Dioscorea villosa (wild yam) induces chronic kidney injury via pro-fibrotic pathways

Ken Wojcikowski a,b,*, Hans Wohlmuth b,c, David W. Johnson d, Glenda Gobe a

a Molecular and Cellular Pathology, School of Medicine, University of Queensland, Brisbane, Queensland, Australia
b Department of Natural and Complementary Medicine, Southern Cross University, Military Road, Lismore, NSW 2480, Australia
c Medicinal Plant Herbarium, Southern Cross University, Lismore, NSW 2480, Australia
d Department of Renal Medicine, University of Queensland, Princess Alexandra Hospital, Woolloongabba, Brisbane, Queensland, Australia

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A B S T R A C T

Dioscorea villosa (wild yam) rhizome extract is a medicinal herb that is commonly used to treat symptoms of menopause and rheumatoid arthritis. We had evidence from previous in vitro experiments that this extract is toxic and pro-fibrotic in renal cells and aimed to test whether this occurs in vivo. Sprague-Dawley rats received 0.79 g/kg/d D. villosa extract in their food or no treatment over 7, 14 and 28d (n = 4 per group). Kidney and liver tissues were collected for protein extraction and Western immunoblots or fixed for special histologic stains, immunohistochemistry (IHC) and microscopy. Collagen deposition was assessed using Masson’s trichrome staining and morphometry. Macrophage infiltration (ED-1), epithelial-to-mesenchymal transdifferentiation or activation of fibroblasts (vimentin, α-SMA), and pro-fibrotic growth factors (TGFβ1, CTGF) were assessed using IHC. Protein expression levels of the pro-inflammatory cytokine, TNF-α, the pro-fibrotic transcription factor, NFκB, a measure of oxidative stress (heme oxygenase-1, α-SMA), vimentin and TGFβ1 were determined. Results showed that kidneys of the treated animals had significantly increased collagen, vimentin, TGFβ1, NFκB, EDI, CTGF and α-SMA by 28d. In the liver, there was increased ED-1 and TGFβ1 in the centrilobular zone at 28d in treated animals. In conclusion, there was no acute reno- or hepato-toxicity associated with administration of D. villosa. However, there was an increase in fibrosis in the kidneys and in inflammation in livers of rats consuming D. villosa for 28 days. Long term supplementation with D. villosa may be best avoided, especially in people with compromised renal function and in those who need to take other drugs which may alter kidney function.

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1. Introduction

The incidence of chronic kidney disease (CKD) in industrialised countries is rapidly increasing partly due to the increase in chronic disorders such as obesity, diabetes, and peripheral artery disease. Other factors contributing to the development or progression of CKD include environmental toxins, recreational drugs, smoking, pharmaceutical therapies and over the counter medications (Blowey, 2005; Costa et al., 2004; Yu, 2003). Importantly, certain herbs may also contribute to the development or progression of CKD by exhibiting direct toxicity, altering inflammatory pathways, and other mechanisms (Blowey, 2005; Wojcikowski et al., 2004). The well-documented example is the presentation of aristolochic acid nephropathy (AAN; formerly referred to as Chinese herbs nephropathy) after consumption of herbs containing aristolochic acid (AA) (Wojcikowski et al., 2006). The nephrotoxic and chronic pro-fibrotic action of AA in the kidneys of humans was found only after their exposure to AA (Wojcikowski et al., 2006). It is therefore important that herbs continue to be investigated for their potential contributions to CKD and its correlate, renal fibrosis.

Dioscorea villosa (wild yam) is a medicinal type of yam that is widely touted to be beneficial for menstrual complaints, perimenopausal symptoms, rheumatoid arthritis and other complaints (Braun and Cohen, 2007; Carroll, 2006; Soffa, 1996). D. villosa belongs to a large genus with 600–700 species, many of which have been proven to exhibit toxic properties (Bhandari and Kawabata, 2005; Su et al., 2003). Investigations of individual components found within most Dioscorea sp. in macrophage cultures have reported increases in the expression of tumour necrosis factor (TNF)-α, interleukin-1 (IL-1), IL-6 and other inflammatory cytokines (Choi and Hwang, 2002; Liu et al., 2007). Despite the popularity of this herb, the potential effect on inflammatory cytokines and the toxicity associated with many Dioscorea sp., there has been little work done to prove the safety of D. villosa. One report

