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Bioassay-guided separation and identification of anticancer compounds in *Tagetes erecta* L. flowers

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Abstract

Investigating bioactive components from marigold (*Tagetes erecta* L.) can provide worthy information for drug development. A rapid and efficient method is needed to obtain available anticancer compounds from the ethanol extract of this plant. Therefore, an offline two-dimensional preparative high performance liquid chromatography method guided by a real time cell analysis system was applied on this study. Syringic acid (**1**), quercetin (**2**), 6-hydroxykaempferol (**3**), protocatechuic acid (**4**) and quercetagetin (**5**) with high purities of over 95 % were prepared by this method, while 6-hydroxykaempferol was firstly found in *Tagetes erecta* L.. Compounds **2, 3** and **5** all were flavonoids. The compounds were evaluated for their

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tumor cell growth inhibitory activities on HEPG2 and A549 cells by a real-time cell-analyzer. There were four compounds able to inhibit proliferation of human cancer cells at a concentration of 50 μg/mL. Compounds **2** and **3** showed significant anticancer activity against A549 and HEPG2 cells. Compounds **4** and **5** were effective against A549 cells. The present study demonstrates that this bioassay-guided offline two-dimensional preparative high performance liquid chromatography method is very conducive to isolating plants rich in flavonoids and leading to the discovery of more worthy natural anticancer compounds.

1. Introduction

Marigold (*Tagetes erecta* L.), one of the most well known species in *Tagetes* L., is used as an ornamental and medicinal plant for the most part.[1] It is originally distributed in Mexico and now cultured widely in China. The predominant use of marigold flowers is to extract lutein.[2] At the same time, the residues of industrial lutein production are not effectively utilized and directly discarded in general. Besides the principal component, characteristical secondary products in the marigold, such as flavonoids, triterpenoids, phenolic derivatives, thiophene derivatives, can be applied in the field of agriculture and possess anti-oxidant, anti-inflammatory and other activity.[3] Flavonoids, as a hot spot in the area of development and utilization of natural drugs due to low toxicity and broad-spectrum pharmacological activities, have been discovered more than 5000 structures separated from different plants. In search for anticancer drugs compelling data from laboratories, epidemiologic investigations, and human clinical trials showed that flavonoids have important effects on cancer chemoprevention and chemotherapy so far.[4] What's more, flavonoids' molecular mechanisms against cancer had been reported.[5] Numerous investigations have demonstrated that extracts of *Tagetes erecta* L. have anticancer activity, and most studies have mainly focused on lutein.[6] Recently more and more researches have found that the ethanol extract of *Tagetes erecta* L.is rich in flavonoids.[7] However, the anticancer activity of the ethanol extract of *Tagetes erecta* L. flowers got rid of the lutein part is rarely researched.

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63 In the present article, marigold ethanol extract was separated by an offline
64 two-dimensional preparative high performance liquid chromatography
65 (2D-prep-HPLC) method based on screening for anticancer activity of constituents.[8]
66 Cellular anticancer activity assay was carried out by iCELLigence real time cell
67 analysis (RTCA) system.[9,10,11] Compared with traditional cytological tests such as
68 MTT/WST methods, immunofluorescence detection, mass spectrometry, flow
69 cytometry and so forth, RTCA is non-invasive, real-time dynamic detection system.
70 And it can save working time, lower working load and recover samples.

71 Preparative HPLC captures growing attention, since it has high performance
72 separation, online monitoring and automatic control.[12] And it accomplishes the task of
73 the efficient preparation. However, considering the complex chemical composition of
74 natural medicine extract, it is impossible to get high purity compounds by one
75 dimensional preparation due to the limited resolution and peak capacity.[13]

76 A major application of 2D-HPLC is to separate peaks that co-eluted in the
77 conventional HPLC environment.[14] 2D-HPLC can not only increase the number of
78 separated compounds, but also separate a group of various samples based on
79 molecular structure. The samples in a group may be eluted with different mobile
80 phases selectively and differences in the location of the two-dimensional retention
81 space. Suitability of HPLC separation system can be characterized by theoretical peak
82 capacity (n_c), which determines the maximum number of peaks that can be
83 accommodated side by side in the chormatogram at desirable resolution. However, the
84 theoretical peak capacity can even be difficult to approximate. In the situation of
85 2D-HPLC, peak capacity ($n_{c,2D}$) notably increases and should be accurately equal to
86 the product of three factors: the respective peak capacity of two columns (n_{c1}, n_{c2}) and
87 the fraction of the separation space which is occupied by the 2D separation, $f_{coverage}$,
88 and taking the degree of separation coverage into account , in accordance with Eq.
89 (1):[15,16]

90 $$n_{c,2D} = n_{c1} \cdot n_{c2} \cdot f_{coverage} \quad (1)$$

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Hydrophilic interaction liquid chromatography (HILIC), the special type in the range of normal phase liquid chromatography (NPLC), is essentially non-aqueous stationary phase with conventional normal phase (NP) or reversed phase (RP) mobile phases.[17] HILIC coupled with RP chromatography had been verified to be extraordinarily useful in the separation of Stevia Rebaudiana Bertoni extracts.[18]

In addition, two-dimensional HPLC can be performed either off-line or on-line. In this work, off-line 2D-LC analysis of natural medicine plants is seemingly time-consuming method, but it provides valuable information for the separation of active compounds in one-dimensional HPLC or even multi-dimensional HPLC analysis. Moreover, considering that the off-line mode has no restrictions on the screening of the bioactive components using RTCA, so the bioactive fractions eluted from the first dimension could be prepared and analyzed with enough time. Therefore, the off-line mode was selected to maximize resolving power of two dimensions.

