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Isolation and inhibitory activity against ERK Phosphorylation of hydroxyanthraquinones from rhubarb

Xia Zhou, Baoan Song,* Linhong Jin, Deyu Hu, Chunling Diao, Guangfang Xu, Zhihui Zou and Song Yang

Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Center for Research and Development of Fine Chemicals, Guizhou University, Guiyang 550025, PR China

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Abstract—Five hydroxyanthraquinone compounds were purified from Chinese medicinal herb rhubarb by microwave-assisted extraction and silica gel column isolation. Their structures were identified by spectroscopic analysis. One of them, emodin, was found to block the ERK phosphorylation of PC3 cells at 20 μM. And two other compounds had potent proliferation inhibitory activity on A431 and PC3 cells at 20 μM. Furthermore, crude extracts revealed an antifungal activity stronger than that of purified compounds against several plant pathogens tested. Together, our study suggests that these compounds are biologically active and may lead to development of anticancer medicine.

Protein tyrosine kinases play a central role in the transduction of proliferative signals in mammalian cells.[1] They mainly transduce signals by catalyzing the phosphorylation of tyrosine residues in protein substrates. Many tyrosine signals lead to the phosphorylation of MAP kinases, ERK (extracellular signal-regulated kinase). Abnormal activation of tyrosine kinases has been implicated in many cancers and other proliferative diseases.[2] Therefore, tyrosine kinases are expected to be attractive targets for cancer chemotherapy. Due to the involvement of tyrosine kinases in many signal transduction pathways, the development of protein kinase inhibitors has received much attention in recent years, and numerous review articles have now appeared on the topic.[3] Specific inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase have received the most attention, and several elaborations of the fundamental 4-anilinoquinazoline pharmacophore have now been reported as potent and selective inhibitors of this class of enzymes.[4]

Plants have many phytochemicals with anticancer bioactivities. Chinese medical herbs are considered to be one of the most promising sources due to their variety of species and applications. In addition, their therapeutic effect has been demonstrated by their clinical uses in Chinese for many decades. The roots and rootstock of rhubarb (*Rheum officinale Baill*, and Chinese name Dahuang), which belongs to *Polygonaceae*, is an important Chinese traditional medicine that has been widely used for the treatment of many kinds of diseases.

There has been great clinical interest in using rhubarb in treatment of many kinds of diseases. Some studies have reported that extracts from rhubarb were used as purgative agent,[5] antipyretic, antimicrobial and antivirus,[6,7] anti-tumor drugs,[8] and stomachic medicine.[9] It can resist high fat of blood,[10] protect the liver,[11] diminish inflammation,[12] accommodate immunity,[13] anti-age,[14] and have antioxidant activities.[15–17] Extracts of rhubarb can reduce alcoholism and lighten hangover by inhibiting alcohol dehydrogenase.[18] The inhibition of hydroxyanthraquinones, especially emodin on tyrosine kinase, may be one of the mechanisms of their anti-melanoma activity.[19,20] Emodin was reported to have effects of anti-mutation,[21] antiliver cancer, and mammary cancer.[22] In the present study, we investigated a variety of active ingredients of rhubarb in seeking to find a new inhibitor against ERK phosphorylation.

There was little application of rhubarb for the control of cancers and various fungal diseases in the present

Keywords: Hydroxyanthraquinones; Antitumor bioactivity; ERK phosphorylation.
* Corresponding author. Tel.: +86 851 3620521; fax: +86 851 3622211; e-mail: songbaoan22@yahoo.com

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research. In order to obtain compounds possessing better anticancer activity and inhibitive activity against ERK phosphorylation, we extracted and isolated known hydroxyanthraquinone ingredients (**1**–**5**) from Chinese medicinal herb rhubarb and tested their anticancer activity. Hence, we report herein a new inhibitor for ERK phosphorylation. All isolated compounds were tested for their anticancer properties, inhibitory activity for ERK phosphorylation, and antifungal activities. To the best of our knowledge, this is the first report on the inhibitory activity against ERK phosphorylation of emodin from rhubarb.