Abbreviations: AA, aristolochic acid; AAN, aristolochic acid nephropathy; α-SMA, α-smooth muscle actin; CKD, chronic kidney disease; CTGF, connective tissue growth factor; EMT, epithelial-to-mesenchymal transdifferentiation; HO-1, heme oxygenase-1; IHC, immunohistochemistry; IL, interleukin; NFκB, nuclear factor κB; TBST, Tris-buffered saline and 0.05% Tween20; TGFβ1, transforming growth factor-β1; TNF-α, tumor necrosis factor alpha.

* Corresponding author. Address: Department of Natural and Complementary Medicine, Southern Cross University, Military Road, Lismore, NSW 2480, Australia. Tel.: +61 2 6620 3059; fax: +61 2 6620 3307.
E-mail address: kwojcikoscu.edu.au (K. Wojcikowski).
described an animal toxicity study in which Sprague-Dawley rats received 500 or 2000 mg/kg oleyl alcohol extract of *D. villosa* in a single dose (*Anonymous*, 2004). Those rats receiving the larger dose experienced hypothermia, piloerection and dyspnoea (*n* = 10) or death (*n* = 1). There was no apparent macroscopic change to the organs. Of note, however, no histological or biochemical parameters were investigated. In our preliminary *in vitro* studies involving aqueous, methanol and ethyl acetate extracts, every extract of *D. villosa* tested was highly toxic to renal tubular epithelial cells (*Wojcikowski et al.*, 2008). Furthermore, lower concentrations of all extracts caused apparent transdifferentiation of the surviving renal tubular epithelial cells into cells that expressed α-smooth muscle actin (α-SMA) and had a fibroblastic phenotype (*epithelial-to-mesenchymal transdifferentiation, or EMT*). The activated fibroblast, the myofibroblast, frequently identified by its expression of α-SMA, is a key cellular mediator of fibrosis, and a primary collagen-producing cell (*Pat et al.*, 2007).

The development of renal fibrosis is a complicated process that involves numerous molecular interactions and various cytokines and growth factors. Among these, it is widely accepted that transforming growth factor-β1 (TGFβ1) plays a key role in renal fibrosis, as up-regulation of TGFβ1 is a universal finding in virtually every type of CKD, no matter the cause (*Prud'homme*, 2007). TGFβ1 stimulates several pathways in mesangial cells, interstitial fibroblasts and tubular epithelial cells to undergo myofibroblastic activation or transition (*Bottinger*, 2007). TGFβ1 also decreases collagenase, thus preventing the breakdown of the newly formed collagen (*Wang et al.*, 2005). The connective tissue growth factor (CTGF) gene expression is strongly induced by activated TGFβ1, and CTGF, together with TGFβ1, stimulates several pathways in mesangial cells, interstitial fibroblasts and tubular epithelial cells to undergo myofibroblastic activation or transition. Furthermore, CTGF inhibits several cell types from undergoing myofibroblastic activation. In addition, CTGF induces the expression of endothelial adhesion molecules and chemokines that attract inflammatory leukocytes and more macrophages to sites of tissue injury and stimulates leukocytes to release chemokines and more inflammatory cytokines. These key molecular players in renal fibrosis are involved in positive reinforcing loops with angiotensin II and the transcription factor, nuclear factor κB (NFκB). Drugs or biologically active substances within herbal therapies have the potential to affect the development of renal fibrosis in many ways, including anywhere along these inflammatory pathways.

The aim of the present study was to determine whether rats ingesting *D. villosa* have altered levels of inflammatory cytokines or growth factors, infiltration of macrophages or fibroblasts or evidence of measurable fibrosis after consuming the herb for 7–28 days. Both kidney and liver were analysed. We also tested the potential of *D. villosa* to alter heme oxygenase-1 (HO-1) expression, which, if raised, would suggest oxidative stress is a mechanism of fibrosis.