In this study, an offline 2D-NPLC/HILIC system was carried out for isolation and purification of marigold residues, while the highlight was the separation bonded with bioactivity evaluation. Hence, the aim of this work was to find out anticancer compounds and verify the feasibility of this strategy.

2. Results and discussion

2.1 Establishment of bioassay-guided off-line 2D chromatography system

The steps of bioassay-guided isolation system includes separation, fractionation, and purification. Every step is systematically directed by a bioassay result. For this reason, rapid, reliable and relatively simple biological detection is indispensable. For anticancer assay, RTCA, which enables rapid measurement of the dynamic biological response of living cells to the presence of compounds having cell growth-inhibiting or promoting properties, meets these demands. The data with high reproducibility can be achieved by RTCA and are real-time, rather than one of a final state. Samples are unmarked and able to apply to other activity assay and structure identification. This point has a crucial significance to medicinal material separation on account of low yield of compounds. Results are obtained as Cell Index (CI), which represents cell

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status based on electrical impedance. The larger the number of healthy cells attaches to the sensor surface, the higher the CI value. If compound cytotoxicity results in cell death, morphological change that affects the contact area between the cell and the surface leads to a CI decrease.[10]

In consideration of the ethanol extract of marigold flowers was slightly soluble in water, non-aqueous solvent and non-aqueous columns seemed to be the better option for the preparative separation. The comparison and selection of different columns were crucial to the construction of an offline 2D-HPLC preparation separation method. Three columns were tested for the second dimensional separation. Eventually, a traditional normal phase column and a HILIC column used in non-aqueous mobile phase were selected for this work. Since two columns have different stationary phase, it was possible for silica and diol column to form an orthogonal separation system.

To achieve the goal of better separation, selection of mobile phases is also important. NP columns use a stationary phase that is more polar than the mobile phase. In a traditional NPLC, mobile phases consist of a very non-polar solvent and small amounts of polar organic solvents.[19] At present, several polar solvents mixed with a non-polar solvent (n-hexane) were investigated. When UV detection was used, the choice of mobile phase was limited. Due to interference of solvent absorption in the UV region, the combinations n-hexane/acetone and n-hexane/ethyl acetate did not provide good baseline separation. However, a mixture of n-hexane and ethanol as binary mobile phases for NPLC had such advantages as wide polarity range by changing the ratio of the two solvents and relatively low environmental toxicity.

Above all, this strategy offered the possibility of finding active compounds and saving separation time. It contributed to discovering quickly targeted fractions and more useful nature chemicals.

2.2 First-dimensional preparation

Fig.1 illustrates the first-dimensional HPLC chromatograms of the concentrated marigold flower ethanol extract on the innoval silica columns. Total preparation procedure took 45 min, which consisted of 10 min for column balance, 10 min for

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column washing, and 25 min for preparation. The sample had been got rid of *luetin* and became simple, so its chromatographic peaks could distribute intensively. A gradient elution combined with isocratic elution was chosen for the first dimensional preparation. In analytical HPLC, the flow rate was 1 mL/min and the sample loading was 4.19 μg. When analytical HPLC was scaled up to preparative scale, the sample loading was increased to 4.19 mg. Meanwhile, the flow rate of preparation (u_p) was in proportion to the square of diameter according to Eq. (2). The diameter of preparation was r_p, and one of analysis was r_a, So u_p w as 100 mL/min.

$$u_p = u_a \cdot (r_p / r_a)^2 \quad (2)$$

Good separation performance was obtained in analytical HPLC (Fig. 1**a**). When using the same mobile phases in the same proportion as in preparative HPLC, resolution and peak capacity decreased obviously. But the sample loading and productivity were increased as a result of reduced cost, and it was more important for preparation. In a word, elution method including 0-10 min for 1 % B, 10-18 min for 1-19 % B, 19-35 min for 19 % B and 35-45 min for 100 % B was used for the preparation. Preparation of the total 41.85 g crude sample was injected 100 times in about 75 h. The fractions were collected according to the UV absorption intensity to reduce the complexity of each fraction as much as possible. As shown in Fig.1, nine fractions were collected in the first-dimensional separation.

2.3 Optimization of the second-dimensional separation of active fractions

For the second-dimensional separation, the analytes should have better retention than the first one. Three columns consisting of X-Amide (4.6 mm × 250 mm i.d., 10 μm, 100 Å, ACCHROM), YMC-Pack Diol-NP (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC) and YMC-Pack CN (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC) were chosen to reanalyze active fractions. Fr. 7 and Fr. 8 expressed significantly higher anticancer activity than other first-dimensional fractions in Fig. 2, so the second-dimensional separation was therefore focused on Fr. 7 and Fr. 8. Two fractions eluted in last 10

