Anti-fibrotic activities of herbal compounds and herbs

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Abstract

Background. We recently developed high-throughput assays of inflammation-independent anti-fibrotic activities based on TGF-β1-induced total collagen accumulation and nodule formation in normal rat kidney fibroblasts.

Methods. These assays were applied to examine the anti-fibrotic activities of 21 compounds isolated from plants used in Chinese medicine and methanol extracts of 12 Chinese herbs. Lactate dehydrogenase release assay and cell detachment index were used to monitor cytotoxicity. Changes in fibrogenic molecular markers were observed by reverse transcriptase quantitative polymerase chain reaction and high-content imaging analysis of immunofluorescence.

Results. Three flavonoids (quercetin, baicalein and baicalin) and two non-flavonoids (salvianolic acid B and emodin) demonstrated anti-fibrotic activities in both total collagen accumulation and nodule formation assays. The remaining 16 compounds had little anti-fibrotic effect or were cytotoxic. The anti-fibrotic compounds suppressed collagen I expression at both mRNA and protein levels and also variably suppressed α-smooth muscle actin expression and bromodeoxyuridine incorporation. Methanol extracts of Scutellaria baicalensis Georgi, Salvia miltiorrhiza Bunge and Rheum palmatum L., which are rich sources of baicalein, baicalin, salvianolic acid B and emodin, respectively, also showed in vitro anti-fibrotic activities.

Conclusions. Five herbal compounds and three herbal extracts have in vitro anti-fibrotic activities. These data warrant further studies on these anti-fibrotic entities and suggest it a promising strategy to discover new anti-fibrotic drugs by screening more plant materials.

Keywords: fibroblasts; herbal compounds; renal fibrosis; TGF-β1

Introduction

Fibrosis, characterized by extracellular matrix (ECM) accumulation and disruption of normal tissue architecture, is the common cause of chronic failure of many organs, including kidneys, and is a leading cause of morbidity and mortality worldwide [1]. Unfortunately, despite the undoubted importance of fibrosis as an independent pathological entity and the everlasting research on fibrogenesis and anti-fibrotic therapy, there are few Western medicines demonstrating convincing clinical specificity and efficacy as anti-fibrotic drugs. Thus, it represents a huge challenge to develop specific and potent anti-fibrotic drugs [1].

In contrast to the lack of anti-fibrotic drugs in Western medicine, there is accumulating evidence suggestive of beneficial effects of plants used in traditional Chinese medicine and compounds isolated from medicinal plants in the treatment of fibrotic diseases [2]. These data, along with the well-documented pro-fibrotic activities of aristolochic acids and Aristolochia [3], form a balanced picture of the ‘herbal power’ to modulate the Yin (suppression) and the Yang (induction) of fibrosis. However, the importance of the reported anti-fibrotic activities of herbal medicines is poorly accepted by mainstream academic and industrial sectors due to a number of reasons, among which the following two are critical: (i) most conclusions in these reports were drawn from inflammation-induced fibrosis in animal models or patients and it is largely undetermined if the effects are due to inhibition of fibrosis per se, or simply due to inhibition of inflammation, the inciting factor of fibrosis; (ii) some reports were based on inflammation-free in vitro models; however, in these cases, the use of selected molecular markers might not truthfully reflect the net activities of the drugs on fibrosis [4]. We hypothesized that, among the herbal compounds suggested to be anti-fibrotic in the literature, at least some of them are genuine inhibitors of fibrosis by blocking transforming growth factor β1 (TGF-β1)-mediated fibrogenesis.

We have recently established TGF-β1-induced in vitro models of fibrosis, including a two-dimensional (2D) model, which visualizes and quantifies total collagen accumulation and a three-dimensional (3D) model, which monitors matrix-mediated cell monolayer disruption representing matrix-mediated disruption of the tissue architecture, and have refined these models for high-throughput detection of anti-fibrotic activities [5]. In the current study, we further characterized the potency and specificity of these models in detecting genuine anti-fibrotic activities from selected herbal compounds and herbal extracts. We have shown that 5 out of 21 herbal compounds and 3 herbs that
are rich sources of some of these compounds have net anti-fibrotic activities in our in vitro models and thus established the rationale to use these in vitro models to discover new anti-fibrotics from plant materials.