Tutin and Clewer[23] had studied the extraction and isolation of hydroxyanthraquinones from rhubarb *officinale Baill* extensively. By far, there are two kinds of separation methods, pH-modulated stepwise elution[24,25] and column chromatography,[26,27] reported. However, we found that pH-modulated stepwise elution gave not monomer but complex that needs to be further purified. Column chromatography can be used to obtain the monomer meanwhile it would use up too much reagent and take too long time. We have developed the purification method by combining the pH-modulated stepwise elution and silica gel column. At the same time, we have also screened an extraction method for application of ultrasound extraction (UE) and microwave-assisted extraction (MAE). The same concentration of starting material and volume of solvent were used in experiments. As shown in Table 1, the extraction of crude product was carried out in 24% extraction rate at 70 °C for 3 h with ultrasound irradiation. Under refluxing extraction condition, time was extended to 12 h at 100 °C and the extraction rate of crude product obtained was 18%. When the extraction was preceded at 70 °C for 1 h by the MAE method, crude product was obtained in 42% extraction rate. Conventional reflux extraction (water bath), UE and MAE of the crude extraction were compared (Table 1).

Table 1. Conventional RE and UE versus MAE of crude hydroxyanthraquinone extraction from rhubarb in $CHCl_3/H_2SO_4$ 20% (5:1,v/v) solvent

Extract method	Temp (°C)	Time (h)	Extraction rate (%)
RE[a]	100	12	18
UE[b]	70	3	24
MAE[c]	70	1	42

[a] RE, refluxing extraction (water bath).
[b] UE, ultrasound extraction.
[c] MAE microwave-assisted extraction. The temperature of MAE monitored directly by a microwave-transparent fluoroptic probe inserted into the solution.

Five hydroxyanthraquinones were purified by the developed method[28] of extraction and isolation (Fig. 1). On the basis of comparison with their spectral data in the literature, their structures were identified as chrysophanol, physcion, aloe-emodin, emodin, and rhein.[29] Structures of five hydroxyanthraquinones are shown in Figure 2.

In order to determine the biological activities of these identified compounds, we carried out several ERK phosphorylation experiments.[30] First we were seeking to determine the effects of these compounds on

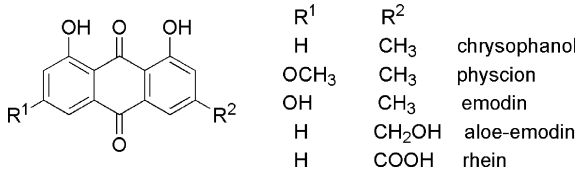

Figure 2. Structures of five hydroxyanthraquinones.