### 2. Materials and methods

#### 2.1. Plant material and extraction

One kilogram of crude *D. villosa* rhizome was obtained from a reliable supplier and authenticated by a pharmacognosist (H. Wohlmuth, Southern Cross University Medicinal Plant Herbarium) by chemical and morphological comparison with an authentic reference specimen. The dried plant material was ground to a powder using a Retsch SM2000 (Germany) mill fitted with a 0.5 mm screen and extracted with a three-solvent sequential process. Ground material was sonicated (10 min) in warm (40 °C) anhydrous ethanol and filtered (Whatman No. 3, gravity filtration). After a second and third extraction with that solvent, the process was repeated with warm (40 °C) aqueous ethanol (1:1; v:v) and finally hot (90 °C) water. All filtrates were combined, reduced (total yield 0.21 mg/g starting material) and distributed equally into the rat chow (0.72% dried *D. villosa* extract). The modified rat chow was stored at 4 °C for less than 30 days before use.

#### 2.2. Animals and experimental protocol

Male Sprague-Dawley rats (175–200 g) were used for the experiments and maintenance according to guidelines approved by the University of Queensland Animal Ethics Committee. Twenty-four rats were randomly divided into six experimental groups (*n* = 4) to receive normal rat chow or the same rat chow with *D. villosa* extract within the chow for 7, 14 or 28 d. The rats were monitored daily for signs of anorexia, weight loss, acute toxicity and average food consumption.

#### 2.3. Functional studies

Blood sampling from the aorta was obtained during sacrifice. Plasma samples were processed for the determination of sodium, chloride, potassium, urea, creatinine, and lactate dehydrogenase.

#### 2.4. Histological analysis

Liver and kidney tissues obtained from the sacrificed rats were fixed in 4% buffered paraformaldehyde, embedded in paraffin using routine histological techniques, sectioned at 4 μm, and mounted on SuperFrost Plus glass slides (Menzel Gläser, Braunschweig, Germany). Sections were dewaxed, rehydrated, stained with hematoxylin and eosin (HE) for morphological assessment and with Massons trichrome for assessment of fibrosis/collagen as previously described (*Pat et al.*, 2007). Massons trichrome staining is a well accepted marker of fibrosis. Its limitations include that it indicates collagen and none of the other extracellular matrix proteins, and does not take into account any oedematous change. For quantification of collagen, Massons trichrome-stained slides were visualized using a Nikon EPI500 microscope (Kanagawa, Japan) fitted with a Spot RT slider cooled CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and captured directly as digital images (magnification ×200). All slides stained with Massons trichrome were photographed on the same day to avoid any variability associated with the light source. Image morphometry was performed using ImageProPlus image-analysis software (Version 4.1.29, Media Cybernetics, Silver Spring, MD), which automatically calculates the area of blue stained colour in each of the sections. For liver slices, a total of 15 fields representing five fields each of medium-sized portal areas, medium-sized central veins, and parenchyma, were randomly chosen for each rat. Each set of five fields was analysed separately. For kidney slices, a total of eight fields, representing two fields from the upper cortex, three fields from the mid cortex and three fields from the medulla were randomly chosen. A field was not considered if it contained more than two small blood vessels which also stain positive for collagen. All slides were photographed by the same investigator (KW), who was blinded to the treatment procedure, and images were reviewed by a second investigator (GG) similarly blinded to treatment procedure. Results were presented as microns of area stained per 376,534 micron fields examined.

#### 2.5. Western blot analysis

The following antibodies were used as primary antibodies for Western blot analysis: Polyclonal goat anti-TNF-α (Santa Cruz Biotechnology; Santa Cruz, CA; 1:200), monoclonal rabbit anti-TNF-α (Santa Cruz Biotechnology; 1:300; molecular weight, 12.5 and 25 kDa, as per protocol by supplier), monoclonal mouse anti-vimentin clone VIM31B4 (Novacatt, Laboratories Ltd., Newkirk, UK; 1:1000), polyclonal rabbit anti-NFκB L-20: sc-146, (Santa Cruz Biotechnology; 1:250), monoclonal mouse anti-HO-1 (Stressgen, Victoria, BC; 1:1000), polyclonal goat anti-TGFβ1 (Santa Cruz Biotechnology; 1:2000), monoclonal mouse anti-β-actin (Stressgen, Victoria, BC; 1:1000), polyclonal mouse anti-α-SMA clone 1 A4 (Sigma, St. Louis, MO; 1:400).