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179 min retained well in NP mode, which suggested that they contained compounds with
180 relatively higher polarity than other fractions.
181 Fig. 3 and Fig. 4 describe that the elution order, the retention time, the shape and
182 the resolution of the peaks in these three columns are quite different from those in the
183 silica column used for the first-dimensional separation, which indicates that one of
184 three columns is a candidate for constructing an orthogonal two-dimensional
185 separation system. Fig. 3**a** and Fig. 3**c** manifest that the amide column and the CN
186 column both have certain retention for Fr. 7, but it is impossible to obtain
187 well-pleasing resolution in the two columns by reason of their close retention times,
188 and the second peak is covered by the first one. Compared with the two columns, the
189 diol column proved to have better selectivity and longer retention time in this study in
190 Fig. 3**b**. Fr. 7 was expectably separated with higher resolution and both Fr. 7-2 and
191 Fr.7-3 could be clearly recognized on this column. Above all, this preparation
192 procedure was able to be scaled and applied to second dimension. Silica and diol
193 columns were seasonably orthogonal as expected. Different HILIC columns bonded
194 with a bare silica column showed different separation properties. Good orthogonality
195 was significant in achieving efficient preparation of compounds with high purity. For
196 samples with different unknown components, the most suitable columns combination
197 was applied by comparison. Hence, an offline 2D-NPLC/HILIC system was
198 established, in which the silica column was employed in first dimension while the diol
199 column was used in second dimension respectively. It not only improved the
200 separation efficiency but also made a remarkable contribution to the purity of the
201 compounds collected.
202 In a similar way, Fr. 8 was analyzed. As described in Fig. 4, the diol column still
203 had the satisfactory resolution for the separation of Fr. 8, while the separation ability
204 of other columns paled in comparison with the diol column. Even if the peak shape of
205 Fig. 4**a** was perfect and these were recognizable as single peaks, the amide column
206 could not be adopted due to too short and centralized retention time. The CN column
207 was worse than the amide one. As a matter of fact, the polarity of the stationary phase
208 of the diol column used in the situation of non-aqueous mobile phase was higher than

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amide and CN columns. By the analytical work, we could confirm that the diol column was the optimal one for the separation of marigold fractions.

2.4 Preparation of active compounds

The 2D-NPLC/HILIC system was further applied to isolate and purify marigold ethanol extraction at preparative scale. On basis of the result of optimization, the YMC-Pack Diol-NP (10 mm × 250 mm i.d., 5 μm, 120 Å, YMC) was effectively carried on the second-dimensional preparative separation. It was discovered that isocratic elution conditions were adequate for the separation of Fr. 7 and Fr. 8, and isocratic elution method would avoid extra time required for coordinating in gradient elution. 28 % of the mobile solvent B made sure that the retention time of the peaks is between 5 and 30 min. The preparative chromatogram of Fr. 7 in Fig. 3**d** was nearly consistent with the analytical chromatogram in Fig. 3**b**. The situation of Fr.8 was just the same as above. It proved to demonstrate the feasibility of the transformation from an analytical scale to a preparative one. Good separation resulted in three major peaks labeled Fr. 7-1, Fr. 7-2 and Fr.7-3, eluting at 10, 14 and 16 min (Fig. 3**d**). At the same time, there were two major fractions labeled from Fr. 8-1 to Fr. 8-2, eluting at 10 and 23 min respectively (Fig. 4**d**). After isolating Fr. 7 and 8, five compounds with high purity were gained. It was noteworthy that heart-cutting was used as the repeated separation strategy to insure the purity of compounds. The cross in each fraction had been minimized because of the good separation repeatability.

Benefiting from good orthogonality and optimized collection operation, compounds **1** (Fr. 7-1, 7.9 mg), **2** (Fr. 7-2, 30.7 mg), **3** (Fr. 7-3, 25.8 mg), **4** (Fr. 8-3, 19.7mg) and **5** (Fr. 8-5, 83.1 mg) were yielded in preparation. All in all, these compounds had enough amounts to be characterized by NMR spectroscopy. The purity of these compounds tested by HPLC was manifested in Fig. 5 and the purity of all compounds was above 95 %. These compounds, whose structures were showed in Fig. 6, were identified as syringic acid (**1**), quercetin (**2**), 6-hydroxykaempferol (**3**), protocatechuic acid (**4**) and quercetagetin (**5**) by comparison with the

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reference.[20,21,22,23,24] The NMR data are supplied in the Supplementary Material. What's more, 6-hydroxykaempferol was firstly found in *Tagetes erecta* L.

Compounds **2**, **3** and **5** all were flavonoids and this 2D system provided complementary selectivity for flavonoids. So this simple method in the present study was extremely useful for preparative separation of plants rich in flavonoids and could lead to the discovery of more useful natural active compounds.

2.5 Detection of Cellular anticancer activity

An important future of RTCA is dynamic monitoring. Fig. 2 shows dynamic responses to different sample groups. As shown in the result, the CI values of every group had various changes after adding fractions. In Fig. 2**a**, the CI value of the blank control group(DMSO) had a continuous increase and the finial CI was about 8.0. At the same time, the values of other groups in Fig. 2**a** rose to about 6.0 too. So, Fr.1 to Fr. 5 had no inhibitory effect on the growth of A549 cells. And Fr.1 to Fr. 5 were also ineffective against the growth of HEPG2 cells in Fig. 2**d**. Nevertheless, the situation of Fig. 2**b** and **e** was different from the one of above pictures. In Fig 2**b**, the CI of Fr. 7 increased to 3.0 in the first 20 hours and entered a stationary phase (CI 2.8 to 3.0) in the following 28 hours. Its finial value was well below one of the blank control group and the trend lines were not similar. And the CI of Fr. 8 increased to 0.5 in the first 2 hours and entered a stationary phase (CI 0.5 to 1.0) in the following 46 hours. In a word, Fr.7 and Fr. 8 had better inhibitory effect on the growth of A549 cells. In a similar way, we could find that Fr. 7 and 8 were effective against the growth of HEPG2 cells compared with the blank control group of DMSO and other fractions. In Fig. 2**e**, Fr. 9 could inhibit the growth of HEPG2 cells, but it was not researched on this study because of its complex constituents and low yield. After second-dimensional preparation of Fr. 7 and Fr. 8, five fractions we obtained were single compounds with high purities. In Fig. 2**c** and **f**, Fr. 7-2 and 7-3 had preferable anticancer activities, and Fr. 8-1 and 8-2 had good inhibitory effect on the growth of A549 cells. It was a simple method that used to preliminary screening of anticancer active fractions and guided our separation. As described above, quercetin (Fr. 7-2) and