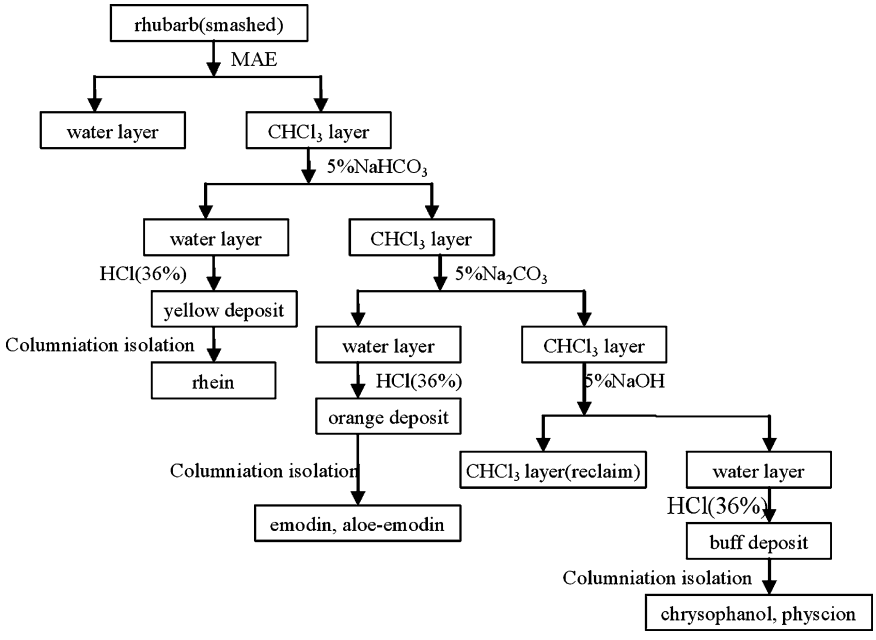

Figure 1. The isolation process of hydroxyanthraquinones in *Rheum*.

(platelet-derived growth factor) PDGF-induced ERK phosphorylation. NIH3T3 cells were pretreated with 20 μM of these compounds, respectively, for 30 min at 37 °C in media with serum, followed by treatment with 40 ng/ml PDGF for 10 min. The result is shown in Figure 3. The blot was in sequence, negative control (control), positive control (PDGF), and compound plus PDGF (**1** + **P**, **2** + **P**, **3** + **P**, **4** + **P**, and **5** + **P**). It can be seen that the compounds **1**–**5** at 20 μM had no significant inhibitory effect on PDGF-induced ERK phosphorylation in NIH3T3 cells. Since there was no effect of these compounds on PDGF-stimulated ERK phosphorylation, we shifted the gear to test the effect on EGF stimulation. PC3 cells were incubated for 30 min in the presence of compounds **1**–**5** as described and then stimulated with 40 ng/ml EGF for 10 min (Fig. 4). The experimental setup was the same as shown in Figure 3. Results in Figure 4 show that compound **4** at 20 μM blocked ERK phosphorylation in PC3 cells induced by EGF. To find out whether the effect observed at 20 μM could be extended to a lower concentration, a dose response was performed at gradient concentration ranging from 1 μM to 20 μM. Figure 5 represents a duplicate of dose response. It reveals that chemical **4** started having some inhibitory activity at 10 μM and totally inhibited EGF-stimulated ERK phosphorylation in PC3 cells at 20 μM shown in Figure 4.

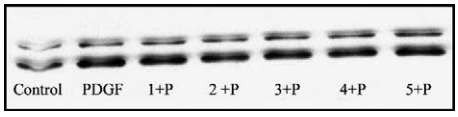

Figure 3. Inhibitory activity of compounds **1**–**5** against ERK1/2 phosphorylation in NIH3T3 cells induced by PDGF. NIH3T3 cells were cultured in 6-well plates to 100% confluence, pretreated, respectively, with 20 μM compound, **1**–**5** for 30 min, and stimulated by PDGF for 10 min. After treatment, the cells were directly dissolved in SDS sample buffer and proteins were separated using SDS–PAGE, transferred to PVDF membrane, and blotted with anti-pERK1/2.

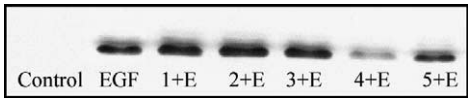

Figure 4. Inhibitory activity of compounds **1**–**5** against ERK1/2 phosphorylation in PC3 cells induced by EGF. PC3 cells were cultured in 6-well plates to 100% confluence, pretreated, respectively, with 20 μM compounds for 30 min, and stimulated by EGF for 10 min. After treatment, the cells were directly dissolved in SDS sample buffer and proteins were separated using SDS–PAGE, transferred to PVDF membrane, and blotted with anti-pERK1/2.

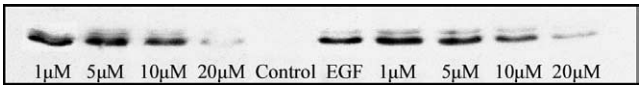

Figure 5. Inhibitory activity of compound **4** against ERK1/2 phosphorylation in PC3 cells induced by EGF. PC3 cells were cultured in 6-well plates to 100% confluence, pretreated with indicated doses of compound **4** for 30 min, and stimulated by EGF for 10 min. After treatment, the cells were directly dissolved in SDS sample buffer and proteins were separated using SDS–PAGE, transferred to PVDF membrane, and blotted with anti-pERK1/2.