A portion of the renal cortex and medulla from each kidney and liver parenchyma was individually homogenized in ice-cold HEPES buffer containing protease inhibitors (50 mM/l Tris HCl [pH 7.5], 150 mM/L NaCl, 5 mM/L EDTA, 1 g/ml leupeptin, 1 mM/L phenylmethylsulfonyl fluoride [PMSF], and aprotinin 1 g/ml). Cell debris was analysed by pelleting centrifugation at 13,000 g for 20 min. The liquid portion was removed, the process repeated, and the protein content of the supernatant was determined using the Bradford assay. Samples containing 40 μg of protein were analysed using SDS–polyacrylamide gel electrophoresis (10% acrylamide gel). Samples were prepared in buffer containing β-mercaptoethanol and boiled for 5 min before loading. After wet transfer at 100 V for 1 h onto PVDF transfer membranes (Perkin-Elmer, Boston MA), the filter was blocked with 5% BLOTTO (5% skim milk, 1 v:v Tris-buffered saline and 0.05% Tween20 [TBST]; Sigma–Aldrich, Sydney Australia) for 1 h at ambient temperature. The blot was then incubated with the primary antibody overnight at 4 °C with gentle rocking. The blots were washed four times in TBST for 5 min, followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase (*Zymed, San Francisco, CA*) for 1 h at ambient temperature. Blots were washed 4 times in TBST for 5 min, three times in TBS for 5 min and developed with SuperSignal® blotting detection reagents according to the protocol provided by the manufacturer (Pierce, Rockford, IL). Equal loading of protein was determined by Coomassie brilliant blue staining and by Western immunoblot using a mouse anti-β-actin antibody (1:5000 dilutions, 1:5000 dilutions).
Table 1
Effect of Dioscorea villosa on metabolic parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Sodium (mmol/L)</th>
<th>Chloride (mmol/L)</th>
<th>Urea (mmol/L)</th>
<th>Creatinine (µmol/L)</th>
<th>Lactate dehydrogenase (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>142.3 ± 1.25</td>
<td>99.5 ± 2.5</td>
<td>6.25 ± 0.3</td>
<td>32.25 ± 2.36</td>
<td>716.3 ± 271.5</td>
</tr>
<tr>
<td>7d D. villosa</td>
<td>142.0 ± 1.22</td>
<td>100.0 ± 0.70</td>
<td>6.5 ± 0.52</td>
<td>32.75 ± 1.89</td>
<td>998.0 ± 184.4</td>
</tr>
<tr>
<td>14d D. villosa</td>
<td>138.3 ± 1.79</td>
<td>99.5 ± 1.04</td>
<td>7.00 ± 0.58</td>
<td>34.75 ± 2.66</td>
<td>660.5 ± 72.9</td>
</tr>
<tr>
<td>28d D. villosa</td>
<td>138.0 ± 1.08</td>
<td>99.0 ± 1.080</td>
<td>6.35 ± 0.37</td>
<td>36.25 ± 2.96</td>
<td>1119 ± 337.2</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM, n = 4. There were no significant differences between groups in any of the serological parameters investigated.

Fig. 1. Representative photomicrographs of staining for fibrosis, myofibroblast activation and differentiation in the kidneys. (A) Masson’s trichrome staining for matrix protein in a control rat showed almost no staining in the interstitium. (B) Masson’s trichrome staining of the 28d treated group revealed mild renal tubulo-interstitial fibrosis with evidence of extra collagen in the interstitium. (C) α-SMA staining in controls found slight staining in the blood vessels (BV). (D) α-SMA of the 28d treated animals found more intense sporadic staining in the interstitium especially in interstitial fibroblasts (arrowheads). Blood vessels stained very intensely in these animals. (E) Expression of vimentin in controls revealed small sporadic staining of arterioles and glomeruli. (F) Vimentin positive cells (arrowheads) in the 28d treated rats markedly increased in diffuse peritubular and glomerular patterns (magnification ×400).
Siga–Aldrich). Band intensities were quantified by using an image analyser and Scion software (Scion Corp., Frederick, MD). All experiments were performed in triplicate.