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6-hydroxykaempferol (Fr. 7-3) seemed to be capable of inhibiting proliferation of human lung cancer and liver cancer at a concentration of 50 μg/mL. At the same time, protocatechuic acid (Fr. 8-1) and quercetagetin (Fr. 8-2) were effective against A549 cells.

3. Experimental

3.1 Apparatus

The preparative HPLC system used in this study was the Agela CHEETAH HP 100 system, which consisted of two high pressure solvent delivery pumps, an UV detector, a manual injection valve, a column and a chromatography workstation (CHEETAH HP 100; Bonna-Agela Technologies Inc., Tianjin, China). Chromatographic analysis was carried on a Hitachi HPLC system including a Hitachi L2400 UV detector, a Hitachi L2130 pump, a Hitachi L2200 autosampler and Hitachi Lachrom Elite HPLC software (Hitachi, Japan).

^{1}H-NMR and ^{13}C-NMR spectrum were recorded on a Bruker 600 NMR spectrometer with CD_3OD as solvent, and chemical shifts were given as δ-values with reference to TMS as internal standard.

The iCELLigence RTCA system was composed of three main components: an iCELLigence RTCA analyzer, and RTCA control unit (iPad with integrated software), and a disposable E-Plate L8 (ACEA Biosciences, USA).

3.2 Reagents

Ethanol and n-hexane of analytical grade and chromatographic grade were purchased from Concord Technology Co. Ltd (Tianjin, China). Human non-small cell lung cancer (A549) cell lines and human liver carcinoma (HEPG2) cell lines were obtained from American Type Culture Collection (USA), cultured in our own laboratory (Tianjin, China). Dimethyl sulfoxide (DMSO) was obtained from Sino-American Biotechnology Company of Beijing (Beijing, China). Minimum essential medium (MEM) and Kaighn's modified medium (F-12K) were obtained from Life Technologies (USA).

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299 **3.3 Preparation of marigold ethanol extract**

300 The powder of marigold (*Tagetes erecta* L.) flower, which had been handled to extract
301 the part of the lutein completely, was offered by Chenguang Biotech Group (Handan,
302 Hebei province, China) and authenticated by Professor Lijun Zhou, School of
303 Pharmaceutical Science and Technology, Tianjin University.

304 The dried powder of marigold flower (1 kg) was extracted respectively with 5 L
305 of 95 % ethanol at 60 for 120 min three times. The ethanol extract of marigold
306 flower was filtered with 0.45 μm membranes by vacuum filter. Then the filtrate was
307 collected and concentrated by a rotary evaporation at 60 under vacuum. The
308 concentrated ethanol extract was dissolved in a 6: 4 v/v mixture of n-hexane and
309 ethanol and placed at room temperature over night in dark. After 24 hours' standing,
310 the solution with a concentration of 83.70 mg/mL and filtered through 0.22 μm
311 membranes by vacuum filter again.

312

313 **3.4 First-dimensional chromatographic conditions**

314 Innoval silica columns (4.6 mm× 250 mm i.d., 5 μm, 100 Å, Agela; 50 mm× 250 mm
315 i.d., 10 μm, 100 Å, Agela) were adopted in the first-dimensional preparation at the
316 temperature of 30. The mobile phase A was n-hexane and B was ethanol. The
317 mobile phase gradient program was from 1 % to 19 % B in 8 min and keeping 19 % B
318 in 17 min at the flow rate of 100 mL/min. The detection wavelength was set at 210
319 nm. In analytical HPLC, the flow rate was 1 mL/min and the injection sample volume
320 was 50 μL. In preparative HPLC, the flow rate was 100 mL/min and the injection
321 sample volume was 5 mL. The fractions collected from the first-dimensional
322 separation were concentrated and stored.

323

324 **3.5 Cellular anti-cancer activity assay**

325 Cellular anti-caner activity assay, using the iCELLigencec RTCA system, was
326 evaluated against A549 cells and HEPG2 cells. In the experiment of A549 cells, 150
327 μL MEM medium was added to every well of E-plates 8, which was immediately

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connected to the iCELLigence RTCA system and checked in the cell culture incubator for proper electrical-contacts. And the background impendance was measured during 2 min. The plates were incubated for 30 min to obtain a stable noise signal. Nine fractions from the first-dimensional separation were dissolved in DMSO and diluted with MEM medium, and then 2×10^4 of A549 cells were cultured in each well. DMSO of the same volume was added in a well as a blank control group and the solution of fractions were added into wells respectively. At last, the plates were placed in the environment of 37□ and 5 % carbon dioxide for 48 h and a cell index (CI) was recorded. The final concentration of samples in wells was 50 μg/mL. For the experiment of HEPG2 cells, some differences from one of A549 cells were as the follows: On the one hand, the medium was F-12K medium. And the other hand, there were 4×10^4 cells in every well.

