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Bioassay guided discovery of apoptosis inducers from gamboge by high-speed counter-current chromatography and high-pressure liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry

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ABSTRACT

A screening system, composed of high-speed counter-current chromatography and high-pressure liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry, was established to find bioactive lead compound. This system succeeded in discovering apoptosis inducers from gamboge, the resin of *Garcinia hanburyi*. High-speed counter-current chromatography was used to provide well-separated fractions for bioassay and the resulted active fractions were rapidly identified using high-pressure liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry. The solvent system of *n*-hexane/ethyl acetate/methanol/water was optimized to the ratio of 7:3:7:3 (v/v/v/v) by a *K* value analysis. As a result, two active fractions were obtained. They showed apoptosis inducing effects as potent as that of taxol (500 nM) at the concentration of 1 μg/ml. Gambogenic acid (72.1%) and epimeric isogambogic acids (25.3%) were identified in one of the fractions. The other active fraction mainly contained two epimeric mixtures, gambogic acids (68.7%) and gambogoic acids (26.9%). Among them, gambogenic acid, without epimerization, has priority to be lead compound.

1. Introduction

Natural products (NP) have played an important role in drug discovery. At least 34 NP and NP-derived drugs have been launched onto the market in the United States, Europe and Japan during the past decade [1,2]. It is, however, not easy to find a drug candidate from thousands of natural materials, such as higher plants, marine organisms and microorganisms, since any of them may contain thousands of constituents. Bioassay guided fractionation therefore becomes increasingly popular in searching lead compounds. To separate an NP extract into many fractions and subfractions for bioassay, means of chromatography are used with a variety of packing material, including both normal- and reversed-phase silica gel, alumina, Celite, Amberlite resins and Sephadex LH-20, etc. [3–6]. However, these liquid-solid separation methods have several disadvantages and limitations: (i) irreversible sample adsorption; (ii) risk of sample denaturation; (iii) peak tailing; (iv) high consumption of solvents and columns/packing materials.

Compared to these conventional liquid-solid methods, the counter-current chromatography (CCC), without solid phases, shows significant advantages. This technique has rapidly further developed to the new generation, high-speed counter-current chromatography (HSCCC). With radical improvement in terms of resolution, separation time and sample loading capacity, HSCCC has been popularly and widely applied in separation and purification of natural products [7–9]. Undoubtedly, HSCCC has great potential in drug discovery by providing ideal fractionation of complex natural products, with 100% theoretical sample recovery. As a matter of fact, HSCCC has been successfully used as such a tool in several efforts to find bioactive compounds from herbal medicines [10–14].

However, all these approaches included a time-consuming step in the identification of target compounds. These compounds need to be isolated in high purity and yield before measuring their NMR spectra. This therefore greatly limited the efficiency of the HSCCC approach in NP drug discovery. On the other hand, liquid chromatography/mass spectroscopy has achieved great success in shortening the time line of quantitative and qualitative anal-

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ysis of natural products [15–17]. The combination of HSCCC and LC/MS should fully utilize the power of both techniques. In this paper, we will report a successful bioassay-guided discovery of apoptosis inducers from gamboge using a combined approach of high-speed counter-current chromatography and high-pressure liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry.

Gamboge, the resin of *Garcinia hanburyi*, has generated increasing research interest in phytochemical, pharmacological, synthetic, and biological communities in recent years. This is attributed to its major ingredient gambogic acid, an interesting polyprenylated xanthone which not only contained a unique caged skeleton of 4-oxa-tricyclo[4.3.1.0^{3,7}]dec-2-one scaffold, but also activated apoptosis in several types of cancer cell lines [18–21]. It has been tested in phase II clinical trial in China as a new anticancer drug candidate [22]. However, gambogic acid was recently separated into two epimers that showed different bioactivities [23,24], and this might cause problems in the quality control of new drug product and might even become an obstacle to drug licensing. As such, the quest for an alternative of gambogic acid from gamboge becomes more important than ever. In this study, gamboge extract was examined using the rapid screening system, resulting in the discovery of an apoptosis inducer similar to gambogic acid but without epimerization.

2. Experimental

2.1. Apparatus

The preparative HSCCC instrument used in this study was TBE-300A high-speed counter-current chromatography (Shanghai, Tauto Biotech, China) with three polytetrafluoroethylene preparative coils (diameter of tube, 2.6 mm, total volume, 260 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). A HX 1050 constant-temperature circulating instrument (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. The HSCCC system was equipped with a model S constant-flow pump, a 3725i-038 injector (Rheodyne, USA), the UV–vis G1365B photodiode array detector and Agilent HPLC workstation of a preparative Agilent 1100 HPLC Series.

The measurement of K values using HPLC analysis was carried out on an analytical Agilent 1100 series and Alltima-C_8 column (4.6 mm × 250 mm, 5 μm) at room temperature. HPLC/MS analyses were performed using a Waters ACQUITY UPLC^{TM} system (Waters Corp., MA, USA), equipped with a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) operating in positive ion mode and a Waters ACQUITY BEH C_8 column (100 mm × 2.1 mm, 1.7 μm, Waters Corp., Ireland).