2.6. Immunohistochemistry (IHC)

The following antibodies were used as primary antibodies for IHC: monoclonal rabbit anti-TGFβ1, (Cell Signaling; Beverly, MA; 1:50), monoclonal mouse anti-vimentin clone V9M3B4 (Novacool Laboratories Ltd., Newcastle, UK; 1:250), monoclonal mouse anti-ED-1 (Serotec Ltd., Oxford, UK; 1:150) and mouse monoclonal anti-α-SMA clone 1 A4 (Sigma, St. Louis, MO; 1:400).

Four micron tissue sections were cut to Superfrost Plus slides (Menzel Glaeser, Braunschweig, Germany), airdried overnight at 37°C, dewaxed, re-hydrated in graded ethanol to phosphate buffered saline (PBS), and immersed into 1.0% hydrogen peroxide, 0.1% sodium azide in PBS for 10 min at ambient temperature to block endogenous peroxidase activity. Sections were washed in PBS (3 × 5 min changes), before being incubated for 20 min with normal goat serum diluted 1:10 with 0.1 mol/L PBS, pH 7.4. Sections were then incubated with the primary antibody with PBS overnight (18 h) at 4°C. The following day the sections were thoroughly washed in PBS (3 × 5 min changes), and incubated with the appropriate secondary antibody (DAKO Envision, DAKO Cytomation, Glostrup, Denmark) for 30 min. Visualization of antibody localization was facilitated with diaminobenzidine hydrochloride (DAB). Sections were rinsed with PBS (2 × 5 min) followed by counterstaining with Mayer’s haemotoxylin, blued in Scott’s tap water, dehydrated, cleared and mounted using DPX mounting medium. Negative controls were prepared without primary antibody and were consistently negative.

For quantification of TNF-α, TGFβ1, vimentin, NFκB, HO-1 and α-SMA, IHC slides were visualized using a Nikon EP600 microscope and image morphometry was analyzed using ImagePro Plus image-analysis software, as described above. For quantification of macrophages in the liver, a total of 15 fields representing five fields each of medium-sized portal areas, medium-sized central veins, and parenchyma were randomly chosen from each rat and the number of ED-1 positive cells from each area was analyzed separately. For glomerular macrophage accumulation, 25 glomeruli were randomly chosen from each rat and the number of ED-1 positive cells was quantified. In the kidney interstitium, macrophages were quantified in two ways to determine global and focal changes. For global changes, 25 fields from each rat were randomly chosen and the number of ED-1 positive cells was quantified for each field. For focal quantification of macrophages in the kidney interstitium, the five areas of greatest ED-1 stain intensity were determined at low resolution (×100). Five fields from each of these areas were then randomly chosen and quantitated by counting positively-stained cells under higher magnification. The number of ED-1 positive cells was analyzed using a magnification of ×400 and scored under blinded conditions.

2.7. Statistical analysis

Student’s t-test was used to analyse differences between the two groups. A one-way ANOVA test (Dunnett method) was used to assess the differences among groups. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. General parameters and histology

There were no significant differences in serological measurements (Table 1), rat weights amongst groups at the beginning or end of the experiment, or food consumed. Rats in the treated groups consumed 0.79 ± 0.06 g/kg/d D. villosa dried extract equivalent, a dose comparable to animal (Chang et al., 2004; Lee et al., 2002) and human studies (Wang et al., 2005) testing the positive effects of rhizomes from the Dioscorea genus. No animals died during the course of the experiment and the animals appeared healthy. There was no macroscopic evidence of frank fibrosis in the kidneys or livers of any single animal or group. Initial inspection of the kidney slides with HE staining from the treated groups by an investigator blinded to the treatment group showed no apoptosis, necrosis or other cell injury in the kidneys of those rats consuming D. villosa.