3.6 Second-dimensional chromatographic conditions

Taking into consideration of the results of bioassay, Fr. 7 and Fr. 8 were analyzed by a Hitachi HPLC system. Three different analytical columns were applied. The mobile phases used for all systems were n-hexane (A) and ethanol (B). The column temperature was 30□ and the UV detector was 210 nm. Isocratic elution procedure for Fr. 7 was 27 % B for 20 min, Fr. 8 was 27 % B for 30 min with 50 μL of the injection volume at a flow rate of 1 mL/min. The diol stationary phase was the optimal choice, and the semi-preparative diol column (10 mm × 250 mm i.d., 5 μm, 120 Å, YMC) was applied with 250 μL of the injection volume at a flow rate of 4 mL/min.

The purity analysis of compounds obtained in this 2D separation system was performed on YMC-Pack Diol-NP (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC) : 0-30 min, A/B, 75:25. The flow rate was 1 mL/min.

4. Conclusion

An efficient and bioassay-guided method for separation and purification of anticancer compounds from marigold flowers was established. On the basis of this novel method,

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358 three kinds of anticancer flavonoids, syringic acid and protocatechuic acid were
359 obtained from the ethanol extract of marigold flowers. Two dimensions provided
360 complementary selectivity for flavonoids, which were the most abundant and
361 biologically active compounds in marigold. So this simple method in the present
362 study was extremely useful for preparative separation of plants rich in flavonoids and
363 could lead to the discovery of more useful natural active compounds.

364

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Figure Legends

Figure 1. **a** First-dimensional analytical chromatogram for the ethanol extract on an innoval silica column (4.6 mm× 250 mm i.d., 5 μm, 100 Å, Agela). Inj. V: 50 μL; flow rate: 1 mL/min; UV: 210 nm. **b** First-dimensional preparative chromatogram for the ethanol extract on an innoval silica column (50 mm× 250 mm i.d., 10 μm, 100 Å, Agela). Conditions: Inj. V: 5 mL; flow rate: 100 mL/min; UV: 210 nm. Mobile phase: (A) n-hexane, (B) ethanol; elution program: 0-8 min for 1-19 % B, 9-25 min for 19 %B.

Figure 2. **a** HPLC analysis of Fr. 7 on X-Amide (4.6 mm × 250 mm i.d., 10 μm, 100 Å, ACCHROM). **b** HPLC analysis of Fr. 7 on YMC-Pack Diol-NP (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC). **c** HPLC analysis of Fr. 7 on YMC-Pack CN (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC). Conditions: Inj. V: 50 μL; flow rate: 1 mL/min; UV: 210 nm. Mobile phase: (A) n-hexane, (B) ethanol; elution program: 0-20 min for 27 % B. **d** Second-dimensional preparative chromatogram for Fr. 7 on an semi-preparative diol column (10 mm × 250 mm i.d., 5 μm, 120 Å, YMC). Conditions: Inj. V: 250 μL; flow rate: 4 mL/min; UV: 210 nm. Mobile phase: (A) n-hexane, (B) ethanol; elution program: 0-20 min for 27 % B.

Figure 3. **a** HPLC analysis of Fr. 8 on X-Amide (4.6 mm × 250 mm i.d., 10 μm, 100 Å, ACCHROM). **b** HPLC analysis of Fr. 8 on YMC-Pack Diol-NP (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC). **c** HPLC analysis of Fr. 8 on YMC-Pack CN (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC). Conditions: Inj. V: 50 μL; flow rate: 1 mL/min; UV: 210 nm. Mobile phase: (A) n-hexane, (B) ethanol; elution program: 0-20 min for 27 % B. **d** Second-dimensional preparative chromatogram for Fr. 8 on an semi-preparative diol column (10 mm × 250 mm i.d., 5 μm, 120 Å, YMC).

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Conditions: Inj. V: 250 μL; flow rate: 4 mL/min; UV: 210 nm. Mobile phase: (A) n-hexane, (B) ethanol; elution program: 0-20 min for 27 % B.

Figure 4. Purity evaluation of prepared compounds on YMC-Pack Diol-NP (4.6 mm × 250 mm i.d., 5 μm, 120 Å, YMC). Conditions: Inj. V: 20 μL; flow rate: 1 mL/min; UV: 210 nm. Mobile phase: (A) n-hexane, (B) ethanol; elution program: 0-30 min for 25 % B.

Figure 5. Structures of the purified compounds.

Figure 6. Effect of fractions on the viability of A549 and HEPG2 cells, determined by use of the iCELLigence RTCA system. A549 cells at a density of 20,000 cells/well in E-Plate L8 were observed for 48 h. HEPG2 cells at a density of 40,000 cells/well in E-Plate L8 were observed for 48 h. a Anti-lung cancer effect of Fr. 1 to Fr. 5. b Anti-lung cancer effect of Fr. 6 to Fr. 9. c Anti-lung cancer effect of Fr. 7-1 to 7-3, 8-1, 8-2. d Anti-liver cancer effect of Fr. 1 to Fr. 5. e Anti-liver cancer effect of Fr. 6 to Fr. 9. f Anti-liver cancer effect of Fr. 7-1 to 7-3, 8-1, 8-2.