Methods

Materials

Twenty-one compounds (Table 1) isolated from different species of herbal plants were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All compounds were purified and used as national standards for quality control purposes in China. The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK). NRK-49F cells were seeded in collagen I-coated 96-well plates and non-tissue culture 96-well plates (BD Biosciences, Oxford, UK) for 2D and 3D in vitro models of fibrosis respectively, as previously reported [5]. NRK-49F cells were seeded at a density of 10^5 cells per well in DMEM supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 5% fetal calf serum (FCS, Sigma-Aldrich, Dorset, UK). NRK-49F cells were seeded in collagen I-coated 96-well plates and non-tissue culture treated 96-well plates (BD Biosciences, Oxford, UK) for 2D and 3D in vitro models of fibrosis respectively, as previously reported [5]. NRK-49F cells were seeded at a density of 10^4 cells per well in DMEM supplemented with 2.5% FCS and 2.5% BDTM Nu-Serum serum replaced with 2.5% FCS and 2.5% BDTM Nu-Serum serum replace

<table>
<thead>
<tr>
<th>Group</th>
<th>Effect</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Effective, with relatively low cytotoxicity</td>
<td>Quercetin, baicalin, baicalein, salvinolonic acid B, emodin</td>
</tr>
<tr>
<td>2</td>
<td>Efficacy coinciding with cytotoxicity</td>
<td>Curcumin, silybin, honokiol</td>
</tr>
<tr>
<td>3</td>
<td>Not effective</td>
<td>Salidroside, astragaloside IV, matrine, oxymatrine, anisodamine, ligustrazine, tashinine IIA, notoginsenoside R1, paeoniflorin, asiaticoside</td>
</tr>
<tr>
<td>4</td>
<td>Highly cytotoxic</td>
<td>Shikonin, tetrandrine</td>
</tr>
</tbody>
</table>

without TGF-β1 (5 ng/ml) in the presence of vehicle, herbal materials or IN1130 for an additional 48 h.

Cytotoxicity assays

Both lactate dehydrogenase (LDH) release and cell detachment index were used to assess the in vitro cytotoxic effects. Fifty microlitre supernatant from each well was collected and tested for LDH release according to the manufacturer’s instructions (Promega, Southampton, UK). Cytotoxicity was determined by the optical density (OD) value of spontaneous LDH release by cells under the applied conditions. As for the cell detachment index, cell detachment in each well was observed under microscope and was categorized using the following scoring criteria: a score of 0 represents no cell detachment and 4 represents a complete disruption of the cell monolayer. Scores 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 stand for detachment of 5%, 10%, 20%, 30%, 40%, 60% and 80% cells, respectively.

Picro-Sirius red (PSR) staining and spectrophotometric analysis

The anti-fibrotic activities of compounds in the 2D model were assessed by PSR staining and quantitative analysis, as we previously reported. In brief, the cells cultured in collagen I-coated 96-well plates were fixed in methanol overnight at -20 °C, carefully washed twice with phosphate buffered saline (PBS) and incubated in the 0.1% PSR staining solution (Sigma-Aldrich) at room temperature for 4 h [5]. The staining solution was then removed and the cells were washed three times with 0.1% acetic acid. The stained cells were dried and subjected to spectrophotometric analysis. PSR was eluted in 0.1 N sodium hydroxide, 200 μl per well. The plates were placed on a rocking platform for 1 h at room temperature before determining the OD value at 540 nm with a Dynex Technologies MRX spectrophotometer (Pior Laboratory Supplies Ltd, East Sussex, UK).