2.2. Reagents and materials

Methanol, acetonitrile, *n*-hexane, ethyl acetate and acetic acid of HPLC grade were purchased from TEDIA company, Inc., USA. Distilled water was prepared using MILLI-Q SP reagent water system (Millipore, MA, USA). The gamboge material was purchased at the local market of Yunnan province, China. The sample was deposited in the Chinese Medicine Laboratory, Hong Kong Jockey Institute of Chinese Medicine.

2.3. Selection of two-phase solvent system

Targeting on five major unknown peaks which had gradually increasing retention time, the two-phase solvent system of *n*-hexane/ethyl acetate/methanol/water was optimized in solvent ratio by analyzing the K values. Approximately 2 mg of the crude extract was weighed in a 10 ml test tube to which 4.0 ml of each phase of the equilibrated two-phase solvent system was added. The tube was shaken vigorously for 2 min to equilibrate the sample thoroughly with the two phases. Then, the upper and lower phases were analyzed using an analytical Agilent 1100 series and Alltima-C_8 column (4.6 mm × 250 mm, 5 μm) at room temperature. Mobile phase: ACN/water (7:3, v/v); flow rate: 1.0 ml/min; UV wavelength: 360 nm. The partition coefficients (K) were expressed as the peak area of target components in the upper phase divided by that in the lower phase.

2.4. Preparation of two-phase solvent system and sample solution

The two-phase solvent system was prepared by adding the solvents to a separation funnel according to the volume ratios and fully equilibrated by shaking repeatedly at room temperature. The upper and lower phases were separated shortly before use and degassed by sonication for 30 min. 300 mg of gamboge was extracted with 20 ml chloroform. The chloroform solution was filtered through a 0.2 μm syringe membrane filter and then was concentrated to yield a yellow dry powder (190 mg). The powder was further dissolved in 10 ml lower phase and the sample solution was filtered through a 0.2 μm syringe membrane filter before HSCCC separation.

2.5. HSCCC separation procedure

The coil column was first entirely filled with the upper phase at the speed of 20 ml/min. Then, the apparatus was rotated at 800 rpm after pausing the pump. Five minutes later, the lower phase was pumped into the column at 1.5 ml/min in the head to tail mode. When the mobile phase front emerged and hydrodynamic equi-

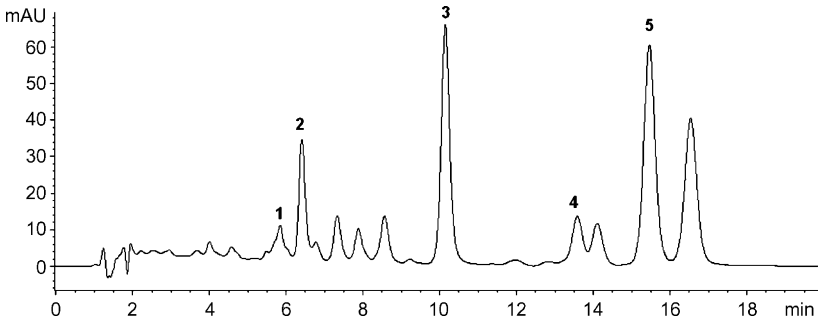

Fig. 1. HPLC chromatogram of gamboge extract. Column: Sunfire C_8 (2.1 mm × 150 mm, 3.5 μm); mobile phase: ACN–water (7:3, v/v); duration: 20 min; flow rate: 0.3 ml/min; UV wavelength: 360 nm.

Table 1
Partition coefficients (*K* values) of five target peaks measured in different solvent system of *n*-hexane-ethyl acetate-MeOH-water.

Volume ratio	Partition coefficients (*K* values, *n* = 3)				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
8:2:8:2	0.14 ± 0.03	0.36 ± 0.02	0.52 ± 0.04	0.57 ± 0.11	1.06 ± 0.06
8:2:7:3	0.42 ± 0.02	1.03 ± 0.05	2.16 ± 0.18	2.42 ± 0.20	4.25 ± 0.36
8:2:6:4	1.80 ± 0.07	3.67 ± 0.08	15.0 ± 0.42	14.71 ± 0.45	24.33 ± 0.54
7:3:8:2	0.16 ± 0.00	0.33 ± 0.01	0.59 ± 0.03	0.62 ± 0.03	1.12 ± 0.05
7:3:7:3	0.55 ± 0.02	1.13 ± 0.02	2.44 ± 0.05	2.51 ± 0.04	4.27 ± 0.04
7:3:6:4	2.32 ± 0.22	3.90 ± 0.26	13.97 ± 1.59	12.30 ± 1.22	23.72 ± 1.26
6:4:8:2	0.32 ± 0.02	0.53 ± 0.02	0.71 ± 0.07	0.72 ± 0.05	1.26 ± 0.12
6:4:7:3	0.81 ± 0.00	1.34 ± 0.01	2.64 ± 0.03	2.50 ± 0.05	4.34 ± 0.08
6:4:6:4	1.94 ± 0.07	5.19 ± 0.13	15.23 ± 0.18	14.46 ± 0.29	21.62 ± 0.42