The inhibitory activity of compound **4** (Figs. 4 and 5) lead us to think that these compounds may have some anticancer properties. The anticancer activity was assayed by the MTT method.[31] The result listed in Table 2 shows that these compounds exhibit certain inhibitory activities against the A431 and PC3 cancer cell proliferation at a concentration of 20 μM. Among them, compounds **2** and **4** had relatively higher anticancer activities than compounds **1**, **3**, and **5**. The anticancer activity of compound **2** at a concentration of 20 μM against A431 cells and PC3 was 80.5% and 73.2%, respectively. Compound **4** had moderate anticancer activity with the anti-proliferation rate of 73.6% and 63.4% against PC3 and A431 at 20 μM, respectively.

At the same time, we also performed antifungal test[32] of the natural products from Rhubarb. In Table 3, assays of crude $CHCl_3$–H_2SO_4 MAE extracts and 5%$NaHCO_3$ extracts against *Fusarium oxysporum*, *Gibberella zaea*, and *Phytophthora infestans* showed strong growth inhibition at a dosage of 1 mg/ml. However, assays of the 5% Na_2CO_3 extracts and 5% NaOH extracts at 1 mg/ml revealed only negligible antifungal activity. Likewise, no or low antifungal activity was detected at 0.5 mg/ml for the pure compounds **1**–**5**. It might be assumed that several certain compounds in the crude extraction acted corporately, which was proved by designing a mixture compounded of two (1:1) or three (1:1:1) different monomers. The mixture containing rhein has obvious activity which confirmed that earlier conclusion. The structure activity relationships for antifungal hydroxyanthraquinones indicated that polar compounds with carboxyl moiety and more hydroxyl moiety were more active than apolar compounds which lack of carboxyl moiety or hydroxyl moiety. However, the content of hydroxyanthraquinones is not parallel with the physiological effects. Hydroxyanthraquinones should not be the only active antifungal component. A new procedure for the detection and isolation of the unknown active part is needed.

In conclusion, extraction and isolation method of rhubarb was developed and five hydroxyanthraquinone ingredients were obtained and their structures were identified. Application of MAE strongly improved the yield and shortened the extraction time. The crude

Table 2. Inhibition rate of compounds **1**–**5** on A431 cells

Compound	Inhibition rate (%) on PC3 cells 20 μM[a]	Inhibition rate (%)[a] on A431 cells 20 μM
1	21.1 ± 3.7*	19.5(±6.1)*
2	80.5(±4.9)**	73.2 ± 6.7**
3	na	na
4	73.6(±6.7)**	63.4(±4.2)**
5	na	54.2(±1.8)*

Inhibition rate (%) = (A1 − A2)/A1 × 100; A1, the mean optical densities of untreated cells; A2, the mean optical densities of drug treated cells.

[a] Values are means of three experiments, standard deviation is given in parentheses (na, not active).

* $P < 0.05$.

** $P < 0.01$.

Table 3. Antifungal activity of natural products from *Rheum*

Natural products extracted and isolated from *Rheum*	Inhibition rate (%)		
	Fusarium oxysporum	*Gibberella zaeae*	*Phytopthora infestans*
Crude $CHCl_3$–H_2SO_4 20%: extracts (1 mg/ml)	63.27	64.10	61.38
5% Na_2CO_3 extracts: (1 mg/ml)	34.05	38.99	23.26
5% $NaHCO_3$ extracts: (1 mg/ml)	74.55	61.99	61.97
5% NaOH extracts: (1 mg/ml)	13.08	26.90	11.97
Compounds **5**, **4**, **3**: 1 mg/ml	48.51	61.16	60.84
Compounds **1**, **4**: 0.5 mg/ml	11.90	11.00	11.14
Compounds **5**, **3**: 0.5 mg/ml	38.67	46.60	42.17
Compounds **1**, **2**: 0.5 mg/ml	0	10.43	19.75
Compound **1**: 0.5 mg/ml	5.40	0	2.40
Compound **2**: 0.5 mg/ml	0	10.12	19.05
Compound **3**: 0.5 mg/ml	9.37	25.68	21.23
Compound **4**: 0.5 mg/ml	28.80	37.06	24.40
Compound **5**: 0.5 mg/ml	39.75	33.39	39.51