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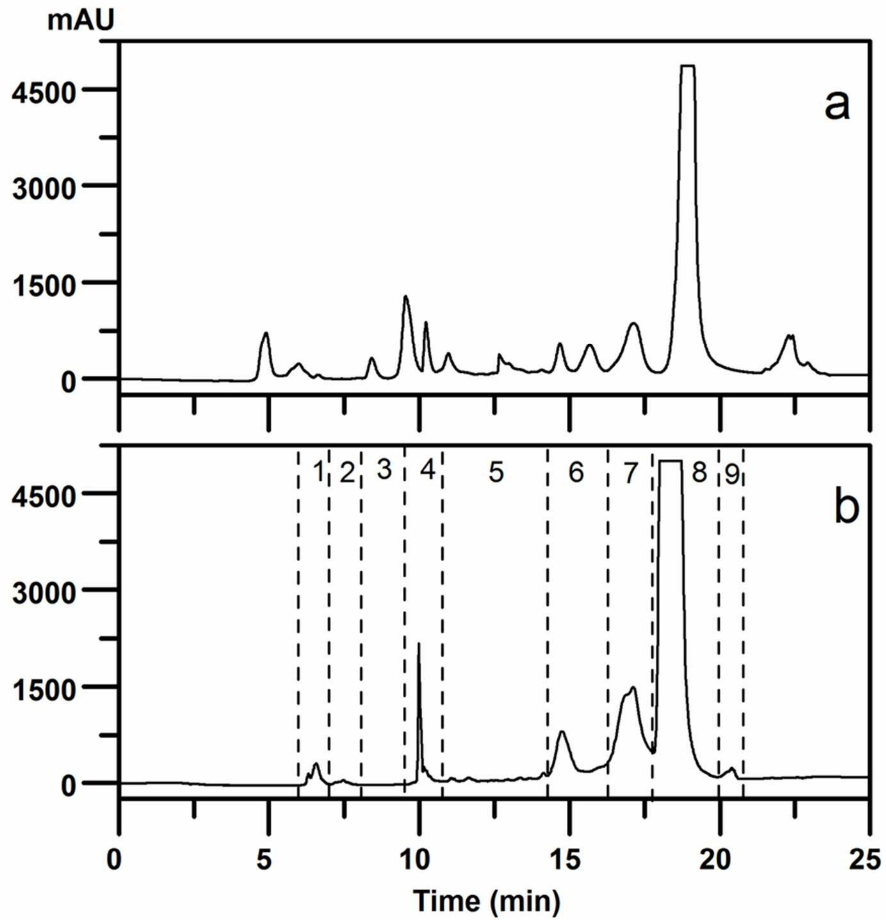

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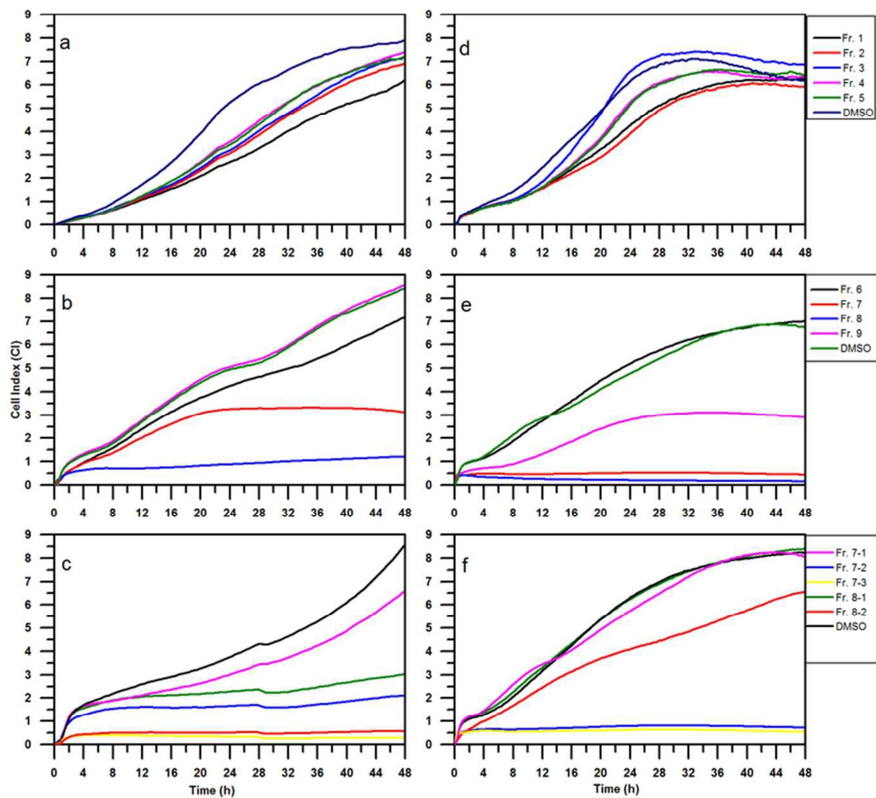