librium was established in the column, 10 ml of sample solution containing 190 mg of crude extract was injected through the injector. When the separation time reached 600 min, the rotation was stopped and water was pumped into the column to push the solvent out at a speed of 10 ml/min. At 630 min, the whole operation was completed. The separation temperature was controlled at 20 °C. The effluent from the outlet of the column was continuously monitored at 360 nm by Agilent 1100 HPLC UV-vis detector and ChemStation and was collected by a Spectra/Chrom (USA) CF-1 collector (5 min/tube).

2.6. Bioassay of HSCCC fractions for their apoptotic effects

The bioassay method was described in our previous paper with some modifications [25]. All the testing samples were dissolved in DMSO to make stock solutions. The concentration of each stock was at least 1000 times higher than the working concentration. HeLa-C3 cells, which can detect apoptotic cell death involving caspase activation, were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, in a 5% CO_2 humidity incubator at 37 °C. The sample well for apoptotic activity testing was prepared by seeding a well on a 96-well plate with 7500 HeLa-C3 cells suspended in 100 μl culture medium. The background well was prepared by only adding 100 μl culture medium. After 12–16 h incubation, the plate was read by a PerkinElmer Victor plate reader with excitation wavelength at 440 ± 10 nm and emission wavelength at 486 ± 8 nm for CFP (cyan fluorescent protein) and 535 ± 8 nm for YFP (yellow fluorescent protein) to obtain the data for time point "0 h". Then immediately, the old medium was removed and 100 μl freshly prepared culture medium containing the testing sample at a certain working concentration was added to both the sample well and the corresponding background well. Culture medium containing 0.1% DMSO was the negative control while 500 nM taxol was the positive control. After that, the plate was read repeatedly at indicated time points. The data acquisition duration was up to 72 h. The YFP/CFP emission ratio was then calculated. If YFP/CFP emission ratio was reduced below 3, the testing sample was considered as a good apoptotic inducer at that concentration. All samples were tested in triplicate. The whole experiment was repeated for three times.

2.7. HPLC/ESI Q-TOF MS analysis of bioactive fractions

HPLC was performed using a Waters ACQUITY UPLC™ system with a Waters ACQUITY BEH C_8 column (100 mm × 2.1 mm, 1.7 μm, Waters Corp., Ireland). The mobile phase consisted of (A) water and (B) ACN containing 0.1% formic acid. The eluting conditions were optimized as follows: 0–0.5 min, 65% B; 0.5–1 min, linear gradient of B from 65% to 75%; 2–6 min, 75% B; 6–7 min, linear gradient of B from 75% to 95%, and 7–8 min, 65% B. The flow rate was 0.3 ml/min. The column and autosampler were maintained at 35 and 10 °C, respectively.

Mass spectrometry was performed on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) operating in positive ion mode. The nebulization gas was set to 650 l/h at 300 °C, the cone gas set to 50 l/h, the source temperature set to 90 °C. The capillary voltage and cone voltage were set to 2700 V and 45 V, respectively. The Q-TOF Premier acquisition rate was set to 0.3 s, with a 0.01 s inter-scan delay. Argon was employed as the collision gas at a pressure of 5.3×10^{-5} Torr. The MS/MS experiments were carried out by setting the Q-TOF Premier quardrupole to allow ions of interest to pass prior to fragmentation in the collision cell with collision energies varying between 10 and 40 eV.

All analyses were acquired using the LockSpray™ to ensure accuracy and reproducibility. Leucine–enkephalin was used as the lock mass (*m/z* 556.2771) in positive mode at a concentration of 50 pg/μl and an infusion flow rate of 10 μl/min. Data were centroided during acquisition, and dynamic range enhancement (DRE™) was applied throughout the MS experiment to ensure accurate mass measurement over a wider dynamic range.

3. Results and discussion

3.1. Selection of the two-phase solvent system

Different from its common application in sample preparation, HSCCC separation for bioassay-use fractions did not target on any specific compound prior to the blind screening. Gamboge extract was designed to be separated into well-proportioned fractions according to their polarity. So we supposed five target compounds (peaks 1–5) in different polarity regions as shown in Fig. 1. Thereby, the conventional method to optimize the sep-

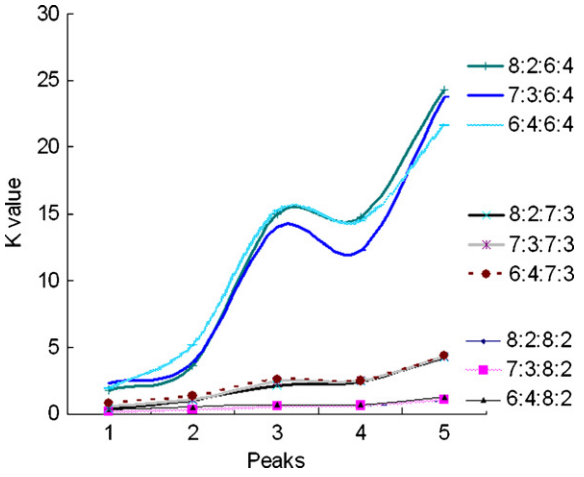

Fig. 2. Partition coefficients (*K* values) of five target peaks measured in different solvent system of *n*-hexane/ethyl acetate/MeOH/water. (For interpretation of the references to color in this artwork, the reader is referred to the web version of the article.)