The quality control of the assays is summarized below. After 48 h exposure to 5 ng/ml TGF-β1, if the model is established upon PSR staining, there will be extracellular collagen deposition visible under microscope as shown in Figures 1–3, 5 and Supplementary Figures 1–3. In such cases, induction of average OD values might vary due to cell condition and some other unknown reasons, but it was always >0.2. In some cases, TGF-β1 only induces a slight increase in PSR staining in the cells, but not in the extracellular compartment; we define them as a failure of model induction, as extracellular collagen deposition rather than intracellular collagen synthesis/deposition is the golden standard for the diagnosis of fibrotic diseases. To set an objective criterion, based on 43 experiments, we selected OD 540 nm induction >0.2 as a cut-off definition to a successful model induction. In our experience, we have 76.7% successful induction in all our experiments. The reasons behind those unsuccessful experiments are not clear, but we think that cell condition, basal extracellular collagen deposition and PSR values, quality of TGF-β1 and occasional technical errors are likely to be the most important causes. Based on the above criteria, in our experience, induction of average OD 540 nm values ranged from 0.23 to 0.65 (0.35±0.019, mean ± SEM; N=33), leading to a relatively high inter-assay CV of 32.04%, which is in contrast to the relatively low intra-assay CV in each experiment (average 5.25%). Probably due to the low intra-assay CV of our assays, inter-assay variation of OD 540 nm values did not appear to significantly affect the reproducibility of screening assays of anti-fibrotic activities. In practice, we analysed 3–5 screening assays by normalizing TGF-β1-induced OD 540 nm values to 100% for all successful experiments and then define all other groups as percentage relative to the relevant TGF-β1 group (as shown in Supplementary Figure 5). As such, suppression of TGF-β1-induced OD 540 nm values to the anti-fibrotic positive control, IN1130, was always highly significant (P < 0.01–0.001), although IN1130 suppression of TGF-β1-induced OD 540 nm ranged from 40%, less than half of the TGF-β1-induced OD value, to 123%, namely 23% lower than the group without TGF-β1 treatment, suggesting a suppression of collagen accumulation under the basal condition. The average suppression was 80.81 ± 5.68% (mean±SEM; N=16), with intra-assay and inter-assay CVs at 6.05% and 28.07%, respectively.

Immunofluorescence assay and automated high-content imaging analysis

Immunofluorescence staining for collagen I and α smooth muscle actin (α-SMA) was performed in the 2D model of fibrogenesis in NRK-49F cells. In brief, the cell monolayer was fixed in cold methanol for 30 min and then blocked with 5% goat serum in PBS at room temperature. After 30 min incubation, the cells were incubated with mouse anti-collagen I antibody (diluted 1:100; Abcam plc, Cambridge, UK) or mouse
### Table 2. Anti-fibrotic herbal compounds and their plants of origin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular structure</th>
<th>ED₅₀ (µM)</th>
<th>Plant source</th>
<th>ED₅₀ of herbal extract (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td><img src="image" alt="Flavonoid Structure" /></td>
<td>22.26</td>
<td>Many fruits and vegetables</td>
<td>Not tested</td>
</tr>
<tr>
<td>Baicalin</td>
<td><img src="image" alt="Flavonoid Structure" /></td>
<td>45.92</td>
<td><em>Scutellaria baicalensis</em> Georgi</td>
<td>51.78</td>
</tr>
<tr>
<td>Baicalein</td>
<td><img src="image" alt="Flavonoid Structure" /></td>
<td>24.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-flavonoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salvianolic acid B</td>
<td><img src="image" alt="Non-flavonoid Structure" /></td>
<td>4.99</td>
<td><em>Salvia miltiorrhiza</em> Bge.</td>
<td>31.84</td>
</tr>
<tr>
<td>Emodin</td>
<td><img src="image" alt="Non-flavonoid Structure" /></td>
<td>13.58</td>
<td><em>Rheum palmantum</em> L.</td>
<td>66.84</td>
</tr>
</tbody>
</table>