In Table 3, compounds **5**, **4**, and **3** represent three compounds, Rhein, emodin, and aloe-emodin mixed in 1:1:1.
Compounds **1**, **4** represent two compounds, chrysophanol, emodin mixed in 1:1.
Compounds **5**, **3** represent compounds Rhein, aloe-emodin mixed in 1:1.
Compounds **1**, **2** represent compounds chrysophanol, physcion mixed in 1:1.

CH_3Cl–H_2SO_4 20% extracts and mixtures containing compound **5** (rhein) were confirmed to exhibit moderate antifungal activity. In the MTT assay, compounds **2** (physion) and **4** (emodin) had a relatively moderate antitumor activity (at 20 μM). ERK phosphorylation inhibition assay suggests that compound **4** (emodin) at 20 μM has inhibitory activity against ERK phosphorylation in PC3 cells induced by EGF. However, no activity for all compounds was observed in inhibiting ERK phosphorylation of NIH3T3 cells induced by PDGF.

Acknowledgments

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28. Extraction and isolation: A sample of 1 kg, dried and smashed root of *Rheum officinale Baill*, was extracted with $CHCl_3$/H_2SO_4 20% (5:1, v/v) for 3× 20 min assisted by microwave. The mixture was then filtered and filtrate was concentrated to proper volume in vacuo, washed with boiling distilled water to neutrality, and evaporated to yield 300 g crude extracts, yellow dried powder. And the crude extracts were dissolved in 400 ml distilled water and extracted subsequently by the solution of 5% $NaHCO_3$ (3× 400 ml), 5% Na_2CO_3 (3× 400 ml), and 5% NaOH (3× 400 ml). And the three extract fractions were acidified with 36% HCl to pH 1–2, filtered, and dried, obtaining 2.20, 3.60, and 2.35 g yellow floccules, respectively. Extraction obtained from the previous step was, respectively, fractionated by silica gel column chromatography eluting with a petroleum ether–ethyl acetate gradient. Five hydroxyanthraquinones were obtained, including 0.85 g rhein

separated from 5% $NaHCO_3$ part (with petroleum ether/ethyl acetate, 1:2, v/v), 0.62 g emodin and 0.45 g aloe-emodin from 5% Na_2CO_3 part (petroleum ether/ethyl acetate, 5:1, v/v), 0.30 g chrysophanol and 0.12 g physcion from 5% NaOH part (petroleum ether/ethyl acetate, 10:1, v/v), In the way as shown in Figure 1, five hydroxyanthraquinones were obtained successfully. In this process, TLC (thin layer chromatography) was introduced to detect and guide the separation. A silica gel foil panel spotted with natural product solution was developed in petroleum ether/ethyl acetate (1:1, v/v). Five spots were marked on the TLC plate under UV light 254 nm or visible light. Judging from the actual spectral data in the order, the spots were proved to be chrysophanol, physcion, emodin, aloe-emodin, and rhein, from top to bottom. Chrysophanol and physcion with two hydroxid moiety of weak polarity gave the largest R_f value, followed by moderate polarity compounds, emodin with three hydroxyl moiety, and aloe-emodin of two hydroxly moiety and one hydroxymethyl moiety. Rhein with two hydroxyl moiety and a carboxyl moiety of strong polarity ran lowest in the panel. The melting points of the componds were determined with a XT-4 binocular microscope (Beijing Tech Instrument Co., China) and are not corrected. The IR spectra were recorded on a Shimadzu IR Prestige-21 spectrometer in KBr disks. ^{1}H NMR (solvent DMSO-d_6) and ^{13}C NMR (solvent DMSO-d_6) spectra were performed on an INOVA-400 (400 MHz) spectrometer at room temperature using TMS as internal standard. An Elementar Vario-III CHN analyzer was used for the elemental analysis. XH-100 A Microwave-assisted extraction instrument was introduced to extract crude products. The reagents were all of analytical reagent-grade or chemically pure. The thin layer chromatography was conducted on GF_{254} plastic sheets at room temperature. The spots were visualized with UV light and with iodine vapor. All solvents were dried, deoxygenated, and redistilled before use. *Rheum officinale Baill* was cultivated from agriculture fields in Guiyang City, Guizhou, China, and the samples were kept in our laboratory.