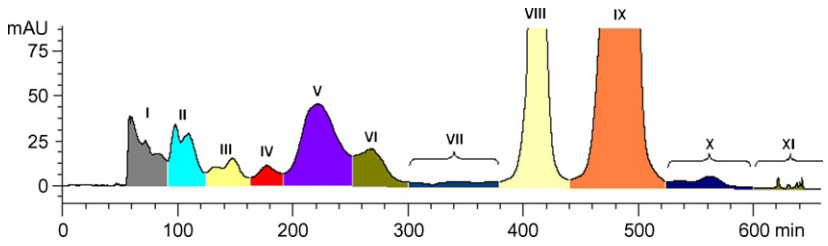

Fig. 3. HSCCC chromatogram of gamboge extract (190 mg). Two solvent system: *n*-hexane/ethyl acetate/MeOH/water (7:3:7:3); stationary phase: upper phase; mobile phase: lower phase; flow rate: 1.5 ml/min; UV wavelength: 360 nm. Fraction I, 17.26 mg, fr. II, 5.48 mg, fr. III, 3.81 mg; fr.3.56 mg, fr. V, 7.66 mg, fr. VI, 3.21 mg, fr. VII, 8.21 mg, fr. VIII, 35.25 mg, fr. IX, 76.78 mg, fr. X, 6.04 mg, fr. XI, 4.34 mg, sample recovery 90.3%.

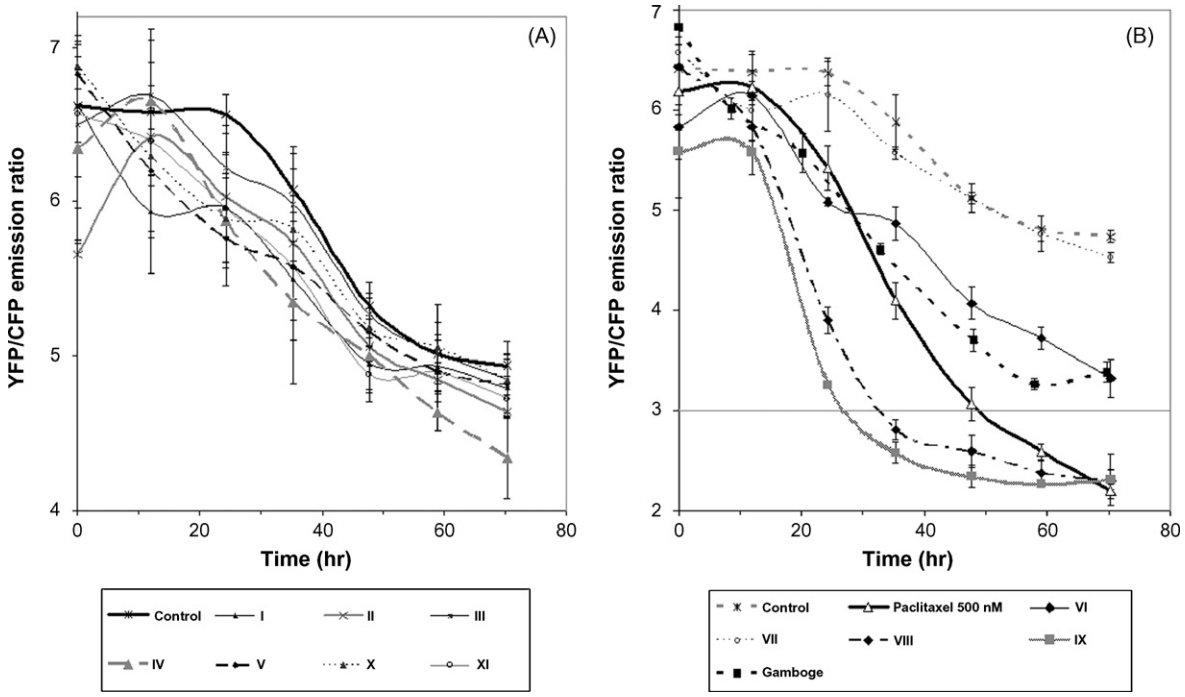

Fig. 4. Y/C emission ratio changes after treatment of 11 HSCCC fractions I–XI and gamboge extract (1 μg/ml). Taxol was used as a positive control (500 nM).