*The circle indicates the core structure of flavonoids.*

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**Fig. 1.** Three flavonoid herbal compounds, quercetin, baicalin and baicalein (Group 1), inhibited TGF-β₁-induced total collagen accumulation in NRK-49F cells. Cells cultured in collagen I-coated 96-well plates were treated with increasing doses of quercetin (0–40 µM), baicalin (0–80 µM), baicalein (0–80 µM) or 1 µM IN1130, with 5 ng/ml TGF-β₁ for 48 h. Total collagen accumulation was visualized by picro-Sirius red (PSR) staining (A, E, I) and quantified by spectrophotometric analysis (B, F, J). Toxicity was determined by recording a mean cell detachment index (C, G, K) and measuring LDH release in conditioned media (D, H, L). N = 4–6 per group; each experiment was repeated at least twice with similar findings. *P < 0.05, **P < 0.01, ***P < 0.001 versus TGF-β₁ alone, respectively.
Fig. 2. Two non-flavonoid herbal compounds, salvianolic acid B (SAB) and emodin (Group 1), inhibited TGF-β1-induced total collagen production in NRK-49F cells. Cells cultured in collagen I-coated 96-well plates were treated with increasing doses of SAB (0–14 μM), emodin (0–40 μM) or 1 μM IN1130, with 5 ng/ml TGF-β1 for 48 h. Total collagen accumulation was visualized by PSR staining (A, E) and quantified by spectrophotometric analysis (B, F). Toxicity was determined by recording mean cell detachment index (C, G) and measuring LDH release in conditioned media (D, H). N = 4–6 per group; each experiment was repeated twice with similar findings. *P < 0.05, **P < 0.001 versus TGF-β1 alone, respectively.

Fig. 3. Herbal compounds down-regulated TGF-β1-induced collagen I and α-SMA expression and BrdU incorporation in NRK-49F cells. (A) Cells cultured in collagen I-coated 96-well plates were exposed to 5 ng/ml TGF-β1 in the presence or absence of 40 μM quercetin, 80 μM baicalin, 80 μM baicalein, 28 μM SAB and 20 μM emodin for 48 h, respectively. 10 μM BrdU was added to the cell culture at the last 14 h of the experiments. Total collagen accumulation was visualized by PSR staining and indirect immunofluorescence staining was used to visualize collagen I expression, α-SMA expression and BrdU incorporation (stained in red; nuclei were in blue). (B) Spectrophotometric analysis of PSR staining shown in (A). (C–E) BD bioimager analysis of the percentage of collagen I, α-SMA and BrdU positive cells shown in (A), respectively. (F) Cells were treated as described in (A) and collected for RT-qPCR analysis of COLA2(I) mRNA which was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. *P < 0.05, **P < 0.01, ***P < 0.001 versus TGF-β1 alone, respectively. Experiments were performed in triplicate and repeated for three times with similar results.
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Fig. 4. Herbal compounds suppressed TGF-β1-induced nodule formation in NRK-49F cells. (A) Cells were seeded in non-tissue culture treated 96-well plates and treated with or without 5 ng/ml TGF-β1 in the presence or absence of quercetin (40 µM), baicalin (80 µM), baicalein (80 µM), SAB (28 µM) and emodin (20 µM), respectively, for 48 h. Nodule formation and disruption of cell monolayer were recorded by photomicroscopy. Due to a high toxicity of emodin at 20 µM, a lower dose (10 µM) of emodin was used in (B). Experiments were performed in quadruplicate and repeated three times with similar results.

anti-α-SMA antibody (diluted 1:400; Sigma-Aldrich) overnight at 4°C. After PBS washing, the cells were incubated with Alexa Fluor 555-conjugated goat anti-mouse IgG antibody (1:400 dilution, Sigma-Aldrich) at room temperature for 1 h, stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/ml, Sigma-Aldrich) for 10 min and then subjected to high-content imaging analysis on a Pathway 435 Bicimager (BD Biosciences). The percentage of collagen I or α-SMA positive cells was determined by automatic scanning, measuring and counting of positive cells (collagen I or α-SMA positive) and total cells (DAPI positive).