3.2. Fibrosis, myofibroblast activation and EMT

Examination of the Masson’s trichrome-stained sections showed that control rats had a normal histological appearance. No increase in the extent of tubulo-interstitial staining for Mas-
control and treated rats, respectively. Quantification of the results showing collagen accumulation is presented as a graph in Fig. 2A. α-SMA was used to analyze for myofibroblast activation and EMT, and vimentin for mesenchymal differentiation. Both proteins were seen routinely in arterioles and small arteries in all sections. In the kidneys of control rats and in the 7 and 14d treated groups, α-SMA was not seen in glomeruli, and vimentin was seen minimally in podocytes. In the tubulo-interstitium, there was infrequent sporadic staining of α-SMA in interstitial fibroblasts. In the treated group, particularly at 28d, increased interstitial α-SMA and vimentin were noted in cells (probably myofibroblasts) in the interstitial spaces. There was also a diffuse pattern of staining in cells around the glomeruli. We detected no definite evidence of EMT, as there was no staining of α-SMA or vimentin in the tubular epithelium. Expression of α-SMA was significantly increased at 28d in the treated animals. There were no significant differences in staining for α-SMA or vimentin in the liver tissue for any group. Representative photomicrographs of these characteristics are presented in Fig. 1C–F while quantification of the differences between control and treated groups by image analysis is presented as graphs in Fig. 2B–C. Western blot analysis confirming the findings is presented in Fig. 3A–B.

### 3.3. Macrophage infiltration

Liver and renal cortical tissue sections were stained for ED-1 to assess the degree of macrophage infiltration during treatment with *D. villosa*. Samples from rats in the control groups and in all 7 and 14d groups exhibited minimal macrophage staining. Focal areas of macrophage infiltration were evident in rats consuming *D. villosa* for 28d. In the kidneys, the ED-1-positive cells were seen strongly in glomeruli and, surrounding these, in focal areas of the renal interstitial space, but not seen invading the tubular epithelium. Global assessment of macrophages in the kidney interstitium did not reach statistical significance but assessment of focal strongly stained regions did. In the liver, macrophage accumulation was seen zonally around the centrilobular veins and within the sinusoids in the 28d treated animals. ED-1 staining in other portions of the liver tissue for any group.

![Western blot analysis](image-url)

**Fig. 3.** Western blot analysis. Expression of (A) α-SMA, (B) vimentin, (C) NFκB, and (D) CTGF at 7, 14 and 28d. Compared to controls, there was a significant increase in expression of α-SMA (**p < 0.001**) and vimentin, NFκB and CTGF (**p < 0.05**) in the kidneys of the 28d treated rats. There were no significant differences in the 7 or 14d groups. The values shown are mean ± SEM (n = 4). C = control; T = treated.
of the liver did not reach statistical significance. These characteristics are presented in Fig. 4A–D. Quantification of ED-1 counts that reached statistical significance is presented in Fig. 4E–G.

3.4. Pro-fibrotic growth or transcription factors, inflammatory cytokines (TGFβ1, CTGF, NFκB and TNF-α) and heme oxygenase-1 (HO-1)

TGFβ1 expression was not detected in glomeruli or arterial blood vessels from the rats in control groups except for cortical arterial walls. However, moderate anti-TGFβ1 reactivity was detected in glomeruli, arterial walls and interstitial fibrotic tissue of the 14d treated group, which became more pronounced in the 28d treated groups. TGFβ1 overexpression was also observed in the centrilobular areas in liver of the 28d treated group but not in the other areas of the liver. Representative photomicrographs of these characteristics are presented in Fig. 5A–D, while quantification by image analysis is presented as a graph in Fig. 5E–F. Western blot analysis confirming these findings is presented in Fig. 6.

Analysis of kidney slices by Western blot also revealed significant increases in NFκB and CTGF in the 28d group. These are graphically presented in Fig. 3C and D, respectively. There were no significant increases in TNF-α or HO-1 in the kidney or liver of any group.