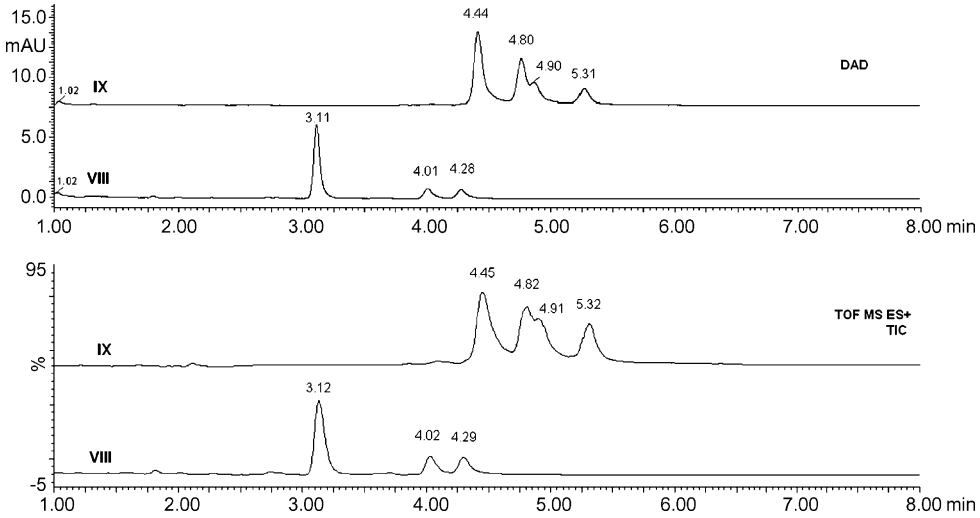

Fig. 5. HPLC–DAD–MS chromatograms of HSCCC fractions VIII and IX, obtained by a Waters ACQUITY UPLC™ system with a Waters ACQUITY BEH C_8 column (100 mm × 2.1 mm, 1.7 μm, Waters Corp., Ireland). The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid. The eluting conditions were optimized as follows: isocratic at 65% B (0–0.5 min), linear gradient from 65% to 75% B (0.5–1 min), isocratic at 75% B (2–6 min), linear gradient from 75% to 95% B (6–7 min), and isocratic at 65% B (7–8 min). The flow rate was 0.3 ml/min.

aration solvent system is still applicable here. In our previous recycling HSCCC separation of gambogic acid epimers [24], we used *n*-hexane/methanol/water (5:4:1, v/v/v) as the two-phase solvent system. Whereas in the current experiment, the commonly used *n*-hexane/ethyl acetate/methanol/water was selected in order to suit more constituents of gamboge. To obtain more accurate results, every *K* value was measured in triplicate and expressed as mean ± SD. The results produced by nine ratios are shown in Table 1. In common operation of HSCCC sample preparation, the *K* value of target compound is always expected to be around 0.5–1.0 [7], and the solvent system is accordingly optimized to generate the expected *K* value. This strategy was modified in our approach for bioassay use. First, the *K* values of different target compounds should increase gradually and significantly so that the crude extract could be well-proportioned. Second, the *K* value should not exceed 5, otherwise the whole separation will be very time-consuming. As displayed in Fig. 2, the *K* values seem to depend on the ratio of methanol/water. On one hand, three solvent systems with methanol/water (8:2) provided highly variable but very large *K* values. On the other hand, those with methanol/water (6:4) generated much smaller *K* values in a narrow range, therefore the target compounds would be eluted out too fast and not well separated. Finally, those three methanol/water (7:3) systems gave suitable *K* values and one of them, *n*-hexane/thyl acetate/methanol/water (7:3:7:3), was selected due to its faster equilibration and equal volume of two-phase solvents.

3.2. HSCCC fractionation and bioassay

The gamboge extract was initially tested for its apoptotic effect using HeLa-C3 cell-based apoptotic detection system. In this system, a stable HeLa cell line (HeLa-C3) expressing a fluorescence resonance energy transfer (FRET)-based biosensor protein CFP-DEVD-YFP was used [25,26]. This engineered protein consisted of