Statistical analysis

Data were expressed as mean ± SE. Statistical differences of multiple groups were assessed by one-way analysis of variance (ANOVA), and the Tukey test was used to compare group pairs; a P-value < 0.05 is considered a significant difference. All analyses were accomplished using the software GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

Results

Twenty-one reported ‘anti-fibrotic’ herbal compounds were categorized into four distinct groups based on their in vitro efficacy and toxicity

A search of the MEDLINE and EMBASE databases using keywords ‘herbal’ and ‘fibrosis’ identified 21 herbal compounds as candidates to be examined in this study. These compounds have been shown to reduce fibrotic lesions in animal models of inflammatory fibrotic diseases, in clinical trials, or to suppress selected molecular markers of fibrosis in cell-based assays. We examined TGF-β1-induced 2D model of fibrogenesis in NRK-49F cells in the absence or presence of different concentrations of these herbal compounds.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from NRK-49F cells using the RNeasy Mini Kit (Qiagen, West Sussex, UK). cDNA samples were generated from the RNA samples using an Omniscript RT kit (Qiagen) and then subjected to qPCR to quantify rat COL1A2 mRNA expression using a TaqMan primer and probe mixture (Assay ID: Collagen_A2-I, Order number 1906874; Applied Biosystems, Warrington, UK). A rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer and probe mixture (Rn99999916_S1; Applied Biosystems) was used to normalize the relative expression levels of COL1A2 mRNA.
Fig. 5. Extracts of 3 herbs, *Scutellaria baicalensis* Georgi (SBG), *Salvia miltiorrhiza* Bunge (SMB) and *Rheum palmatum* L. (RPL), inhibited TGF-β1-induced total collagen accumulation in NRK-49F cells. Cells cultured in collagen I-coated 96-well plates were treated with increasing doses of herbal extracts (0–160 µg/ml) or 1 µM IN1130, with 5 ng/ml TGF-β1 for 48 h. Total collagen accumulation was visualized by picro-Sirius red (PSR) staining (A, D, G) and quantified by spectrophotometric analysis (B, E, H). Toxicity was recorded by a mean cell detachment index (C, F, I). *N* = 6 per group; each experiment was repeated at least twice with similar findings. *P* < 0.05, **P** < 0.01, ***P*** < 0.001 versus TGF-β1 alone, respectively.

**In vitro anti-fibrotic efficacy of five compounds**

As shown in Table 1, quercetin, baicalin, baicalein, salvianolic acid B (SAB) and emodin showed *in vitro* anti-fibrotic activities. Among these compounds, quercetin, baicalin and baicalein showed similar *in vitro* efficacy, suppressing total collagen deposition at concentrations ranging from 5 µM to 80 µM, with relatively low cytotoxicity (Figure 1A–L, respectively). SAB was more potent, demonstrating *in vitro* anti-fibrotic efficacy at concentrations as low as 1.75–14 µM, with little cytotoxicity (Figure 2A–D). Emodin showed a strong anti-fibrotic effect at concentrations around 20 µM, which were very close to concentrations that induced cell detachment, suggestive of cytotoxicity (Figure 2E–H). Interestingly, the three compounds that showed concentration-dependent *in vitro* anti-fibrotic efficacy share the same molecular core and belong to the family of flavonoids (Figure 1, Table 2). Both non-flavonoids showed poor dose dependence, but differed from each other in their range of non-toxic effective concentrations. SAB had a moderate efficacy but a wider effective concentration range (Figure 2A–D), while emodin was characterized by a potent anti-fibrotic effect observed at concentrations close to a toxic range (Figure 2E–H).

**Effects of the five anti-fibrotic herbal compounds on molecular markers of fibrogenesis**

To further confirm our results at the molecular level, we investigated the effects of 40 µM quercetin, 80 µM baicalin, 80 µM baicalein, 28 µM SAB and 20 µM emodin on TGF-β1-induced total collagen accumulation, collagen I expression, α-SMA expression and BrdU incorporation in NRK-49F cells. As expected, these compounds inhibited TGF-β1-induced total collagen accumulation (Figure 3A and B). Immunostaining and high-content imaging analysis revealed that TGF-β1 significantly increased collagen I expression, which was similarly suppressed by all five compounds (Figure 3C). Although baicalin was fairly potent in suppressing total collagen and collagen I accumulation, it was not as effective in suppressing α-SMA expression (Figure 3D). Quercetin was among the most potent in suppressing protein expression of collagen I (Figure 3A and C) and α-SMA (Figure 3A and D), as well as COLA2 (I) mRNA expression (Figure 3F), but it was among the least potent in inhibiting accumulation of total collagen (Figure 3A and B) and BrdU incorporation (Figure 3A and E). Emodin was moderately effective in suppressing total collagen accumulation (Figure 3A and B), although it potently suppressed TGF-β1-induced collagen I accumulation and COLA2 (I) mRNA expression (Figure 3C and F), as well as...
α-SMA expression and BrdU incorporation (Figure 3D and E). The efficacy of herbal compounds to suppress α-SMA and collagen I expression has been confirmed by Western hybridization (Supplementary Figure 4).