29. Compound **1**: Buff platelets, able to sublime, mp: 195–196 °C, molecular formula $C_{15}H_{10}O_4$, M = 254. IR (film): 3049(α-OH concluded with C═O), 1676, 1627, 1606 (characteristic absorbance peaks of anthraquinone), 1475, 1452 (Ar-), 754. ^{1}H NMR (DMSO-d_6) δ ppm: 2.42 (3H, s, 3-CH_3), 7.20 (1H, s, 2-H), 7.37 (1H, dd, J = 1.2, 8.0 Hz 7-H), 7.53 (1H, d, J = 1.2 Hz, 4-H), 7.69 (1H, dd, J = 1.2, 7.6 Hz, 6-H), 7.80 (1H, t, J = 7.6, 8.4 Hz, 5-H), 11.93 (2H, s, OH). ^{13}C NMR (DMSO-d_6) δ ppm: 21.6 (CH_3), 113.7 (CH-7), 115.8 (CH-2), 119.3 (CH-5), 120.5 (CH-4), 124.1 (C-11), 124.4 (C-11a), 133.9 (C-12), 133.3 (C-12a), 137.3 (CH-6), 149.1 (C-3), 161.3 (C-1), 161.6 (C-8), 181.4 (C═O), 191.6 (C═O); Elemental analysis calcd: C, 70.86; H, 3.96. Found: C, 70.47; H, 3.66. On the basis of these spectral data, compound **1** was identified as 1,8-dihydroxy-3-methyl anthraquinone (chrysophanol).
Compound **2**: Buff needles, able to sublime, mp: 207–209 °C, molecular formula $C_{15}H_{12}O_5$, M = 284. IR (film): 2979 (α-OH concluded with C═O), 1671 (dissociate C═O), 1625 (C═O concluded), 1564, 1475, 1383 (Ar-), 756. ^{1}H NMR (DMSO-d_6) δ ppm: 2.42 (3H, s, 3-CH_3), 3.92 (3H, s, 6-OCH_3), 6.86 (1H, s, 7-H), 7.17 (1H, s, 5-H), 7.26 (1H, s, 2-H), 7.51 (1H, s, 4-H), 11.95 (1H, s, 8-OH), 12.4 (1H, s, 1-OH). ^{13}C NMR (DMSO-d_6) δ ppm: 21.6 (CH_3), 56.5 (OCH_3), 104.8 (CH-7), 108.7 (CH-5), 118.9 (CH-11a), 119.8 (CH-2), 123.1 (CH-4), 124.5 (CH-11), 140.9 (CH-12), 142.0 (CH-12a), 142.8 (C-3), 158.5 (C-1), 159.9 (C-8), 168.0 (C-6), 187.5 (C═O), 187.9 (C═O). Elemental analysis calcd: C, 67.60; H, 4.25. Found: C, 67.99; H, 4.34. On the basis of these spectral data, compound **2** was identified as **1**, 8-dihydroxyl-3-methyl-6-methoxyl anthraquinone (physion).
Compound **3**: orange needles, able to sublime, mp: 224–225 °C, molecular formula: $C_{15}H_{10}O_5$, M = 270. IR (film), 3353 (OH, weak), 1720, 1670, 1622 (characteristic absorbance peaks of anthraquinone), 1564, 1471, 1456 (Ar-), 754. ^{1}H NMR (DMSO-d_6) δ ppm: 4.63 (2H, s, 3-CH_2OH), 7.28 (1H, s, 2-H), 7.37 (1H, dd, J = 1.2, 8.4 Hz, 7-H), 7.70 (1H, dd, J = 6.2, 6.0 Hz, 4-H), 7.72 (1H, dd, J = 1.2, 8.4 Hz, 6-H), 7.80 (1H, t, J = 7.2, 8.0 Hz, 5-H), 11.93 (2H, s, 1, 8-OH). ^{13}C NMR (DMSO-d_6) δ ppm: 62.0 (CH_2OH), 114.4 (CH-2), 115.9(CH-7), 117.1 (CH-4) 119.3 (CH-5), 120.6 (C-11), 124.4 (C-11a), 133 (C-12, 12a), 137.3 (C-6), 153.7 (C-3), 161 (C═O), 191.6 (C═O). Elemental analysis calcd: C, 66.67; H, 3.73. Found: C, 66.44; H, 3.95. On the basis of these spectral data, compound **3** was identified as 1,8-dihydroxy-3-hydroxymethyl anthraquinone (aloe-emodin).
Compound **4**: orange needles, mp: 254–256 °C, molecular formula: $C_{15}H_{10}O_5$. M = 270. IR (film), 3353, 3061 (α-OH), 1730, 1670, 1624(characteristic absorbance peaks of anthraquinone), 1558, 1475, 1451 (Ar-), 759; ^{1}H NMR (DMSO-d_6) δ ppm: 2.40 (3H, s, 3-CH_3), 6.56 (1H, s, 7-H), 7.09 (1H, s, 5-H), 7.12 (1H, s, 2-H), 7.45 (1H, s, 4H), 11.19 (1H, s, 6-OH), 11.99 (H, s, 1-OH), 12.07 (H, s, 8-OH). ^{13}C NMR (DMSO-d_6) δ ppm: 21.5 (CH_3), 107.9 (CH-7), 108.7 (CH-5), 108.9 (C-2), 120.4 (C-11a), 124.1 (C-11), 132.7 (C-12), 135.0 (C-12a), 148.2 (C-3), 161.4 (C-1), 164.4 (C-8), 165.5 (C-6), 181.2 (C═O-10), 189.6 (C═O-9). Elemental analysis calcd: C, 66.67; H, 3.73. Found: C, 67.15; H, 4.24. On the basis of these spectral data, compound **4** was identified as 1,6,8-trihydroxy-3-meththyl anthraquinone (emodin).
Compound **5**: yellow fine needles, mp: 320–321 °C, molecular formula: $C_{15}H_8O_6$, M = 284; IR (film): 3441 (COOH), 3407 (OH), 1693, 1673, 1624(C═O concluded), 1560, 1449, (Ar-), 750; ^{1}H NMR (DMSO-d_6) δ ppm: 7.38 (1H, d, J = 8.4 Hz, 7-H), 7.69 (1H, s, 5-H), 7.71 (1H, d, J = 4.8 Hz, 2-H), 7.81 (1H, t, J = 8.0, 8.0 Hz, 6-H), 8.07 (1H, s, 4-H), 11.87 (2H, s, 1, 8-OH); ^{13}C NMR (DMSO-d_6) δ ppm: 116.2 (2,7-CH), 119.0 (11′-C), 119.5 (11-C), 124.2 (4-C), 124.6 (5-C), 133.2 (3-C), 133.9 (6-C), 137.6 (12′-C), 138.0 (12-C), 161.1 (1-C), 161.4 (8-C), 165.5 (COOH), 181.0 (1-C═O), 191.3 (8-C═O). Elemental analysis calcd: C, 63.39; H, 2.84; O, 33.78. Found: C, 63.81; H, 3.15. On the basis of these spectral data, compound **5** was identified as 1,8-dihydroxy-3-carboxyl anthraquinone (rhein).