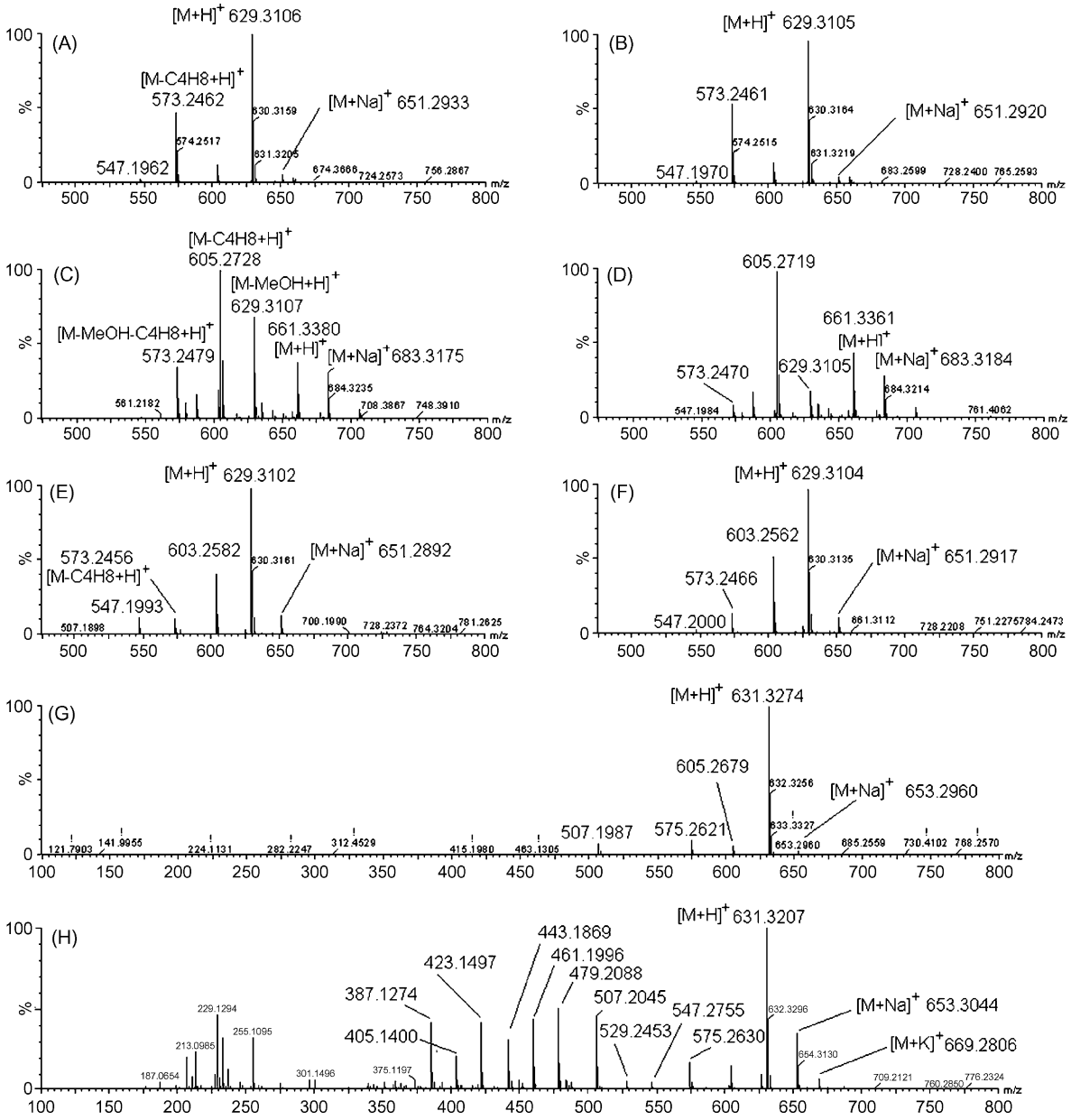

Fig. 6. HRESIMS spectra of ingredients of fractions VIII and IX measured on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, UK) operating in positive ion mode. (A) peak at 4.44 min; (B) peak at 4.80 min; (C) peak at 4.90 min; (D) peak at 5.31 min; (E) peak at 3.11 min; (F) peak at 4.01 min; (G) peak at 4.28 min; (H) peak at 4.28 min performed with CID.

three parts: a donor cyan fluorescent protein (CFP), a peptide linker containing the caspase-3 cleavage site (DEVD), and an acceptor yellow fluorescent protein (YFP). The detection was based on the FRET effects. Before caspase was activated, energy could be transferred directly from the donor (CFP) to the acceptor (YFP), so that when the donor was excited, fluorescence emission from the acceptor could be detected. When caspase-3 was activated during apoptosis, it cleaved the biosensor (CFP-DEVD-YFP) and caused the separation of donor and acceptor proteins. Thus, the FRET effect was effectively eliminated, resulting in a reduced emission from YFP and an increased emission from CFP. By measuring the fluorescence emission ratio between YFP and CFP, the activation of caspase-3 in living cells during apoptosis could be detected [26]. According to our previous studies using known anti-cancer drugs such as taxol, any stimulus that could reduce the fluorescence emission ratio of YFP/CFP to a value lower than 3.0, was considered as a good apoptotic inducer [26].

The gamboge extract showed potent apoptosis inducing effect in a similar trend as taxol in the bioassay. Its apoptosis inducing effect displayed a drug concentration dependent relationship. When the drug concentration was below 2 μg/ml, the effect was much reduced and the Y/C ratio was near but still above 3 about 70 h after drug treatment. The gamboge extract was further separated using the HSCCC method. The lower phase of n-hexane/ethyl acetate/methanol/water (7:3:7:3, v/v/v/v) was used as the eluting solvent at a flow rate of 1.5 ml/min. As a result, the gamboge extract was well fractioned as expected after a separation of more than 600 min. As illustrated in Fig. 3, 11 fractions were obtained according to the HSCCC chromatogram, with a good sample recovery of 90.3%. Among them, fractions VIII (35.25 mg) and IX (76.78 mg) were two major parts, possessing 65% of the total elute. The sample loss was mainly caused by the concentration of collected fractions in vacuum at 55 °C since these caged xanthones were a little volatile.