**Herbal compounds inhibited 3D models of fibrogenesis in NRK-49F cells**

In agreement with the anti-fibrotic effects of quercetin, baikalin, baicalein and SAB in the 2D model, these compounds also suppressed TGF-β1-induced nodule formation [5]. Baicalein was the most potent in this assay (Figure 4A). On non-tissue culture-treated surfaces, NRK-49F cells tolerated emodin poorly and 20 µM emodin induced dramatic cell detachment, again suggestive of its higher cytotoxicity (Figure 4A). However, lower concentrations of emodin (10 µM) did prevent TGF-β1-induced nodule formation, although it did not prevent the spindle-like cell morphologic changes induced by TGF-β1 (Figure 4B).

**Extracts of three herbs rich in baikalin, baicalein, SAB or emodin, respectively, also showed anti-fibrotic activities**

As part of an ongoing project to screen the anti-fibrotic activities in methanol extracts of herbs and herbal mixtures, which are potential anti-fibrotic candidates based on literature reports, we have screened 12 authenticated herbs, including **Scutellaria baicalensis** Georgi, which is rich in baikalin and baicalein, **Salvia miltiorrhiza** Bunge, which is rich in SAB, as well as **Rheum palmatum** L., which is rich in emodin (Table 2). As shown in Figure 5, these three herbal extracts showed anti-fibrotic activities in contrast to the remaining nine herbal extracts that either lacked anti-fibrotic activity or exerted high toxicity (data not shown). In brief, **Scutellaria baicalensis** Georgi showed excellent dose-dependent anti-fibrotic activity in a wide range of concentrations (10–160 µg/ml), without inducing significant cell detachment at concentrations as high as 160 µg/ml (Figure 5A–C). **Rheum palmatum** L. showed weak suppression of total collagen accumulation at concentrations of 10–40 µg/ml, but higher concentrations induced significant cell toxicity although total collagen staining was more potently reduced (Figure 5D–F). **Salvia miltiorrhiza** Bunge showed less dose-dependent anti-fibrotic effect at concentrations of 10–160 µg/ml, but significant cell detachment was also documented at the concentration of 80 µg/ml and above (Figure 5G–I). Since herbal remedies comprising two or more herbs are commonly used in the clinical practice of Chinese medicine, we have also screened the in vitro anti-fibrotic activities of 27 herbal remedy extracts, and the results are shown in Supplementary Figure 5 and summarized in Supplementary Results.

**Discussion**

**In vitro anti-fibrotic activities of herbs and their potential in treatment of fibrotic diseases**

In traditional medicines there have been reports of both profibrotic and anti-fibrotic herbs. Pro-fibrotic herbs are best illustrated by aristolochic acid nephropathy, which was first reported in Belgium but is now appreciated as a worldwide problem [3]. In this study, we have examined 21 candidate anti-fibrotic herbal compounds and 12 herbal extracts in our in vitro models of renal fibrosis and have identified 5 compounds and 3 herbs, which show in vitro anti-fibrotic activities. **Scutellaria baicalensis** Georgi, which is a rich source of baikalin and baicalein, has been widely reported to treat liver diseases [2], although seldom been tested in the setting of kidney diseases. Whereas, **Rheum palmatum** L., which contains emodin, is widely used in China to treat patients with chronic renal failure and has been documented as anti-fibrotic in kidneys [7]. Our present data (Figures 1, 2, 4) suggest that the in vitro anti-fibrotic activity of these herbal compounds and extracts should, at least in part, be mediated via inflammation-independent pathway. These data, together with previous reports on the anti-fibrotic efficacy of emodin in liver and pancreas [8,9], support the notion that although fibrosis of different organs may involve different mechanisms, common mechanisms and thus common preventive strategies may exist for fibrotic and sclerotic diseases of different organs, such as liver and kidneys [1].

