2$), 3.94, 3.85, 3.67, 3.66 (4s, 12H, 4OCH$_3$), 3.54 (s, 3H), 3.16 (d, J = 9.7 Hz, 2H, CH$_2$Ar). ^{13}C NMR (101 MHz, CDCl$_3$): δ 177.54, 172.12, 169.41, 162.44, 161.43, 151.75, 147.69, 146.60, 142.04, 137.66, 137.18, 129.20, 128.23, 127.65, 126.55, 123.12, 114.50, 112.82, 106.44, 73.50, 61.99, 61.52, 61.28, 55.42, 53.76, 52.05, 37.63.

5-(*O*-Acetylphenylalanine methyl)-3',4',6,7,8-pentamethoxyflavone (14b) yellow solid; 84% yield; m.p.110-112 °C;^{1}H NMR (400 MHz, CDCl$_3$): δ 9.92 (d, J = 7.9 Hz, 1H, CONH), 7.52 (d, J = 8.4 Hz, 1H, 6'-H), 7.34 (s, 1H, 2'-H), 7.22-7.06 (m, 5H, C$_6$H$_5$), 6.93 (d, J = 8.5 Hz, 1H, 5'-H), 6.59 (s, 1H, 3-H), 4.83-4.77 (m, 1H, CH), 4.64 (s, 2H, 5-OCH$_2$), 4.03, 3.95, 3.91, 3.89, 3.76 (5s, 15H, 5OCH$_3$), 3.61 (s, 3H, CH$_3$), 3.21 (d, J = 16.2 Hz, 2H, CH$_2$Ar). ^{13}C NMR (101 MHz, CDCl$_3$): δ 176.76, 171.21, 168.36, 160.46, 151.20, 150.95, 148.35, 146.81, 145.86, 141.19, 136.66, 136.14, 128.25, 127.26, 125.56, 122.58, 118.77, 111.96, 110.29, 107.54, 105.81, 72.56, 61.03, 60.64, 60.37, 55.10, 54.98, 52.69, 51.13, 36.81.

Synthesis of polymethylflavones amino acid derivatives 5a, 5b, 6a and 6b

The solid of **13** or **14** (0.21 mmol) was dissolved in 10 mL methanol, in a 100 mL round bottom flask, and 4 mL of

NaOH (1 mol/L) solution were added dropwise. The reaction was carried out at room temperature and the reaction progress was tracked by TLC. After the reaction was complete, the solvent of the reaction solution was removed and water was added followed by HCl (1 mol/L) to adjust the pH to 2-3, The mixture was then placed in a refrigerator and allowed to stand for filtration. The filter cake was dried and recrystallized from anhydrous C_2H_5OH to give a yellow solid.

5-(*O*-Acetylglycine)-4',6,7,8-tetramethoxyflavone (5a) yellow solid; 89% yield; m.p. 180-182 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.69 (t, J = 5.6 Hz, 1H, CONH), 7.94 (d, J = 8.5 Hz, 2H, 2'-H and 6'-H), 7.07 (d, J = 8.5 Hz, 2H, 3'-H and 5'-H), 6.79 (s, 1H, 3-H), 4.62 (s, 2H, 5-OCH_2), 4.06 (s, 3H, OCH_3), 3.98 (d, J = 8.6 Hz, 2H, CH_2COOH), 3.96, 3.86, 3.83 (3s, 9H, 3OCH_3). ^{13}C NMR (101MHz, DMSO-d_6): δ 177.30, 171.45, 169.27, 162.58, 161.37, 151.85, 147.60, 146.18, 142.57, 137.89, 128.25, 123.12, 115.04, 112.95, 106.36, 73.53, 62.33, 61.86, 61.77, 55.92, 40.85; ESI-MS: m/z 473 $[M]^+$. calc:473.13.