30. Cell culture and protein sample preparation: PC3 cells (prostate cancer cell lines) were seeded on a 6-well plate and were incubated in RPMI 1640 medium tamine plus 10% FBS at 37 °C (NIH/3T3 cells, in DMEM). After incubation for 36–48 h, the RPMI 1640 medium was removed and the cells were incubated with serum free medium for 24 h. Then cells were treated with the compounds at the concentration of 20 μM for 30 min followed with 40 ng/ml EGF for 10 min. The plate was then placed on the ice instantaneously to quench the phosphorylation process. Medium was sucked out and cells were then rinsed with ice-cold PBS buffer twice. Then cells were treated with lyss buffer (1%NP-40, 0.1% SDS, 150 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, 0.6 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 40 μg/ml PMSF) and sample buffer, respectively, followed by immunoblotting using P-ERK (E-4) (sc-7383, lot# J0803, Stanta Cruz Biotechnology). A431 cells, a human epidermoid carcinoma cell line (Cell Bank of Committee on

Type Culture Collection of Chinese Academy of Science), were cultivated in F-12 medium supplemented with 10% fetal bovine serum (TBD & HY Bio. Co) and 2 mM L-glutamine. Tissue culture reagents were obtained from Gibco Co. Western blot analysis: The cell lysates prepared above were subjected to 10% SDS–PAGE and proteins were transferred to PVDF membranes (Bio-Rad). The membrane was blocked with 5% nonfat dried milk freshly made in PBS plus 0.2% Tween 20 then incubated with monoclonal antibody against P-ERK1/2 (E-4) overnight at 4 °C. Then the membrane was washed 3×5 min with PBS plus 0.2% Tween 20. The membrane was incubated again with second antibody for 2–3 h at 25 °C, washed three times with PBS plus 0.2% Tween 20, and the signal was detected by enhanced chemiluminescence (ECL) detection system (PIERCE). Likewise NIH/3T3 cells induced by PDGF were assayed in the same way as PC3 cells presented above, except, the cell medium was DMEM and the growth factor was PDGF. Egger, D.; Bienz, K. *Mol. Biotechnol.* **1994**, *1*, 289–305.

31. MTT assay of cell proliferation: The viability of cultured cells was determined by assaying for the reduction of MTT [3-(4,5-dimethylthiazol-2yl)-2-5-diphenyl-tetrazolium bromide] to formazan. In brief, tested cells (2×10^3 cells/well/100 μL) in 96-well plates of the proper culture medium were treated with the compounds (all compounds tested were dissolved in 0.1% DMSO solution and subsequently diluted in the culture medium) at a concentration of 1–20 μM for 72 h. In parallel, the cells were treated with 0.1% DMSO as control. And DMSO was added to dissolve the purple formazan converted from MTT by a mitochondrial enzyme in living cell. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. Absorbance caused by formazan was measured at 595 nm with a microplate reader (BIO-RAD, model-680). Denizot, F.; Long, R. J. *Immunol. Methods* **1986**, *89*, 271.
32. Measurement of antifungal activity: The crude extracts and pure compounds were dissolved in 10 ml of 10% DMSO before being mixed with PDA (90 ml). The final concentration of extracts and pure compounds in the medium was fixed at 1 and 0.5 mg/ml, respectively. Fungi, *Gibberella zaeae*, *Fusarum oxysporum*, and *phytophthora infestans* were incubated in PDA (potato dextrose agar) at 27 °C for 6 days to get new mycelium for antifungal assay. Then a mycelial disk of approximately 0.45 cm diameter cut from culture medium was picked up with a sterile inoculation needle and inoculated in the center of PDA plate. These plates were then inoculated at about 25 °C and the data on diameter were reported on the fourth day. 1% DMSO in sterile distilled water served as control. For each treatment, three replicates were maintained to verify the result.