4. Discussion

Extracts from *D. villosa* (wild yam) rhizome are commonly used in people seeking relief from a variety of disorders. It appears to be well tolerated, and the only adverse reactions reported in the literature are nausea, vomiting and diarrhoea following the ingestion of large doses (Braun and Cohen, 2007). The Australian Drug Reactions Advisory Committee (ADRAC) database, which contains reports of suspected reactions to drugs in Australia since 1972, was
queried for entries involving “Dioscorea” or “wild yam”. A total of 12 reports were identified (Adverse Drug Reaction Unit (2008), Therapeutic Goods Administration). Two of these reports concerned medicines with wild yam as the sole active ingredient (D. dioscorea specified in one case; species unknown in the other case). Only one of the 12 reports involved nephrotoxicity (acute renal failure). According to the report, the medicine taken in this case contained Dioscorea opposita as well as five other medicinal plants (amounts not provided). Although the likelihood of this medicine being the cause of the adverse event was rated as “probable”, it is impossible to ascertain whether its content of D. opposita played any part in the adverse reaction. Our preliminary in vitro results suggested that D. villosa exhibited a direct toxicity to renal cells as well as caused apparent transformation of the surviving renal tubular epithelial cells into cells that expressed α-SMA (Wojcikowski et al., 2008), suggesting EMT had occurred. We tested the hypothesis that similar pro-fibrotic changes would be seen in the kidneys of rats fed a diet that included D. villosa.

In the present study, the most profound effect noted in rats consuming D. villosa was a 10-fold increase in TGFβ1 in the 28d group. TGFβ1 is commonly increased when tissue injury signals the release of inflammatory cytokines and growth factors from macrophages and other inflammatory cells. In this role, TGFβ1 is initially involved in normal repair and regeneration, through its ability to increase the expression of extracellular matrix proteins. Concurrently with the marked increase in TGFβ1 expression, there were only small focal areas of macrophage infiltration. This suggests that D. villosa may directly upregulate pro-fibrotic TGFβ1 in renal epithelial cells and this may be a mechanism not involving chronic inflammatory cells, such as macrophages. Local production of
TGFβ1 stimulates extracellular matrix synthesis, affecting numerous proteins including collagen types I, II, III, IV and V, fibronectin, osteopontin, thrombospondin, tenacin, elastin, hyaluronic acid, and numerous others (Prud’homme, 2007). In turn, overproduction of extracellular matrix molecules like collagen type I and III and fibronectin generates fibrosis, leading to the permanent loss of normal structure and function in the kidney (Groma, 1998).

With such a strong increase in TGFβ1, it was not surprising to find profound increases in the α-SMA and Masson’s trichrome staining. Masson’s trichrome staining is used as a general indicator of increased collagen synthesis and can help assess the degree of interstitial fibrosis. The contractile protein, α-SMA, is a specific marker for myofibroblasts, which make a major contribution to the development of tubulo-interstitial fibrosis (Ng et al., 1998). In the normal kidney of the control rats, staining for α-SMA was rare. In contrast, myofibroblasts, as determined by the expression of α-SMA, were found in significantly increased numbers in kidneys of rats ingesting D. villosa for 28d. This is a growing body of evidence in both experimental animals and humans that suggests a positive correlation between the presence of myofibroblasts and deterioration of renal function (Fujigaki et al., 2005). It is not known why there was sporadic, intense staining of Masson’s trichrome and α-SMA around the blood vessels in treated animals, but a possible cause is that the active components within D. villosa are transported in the vasculature and so have their most intense effect in the cells and tissue surrounding the vessels prior to infiltration of the toxins into the interstitium.

Further supporting our hypothesis that D. villosa is pro-fibrotic to the kidneys, we found smaller but statistically significant increases in vimentin. Whereas, α-SMA acts as a contractile element, vimentin is an intermediate protein that is expressed almost exclusively in mesenchymal cells and serves as mechanical support to retain the cell configuration against tension (Gonlusen et al., 2001; Villanueva et al., 2006). Our control animals, therefore, had small normal amounts of vimentin in the glomeruli and blood vessels, but none in the interstitium. In animals ingesting D. villosa for 28d, the increased vimentin expression at relatively high concentrations within the tubulo-interstitium supports that mesenchymal differentiation had occurred in cells in the interstitial space and glomeruli.