5-(*O*-Acetylglycine)-3',4',6,7,8-tetramethoxyflavone (5b) yellow solid; 85% yield; m.p.110-112 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.71 (t, J = 5.7 Hz, 1H, CONH), 7.63 (d, J = 8.5 Hz, 1H, 6'-H), 7.51 (s, 1H, 2'-H), 7.13 (d, J = 8.6 Hz, 1H, 5'-H), 6.94 (s, 1H, 3-H), 4.63 (s, 2H, 5-OCH_2), 4.07, 3.98 (2s, 6H, 2OCH_3), 3.96 (d, J = 5.8 Hz, 2H, CH_2COOH), 3.87, 3.86, 3.84 (3s, 9H, 3OCH_3). ^{13}C NMR (101MHz, DMSO-d_6) δ 177.41, 171.40, 169.28, 161.35, 152.41, 151.90, 149.45, 147.64, 146.22, 142.59, 137.84, 123.20, 119.95, 112.95, 112.24, 109.30, 106.62, 73.55, 62.32, 61.90, 61.78, 56.15, 56.09, 40.81. ESI-MS:m/z 503 $[M]^+$. calc:503.14.

5-(*O*-Acetylphenylalanine)-4',6,7,8-tetramethoxyflavone (6a) yellow solid; 82% yield; m.p.106-108 oC; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.74 (d, J = 8.0 Hz, 1H, CONH), 8.04 (d, J = 8.4 Hz, 2H, 2′-H and 6′-H), 7.35-7.16 (m, 5H,C_6H_5), 7.13 (d, J = 8.5 Hz, 2H, 3′-H and 5′-H), 6.93 (s, 1H, 3-H), 4.58 (d, J = 9.0 Hz, 2H, 5-OCH_2), 4.07, 3.99, 3.86, 3.80 (4s, 12H, 4OCH_3), 3.19 (d, J = 4.8 Hz, 2H, CH_2Ar). ^{13}C NMR (101 MHz, DMSO-d_6): δ 177.48, 173.19, 168.85, 162.67, 161.48, 151.94, 147.65, 146.29, 142.42, 138.11, 137.85, 129.52, 128.65, 128.41, 126.89, 123.20, 115.15, 112.84, 106.53, 73.52, 62.39, 61.91, 61.71, 55.99, 53.90, 37.24. ESI-MS: m/z 563 $[M]^+$., calc:563.18.

5-(*O*-Acetylphenylalanine)-3',4',6,7,8-pentamethoxyflavone (6b) yellow solid; 82% yield; m.p.106-108 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.74 (d, J = 8.0 Hz, 1H, CONH), 8.04 (d, J = 8.4 Hz, 2H, 2'-H and 6'-H), 7.35-7.16 (m, 5H C_6H_5), 7.13 (d, J = 8.5 Hz, 2H, 3'-H and 5'-H), 6.93 (s, 1H, 3-H), 4.58 (d, J = 9.0 Hz, 2H, 5-OCH_2), 4.07, 3.99, 3.86, 3.80 (4s, 12H, 4OCH_3), 3.19 (d, J = 4.8 Hz, 2H, CH_2Ar). ^{13}C NMR (101 MHz, DMSO-d_6): δ 177.48, 173.19, 168.85, 162.67, 161.48, 151.94, 147.65, 146.29, 142.42, 138.11, 137.85, 129.52, 128.65, 128.41, 126.89, 123.20, 115.15, 112.84, 106.53, 73.52, 62.39, 61.91, 61.71, 55.99, 53.90, 37.24. ESI-MS: m/z 593 $[M]^+$. calc:593.19.

Antiproliferative Activity

The antiproliferative activities of synthetic compounds against four human cancer cell lines (Aspc-1, SUN5, HepG-2 and HCT116) were evaluated by CCK-8 assay *in vitro* using Staurosporine as the positive control drug. All the cells were cultured in an RPMI 1640 medium containing 10% FBS, incubated at 37 °C in a 5% CO_2 humidified incubator to keep the cells growing in the exponential phase. Briefly, Aspc-1, SUN5, HepG-2 and HCT116 cells in a 100 μL culture medium were plated into a 96-well plate at 4000-5000 cells per well, respectively, and subsequently cultured in the RPMI 1640 medium containing 10% FBS, incubated at 37 °C for 24h prior to drug exposure. The selected compounds were weighed and dissolved in DMSO and then diluted with medium to the needed concentrations. Cells were treated with the final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 μmol/L of the tested compounds and incubated for 48h and then 20μL of 0.5% MTT solution was added to each well simultaneously and incubated at 37 °C for 4h. The formed formazan crystals were dissolved by adding 150 μL of DMSO. The optical density at 570 nm was determined on a microtiter plate reader. According to the inhibition ratios, the IC_{50} value was obtained.

Conflict of interest: The authors confirm that this article content has no conflict of interest.

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