Around 2 mg of sample was accurately weighed from each fraction and was tested for its apoptosis inducing effect at the concentration of 1 μg/ml. The results (Fig. 4) showed that only fractions VIII and IX had stronger activities than gamboge extract. Both fractions were even stronger than the positive control (taxol, 500 nM). They produced YFP/CFP ratios lower than 3 after about 36 h compound treatment while that of taxol (500 nM) was still above 4. Fraction IX was more potent than VIII since it resulted a YFP/CFP ratio lower than 3 after about 28 h compound treatment, while that of fraction VIII was above 3. Based on these bioassay results, fractions VIII and IX were revealed as the bioactive fractions. Both fractions possessed the majority of the gamboge extract and therefore had advantage in term of biological resource.

3.3. HPLC/ESI Q-TOF MS identification of bioactive ingredients

The corresponding ingredients of bioactive fractions were identified by subsequent HPLC/ESI Q-TOF MS analyses. The most potent fraction IX was analyzed first. As illustrated in Fig. 5, this fraction showed mainly four peaks at 4.44, 4.80, 4.90, and 5.31 min in the DAD and TIC chromatograms. The two foregoing peaks at 4.44 and 4.80 min were induced to be a pair of epimers since they had the same ESI-MS spectra. The molecular ions of $[M+H]^+$ at *m/z* 629.3106 and 629.3105 and $[M+Na]^+$ at *m/z* 651.2933 and 651.2920 indicated that the molecular formula was $C_{38}H_{44}O_8$ (Fig. 6A and B). As displayed in Figs. 3 and 5, these two peaks should be two major components of Gamboge extract due to their predominant contents. According to the above observations, they were elucidated to be the above-mentioned lead compound gambogic acid and its epimer [23]. In a similar way, the two latter peaks were indicated to be another pair of epimers exhibiting identical $[M+H]^+$ at *m/z* 661.3380 and 661.3361 and $[M+Na]^+$ at *m/z* 683.3175 and 683.3184 (Fig. 6C and D). Their molecular formulae were elucidated