**In vitro anti-fibrotic activity of quercetin and its nutritional and pharmaceutical implications**

Our observation may provide insights into the potential of nutritional treatment in chronic renal diseases and possibly other fibrotic diseases. As shown in Figure 1A–D and Table 2, quercetin, which is one of the most abundant flavonoids in the human diet [10], showed dose-dependent anti-fibrotic activity and relatively low cytotoxicity. Parallel to our data, quercetin has been previously reported to be renoprotective in a number of in vivo models of renal injury including cyclosporine A nephropathy [11], cadmium nephropathy [12], myoglobinuric acute renal failure [13], diabetic nephropathy [14], ferric nitrilotriacetate nephropathy [15], ischaemia-reperfusion renal injury [16], as well as ureteral obstruction [17] and sick serum nephritis rat models [18]. In addition, quercetin has been reported to reduce oxidative stress and oxidative stress-induced signaling in in vitro models such as cultured renal tubular cells [19] and rat mesangial cells [20,21]. Apart from its renoprotective effects, quercetin also exhibited hepatoprotective and anti-fibrogenic effects against chronic biliary-obliterative liver injury [22]. If the anti-fibrotic property of quercetin is applicable to patients, it will be reasonable to propose increasing intake of fruits and vegetables rich in quercetin for patients with kidney diseases to prevent the onset and progression of fibrosis.

**In vitro anti-fibrotic activities of baikalin and baicalein: pharmaceutical implications on flavonoids as a family of anti-fibrotics?**

Besides quercetin, two additional flavonoids extracted from **Scutellaria baicalensis** Georgi, i.e. baikalin and baicalein, which share a similar core structure to quercetin (Table 2) showed similar in vitro anti-fibrotic activities. Both
compounds and *Scutellaria baicalensis* Georgi have been widely studied in protection against inflammation and fibrosis of liver [2,23,24] and lung [25]. Our data further showed that these compounds have inflammation-independent anti-fibrotic activities in renal fibroblasts. It remains obscure if the shared core structure is necessary for the anti-fibrotic activities of flavonoids, and if anti-fibrotic efficacy is a shared pharmacological action of all flavonoids, but we have noted that Bang Qiang Gong et al. have filed a US patent application on the value of flavonoids in the treatment of fibrotic diseases (US Patent Application number 10746632; filed on 23 December 2003). In view of beneficial actions of flavonoids in maintaining health and fighting against diseases [26] and their potential anti-fibrotic effect, we are tempted to propose that this group of small biomolecules might have an important value as both essential nutrients and potential drugs for patients with fibrotic diseases.

**Distinct anti-fibrotic activities of two non-flavonoids and the cytotoxicity issue**

The *in vitro* assays we used in this study also discovered anti-fibrotic activities of two non-flavonoids, emodin and SAB. These non-flavonoids were generally more potent than the three flavonoids, with lower ED$_{50}$ values in suppressing TGF-β1-induced total collagen accumulation (Table 2) but were interestingly less dose-dependent than flavonoids (Figure 2 versus Figure 1). This all-or-none pattern of action in contrast to the concentration-dependent effects of flavonoids might implicate different mechanisms of action. Emodin and SAB also differ from each other in that emodin shows a much higher toxicity. This is not surprising as emodin was first isolated as a toxin in 1975 [27] and since then its cytotoxicity has been well documented in cellular systems [28,29]. Although its toxicity *in vivo* (including during development) has been reported to be minimal [30], its toxicity in the setting of long-term treatment of renal fibrosis deserves special attention. However, toxicity does not necessarily lead to side effects but could, on the contrary, represent therapeutic actions. Taking emodin for example, it induces apoptosis of cancer cells and this might have made it a potential anti-cancer drug [31]. In the setting of fibrogenesis, if it selectively induces apoptosis of proliferative fibroblasts and myofibroblasts, it might well reduce the number of fibrogenic mesenchymal cells and thus suppress fibrosis. However, the case seems to be more complicated as it also leads to apoptosis of cultured human proximal tubular cells *in vitro* [29].