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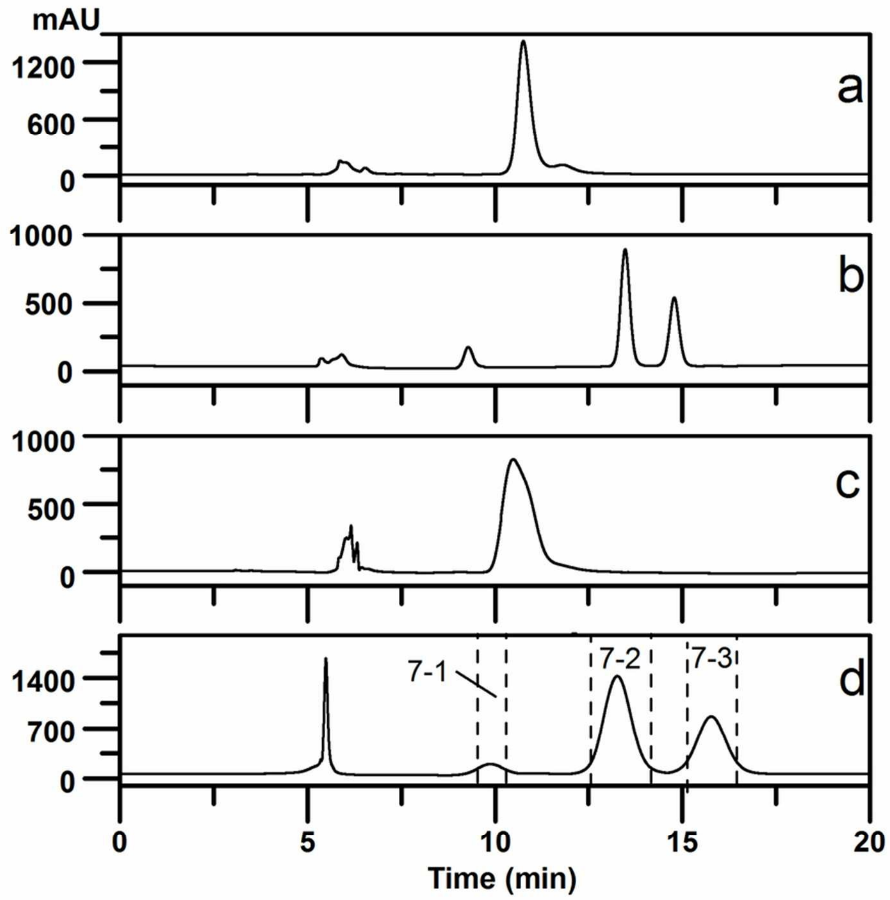

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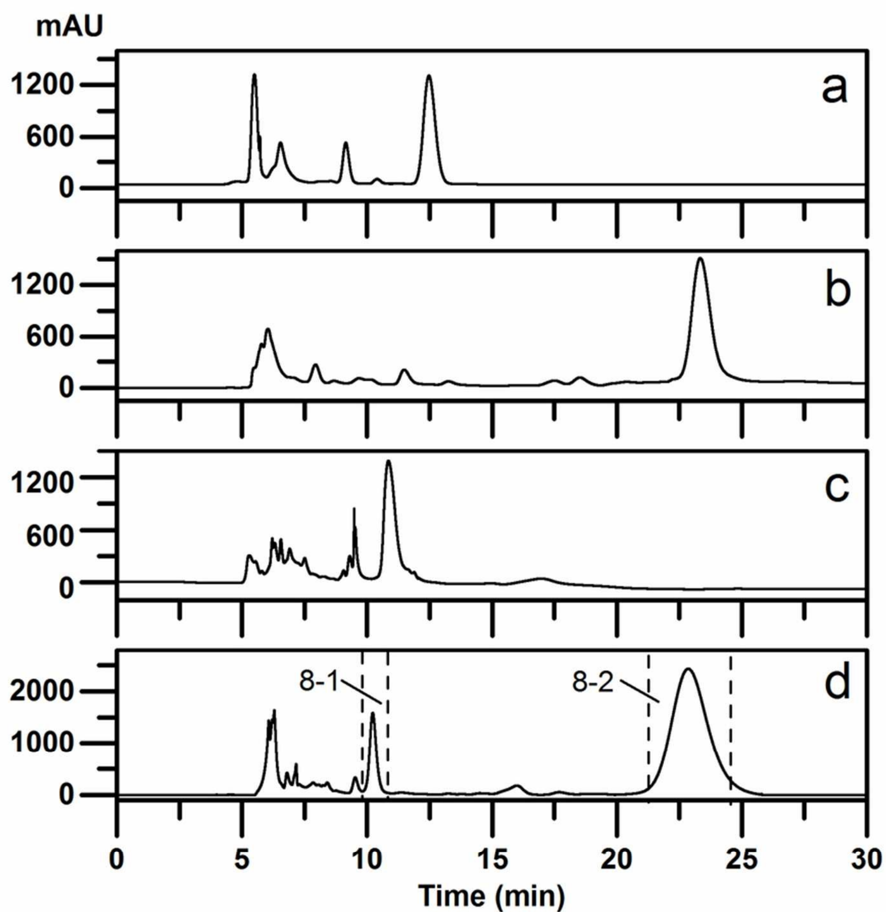