CTGF, another fibrogenic cytokine, was increased in the group consuming D. villosa for 28d. This cytokine facilitates fibrogenesis and acts as a fibroblast chemoattractant, promoting their differentiation, which appears to be crucial in the pathogenesis of renal interstitial fibrosis (Qi et al., 2006). CTGF also stimulates a broad range of cellular events, such as proliferation, differentiation and apoptosis in several cell types, playing a vital role in renal fibrosis (Liu et al., 2006).

Macrophage accumulation was also significantly increased in both the glomeruli and interstitium in focal areas of the kidneys. An increase in macrophages was expected, as TGFβ1 has potent chemoattractive properties and can lead to the rapid accumulation and activation of macrophages. D. villosa may also be directly toxic to renal cells in vivo, which is supported by its strong in vitro toxicity. It is noteworthy that macrophage accumulation was the only parameter, other than TGFβ1, which was increased in the liver, although both of these increases were restricted to the centrilobular zones. Macrophage accumulation in the glomerulous is unusual. Acute tubular necrosis, Fanconi’s syndrome, aristolochic acid nephropathy, and various forms of interstitial nephritis have been associated with consumption of toxic herbs, but these syndromes normally lack glomerular involvement (Wojcikowski et al., 2004). In comparison, D. villosa induced both fibrosing interstitial nephritis and glomerular inflammation.

We did not find an increase in TNF-α. One of the reasons for our initial interest in the fibrotic potential of herbs within the Dioscorea genera was that in vitro studies by others had found increases in TNF-α in macrophage cell cultures with components commonly found within Dioscorea sp. (Choi and Hwang, 2002; Liu et al., 2007). Possible explanations for the lack of effect on TNF-α include that this action may not occur in vivo or that D. villosa does not contain the components that affect TNF-α in a rat kidney, while some other Dioscorea sp. do. TNF-α is produced mostly by activated macrophages, in response to lipopolysaccharides and endotoxins.
Therefore, we cannot rule out that *D. villosa* has significance in Western blot studies using whole tissue homogenates. Increases in TNF-α activates the transcription of genes involved in inflammation (Jiang et al., 2007). The mechanisms that are responsible for increased TNF-α expression and activation, in the absence of significant increases in TNF-α, were not studied here but may include direct binding of a component within *D. villosa* to Toll-like receptors (Tato et al., 2007), stimulation of the renin-angiotensin system (Lee et al., 2004), or direct ischaemic or toxic injury (Jiang et al., 2007). This needs further study.

Taken together, the results of this experiment suggest that *D. villosa* is pro-fibrotic to the rat kidney. If a similar effect occurs in humans, one of the greatest health concerns involves the individual who takes *D. villosa* along with another nephrotoxic substance, as the toxicity of biologically active components often acts additively or even synergistically (Elseviers and De Broe, 1999). Another health concern pertains to patients who consume *D. villosa* for extended lengths of time, as TGFβ1 not only upregulates the transcription of matrix proteins but also decreases collagenases that help regulate interstitial fibrosis (Klahr and Morrissey, 2000). Continuous renal expression of TGFβ1 may also lead to decreases in endogenous anti-inflammatory cytokines such as hepatocyte growth factor and result in irreversible renal insufficiency (Yazawa et al., 2004). Given the lower, focal expression of TGFβ1 in the liver combined with the greater ability of the liver to recover from high levels of TGFβ1 (Lin et al., 1998), the hepatic health implications of long term supplementation with *D. villosa* does not appear to be important and may only be of concern to those with pre-existing liver inflammation or fibrosis.

In conclusion, although rats ingesting *D. villosa* extract for 4 weeks produced no alterations in the serological parameters that would indicate decreased kidney and liver function, there were marked increases in fibrotic markers in the kidneys and to a lesser extent, livers. It is therefore recommended that *D. villosa* is not consumed for extended lengths of time, especially by people with compromised kidney function or in those taking other nephrotoxic agents. Other species in the genus *Dioscorea* should be tested individually, as the individual species are chemically quite diverse.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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