**Limitation and promises of our *in vitro* assays in the discovery of novel anti-fibrotic herbal compounds**

As discussed above, cytotoxicity might contribute towards anti-fibrotic activity. Our *in vitro* models of fibrosis rely on cells remaining in the culturing system for reliable data on total collagen accumulation and nodule formation [5]. Thus, throughout our study, we monitor cell attachment to control the credibility of our assays. However, while our data help establish the genuine anti-fibrotic effects of three flavonoids and two non-flavonoids (Group 1 in Table 1), we do not exclude the possibility of compounds in Groups 2 and 4 playing an anti-fibrotic role by clearing up matrix-producing cells; neither do we exclude compounds in Groups 2–4 as being anti-fibrotic via inhibiting the upstream of TGF-β-induced fibrogenesis, inhibiting epithelial-to-mesenchymal transition of tubular epithelial cells, or by directly inhibiting the inciting factors of fibrosis such as hypertrophy and inflammation. For instance, although our assays did not support curcumin as a good suppressor of TGF-β-induced total collagen accumulation, we do not exclude the possibility that it has an anti-fibrotic role via other mechanisms. In fact, it has been recently demonstrated that curcumin prevented and even reversed experimental cardiac fibrosis by inhibition of TGF-β1 expression, cardiac hypertrophy and inflammation [32], and it has also been demonstrated that it can correct cystic fibrosis defects in mice [33].

Despite certain limitations, our 2D model is so far the most clinically relevant *in vitro* model of fibrosis, as it is validated based on elution and spectrophotometric analysis of PSR staining, which is one of the gold standards for clinical diagnosis of fibrosis [34,35]. Our 3D model is complementary to the 2D model and represents excessive extracellular matrix-induced tissue contraction, which leads to distortion of tissue architecture and contributes to progressive loss of organ functions [5]. By showing all five identified herbal compounds capable of reducing TGF-β1-induced total collagen accumulation and nodule formation, our data thus predict that these compounds might reduce fibrotic lesions and reserve renal functions *in vivo*. It might seem disappointing that all herbal compounds identified in this study worked through inhibiting traditional fibrosis markers like collagen I and α-SMA or fibroblast proliferation (Figure 3), but this is not surprising as these compounds were originally selected as anti-fibrotic candidates largely based on their impact on these traditional parameters. Furthermore, all compounds identified in this project so far were only moderately effective as none was as effective, as IN1130, an anti-fibrotic through inhibition of TGF-β type I receptor Alk5 [6]. Given that we have proven the feasibility to detect anti-fibrotic activities from crude herbal extracts, we hope that, through further activity-guided screening of additional herbs and isolation of compounds from hopeful extracts, our approach might ultimately lead to discovery of novel, robust and less toxic anti-fibrotic herbal compounds through known or novel mechanisms.

In summary, our data have established *in vitro* anti-fibrotic activities of five compounds and three herbs and have proven our *in vitro* models of renal fibrogenesis useful in the discovery of inflammation-independent net anti-fibrotic activities from herbal medicines. Although our screening data of herbal remedies, shown in Supplementary Figure 5, used commercial herbs that meet a lower stringency in authentication and quality control, we found were that most herbal remedies containing the three anti-fibrotic herbs also showed anti-fibrotic efficacy. These results support the idea that herbs with anti-fibrotic activities used in herbal remedies might play an anti-fibrotic role in patients, and thus our preliminary data on herbal remedies might serve as a reference for Chinese medicine.
practitioners in remedy selection. The results also showed that some remedies with in vitro efficacy do not contain the three anti-fibrotic herbs. Most likely, they contain other herbs with similar efficacy. The present data warrant further studies to explore the effects and mechanisms of the identified anti-fibrotic herbal compounds in preventing renal fibrosis and urge us to screen more plant materials to discover novel anti-fibrotic drugs.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data are available online at http://ndt.oxfordjournals.org.

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