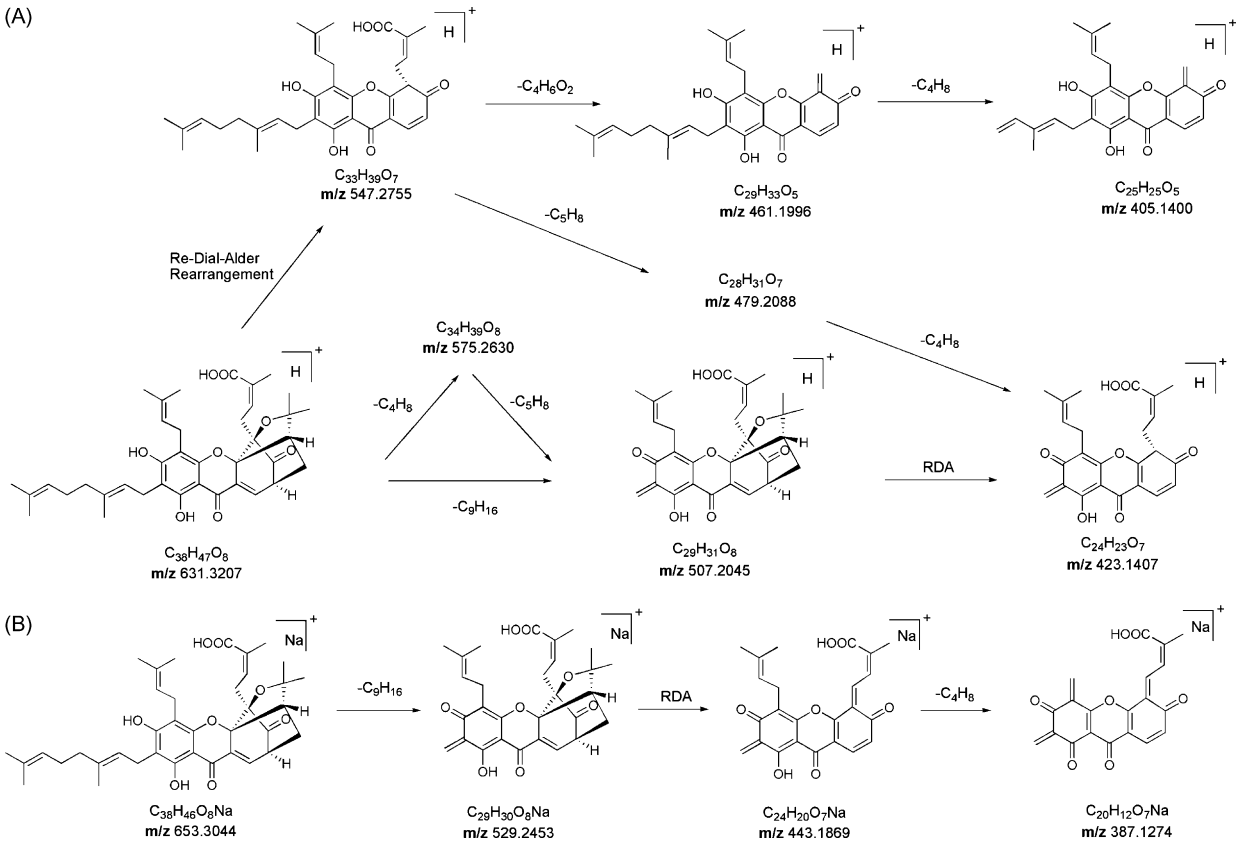

Fig. 7. Fractionation pathways of $[M+H]^+$ (A) and $[M+Na]^+$ (B) of the peak at 4.28 min.

to be $C_{39}H_{48}O_9$, which suggested that these two compounds might be methanol adducts of gambogic acid [27]. This was confirmed by comparison of their MS spectra with those of gambogic acids. Two diagnostic ions that were absent in gambogic acids were found at *m/z* 629.3107 and 573.2479. These signals were assigned to $[M\text{-}MeOH+H]^+$ and $[M\text{-}MeOH\text{-}C_4H_8+H]^+$, respectively. Therefore, these two peaks were elucidated to be gambogoic acid A and its epimer [27]. As determined by HPLC, fraction IX mainly contained epimeric mixtures of gambogic acids (68.7%) and gambogoic acids (26.9%).

Thereafter, fraction XIII was examined in the same way. This fraction displayed three peaks at 3.11, 4.01, and 4.28 min in the DAD and TIC chromatogram. These peaks showed their individual $[M+H]^+$ at *m/z* 631.3274, 629.3102, 629.3104, and $[M+Na]^+$ at 653.2960, 651.2892 and 651.2917 in the positive ESI-HRMS spectra (Fig. 6E, F and G). Like gambogic acid and its epimer, the latter two peaks, having identical MS spectra, were deduced to be a pair of epimers. Furthermore, they had the same molecular formula as gambogic acids, suggesting they might be isomers of gambogic acids.[24] Isomerization and epimerization are phytochemical characteristics of the gamboge extract, which makes its chemical profile seem very complex and brings problems of quality control. As such, our current study is to target gambogic acid analogues that do not have isomerization or epimerization.

Furthermore we examined the peak at 3.11 min which had the molecular formula $C_{38}H_{46}O_8$ as determined by its HRMS. In the positive ESI-MS (Fig. 6G), the pseudomolecular ion peaks were observed together with two fragment ion peaks at *m/z* 605.2679 and 575.2621. These signals resembled those of gambogic acids (Figs. 6A and B) except for the difference of 2 Da, suggesting this should be an analogue of gambogic acid. More structural information was obtained when this peak was measured using Collision Induced Dissociation (CID) (Fig. 6H). As we reported previously [28], gambogic acid and its derivatives had several typical fractionation pathways. Since they were polyprenylated, one of the typical cleavages was the loss of C_4H_8 (56 Da) which was attributable to a prenyl substitute. Another was the Retro-Diels-Alder (RDA) rearrangement, which caused the cleavage of the characteristic caged skeleton, corresponding to the loss of C_5H_8O or $C_5H_{10}O$ (84 or 86 Da). After the RDA rearrangement, there was often a subsequent loss of $C_4H_6O_2$ (86 Da) assignable to a prenyl substitute with carboxyl group. As illustrated in Figs. 6H and A, all these fractionation were clearly observed in the current study. An additional loss of 124 Da due to the cleavage of a geranyl group provided the key information for the structure elucidation. As illustrated in Figs. 6H and 7B, this fractionation pathway was confirmed by those signals from the molecular ion $[M+K]^+$. Thus, this compound should have a caged xanthone skeleton, a prenyl substitute with carboxyl group, and a geranyl group. Taking its molecular formula into consideration, this compound was elucidated to be gambogenic acid [29], a derivative of opening the pyran-ring of gambogic acid which just produced the geranyl group. As determined by HPLC, fraction VIII mainly contained gambogenic acid (72.1%) and epimeric isogambogic acids (25.3%).

In conclusion, this system consisting of HSCCC fractionation, bioassay, and HPLC/MS identification proved to be successful in discovering apoptosis inducers from gamboge. Gambogenic acid was found as an alternative of gambogic acid but had no epimerization. The combination of HSCCC and LC/MS fully demonstrated their powers in the rapid discovery of lead compounds with the aid of sensitive bioassay. The applicability of this system could be extended to a wider range if equipped with various bioassay models.

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