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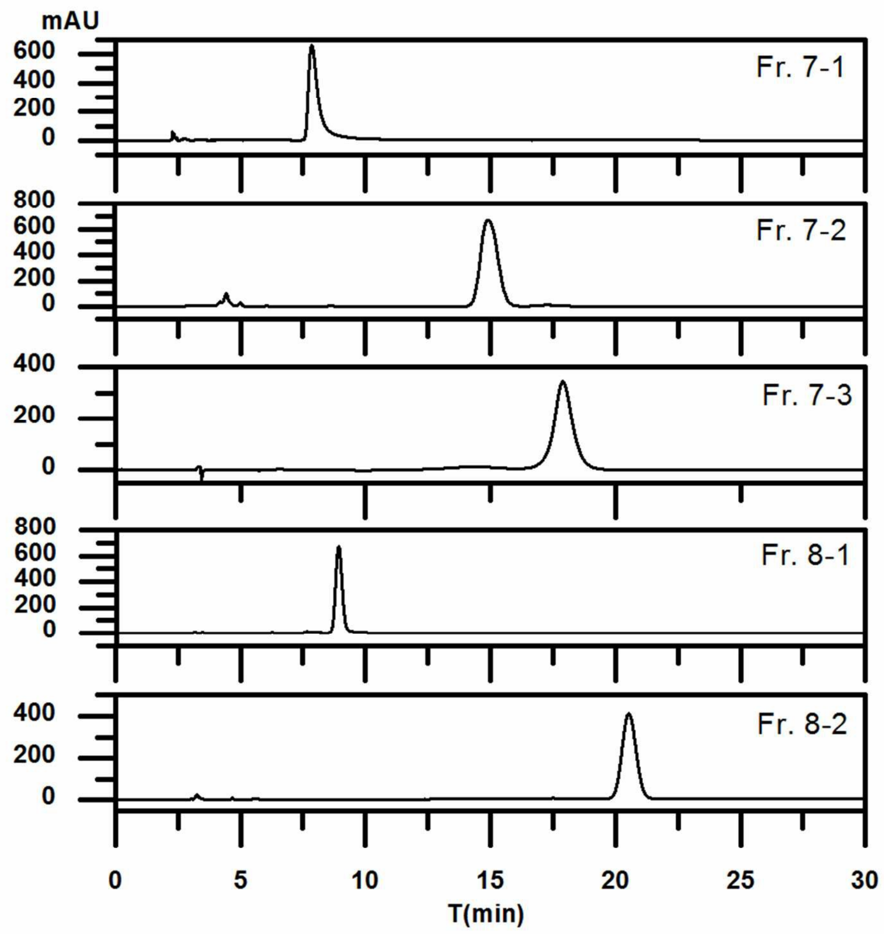

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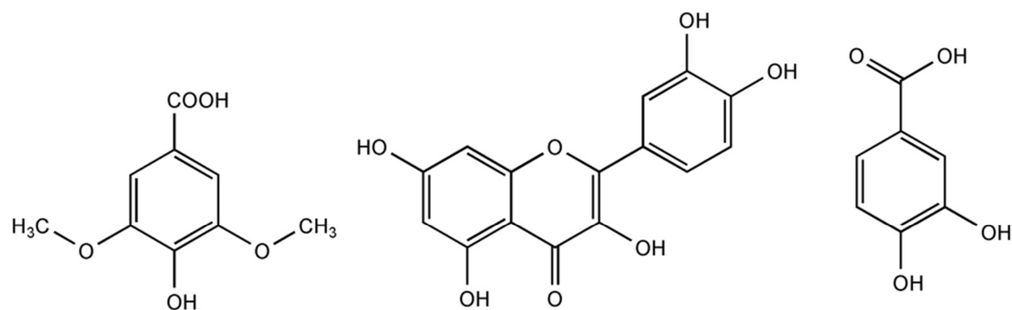
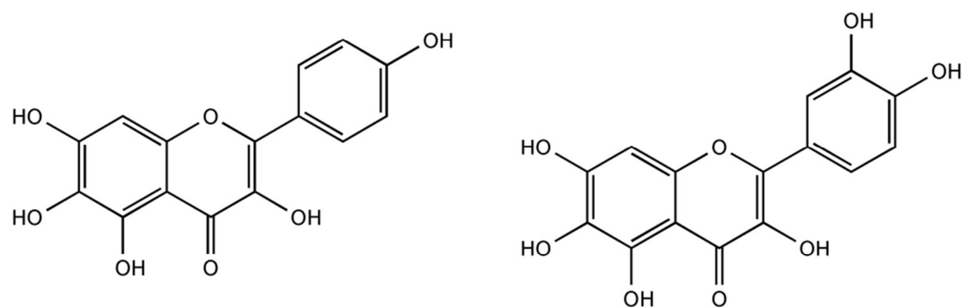


1. Syringic acid **2**. Quercetin **4**. Protocatechuic acid



3. 6-Hydroxykaempferol **5**. Quercetagetin

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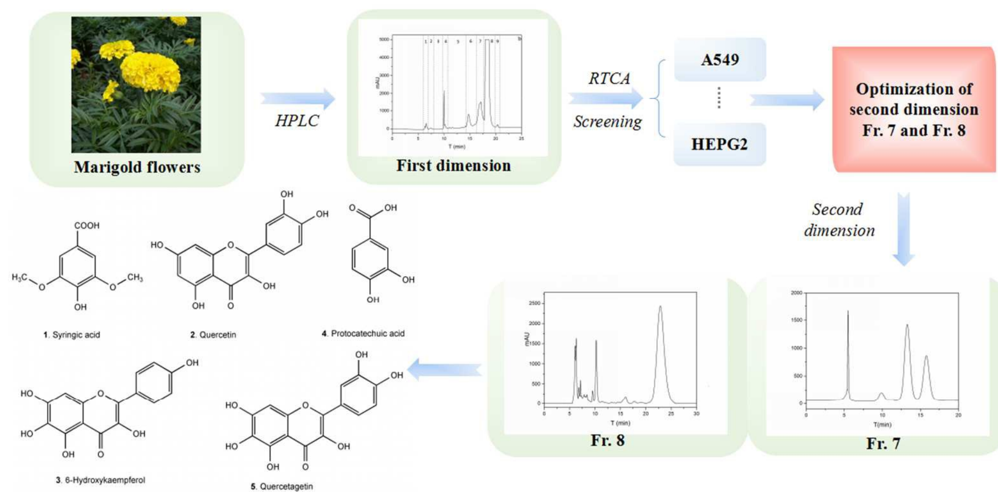

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