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Journal of Ethnopharmacology 116 (2008) 397–402
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Effects of *Bupleurum scorzoneraefolium*, *Bupleurum falcatum*, and saponins on nephrotoxic serum nephritis in mice

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Received 12 April 2007; received in revised form 13 November 2007; accepted 15 November 2007
Available online 23 November 2007

Abstract

Ethnopharmacological significance: Bupleuri radix is a commonly prescribed Oriental herbal medicine containing extracts of different Bupleuri species. We wished to determine whether two of these species, *Bupleurum scorzoneraefolium* and *Bupleurum falcatum*, or their active ingredients, saikosaponins a, c, and d, could prevent the development of immune-complex nephritis in nephrotoxic serum treated mice.
Materials and methods: Immune-complex nephritis was created in C57BL/6 mice by administration of nephrotoxic serum containing anti-basement membrane antibodies. Mice were next given one of five treatments: *Bupleurum scorzoneraefolium*, *Bupleurum falcatum*, saikosaponin a, saikosaponin c, or saikosaponin d. Proteinuria, blood urea nitrogen, creatinine, and renal histological changes were then examined.
Results: Saikosaponin c almost completely prevented the development of nephritis, although immune-complex deposition was not affected. *Bupleurum falcatum* and saikosaponin d had a significant, although lesser effect, and *Bupleurum falcatum* and saikosaponin a showed no effect.
Conclusions: The mechanism of action of saikosaponin c and the reasons for the difference between the two bupleuri species should be investigated further in order to find the best way to utilize the therapeutic effect of Bupleuri radix on nephritis.

Keywords: Chinese herbal medicine; Bupleuri radix; Immune-complex nephritis; Saikosaponin

1. Introduction

Extracts of Bupleuri radix are widely used as primary ingredients in Oriental medicines. Prescriptions including such extracts have long been used in treatments for hepatitis and renal disease. Many plant species called "Bupleuri radix" are available on the market. In particular, the species *Bupleurum falcatum* L. and *Bupleurum scorzoneraefolium* W. are widely used in Japan. Studies of several *Blupleurum* species and their active components, the saikosaponins a, c, and d, have reported biological activities such as inhibition of hepatic fibrosis (Yen et al., 2005), inhibition of cell proliferation in hepatic and lung cancer cell lines (Wu and Hsu, 2001; Hsu et al., 2004), inhibition of tumor cell adhesion (Ahn et al., 1998), anti-inflammatory activity (Takagi and Shibata, 1969; Kyo et al., 1999), and alleviation of hyperlipidemia (Yamamoto et al., 1982), and a beneficial effect on hepatic injury (Abe et al., 1978, 1980) and chronic hepatitis (Arichi, 1979; Yamamoto et al., 1981). In the current investigation, we examined whether *Bupleurum scorzoneraefolium*, *Bupleurum falcatum*, and their three active saikosaponin components could suppress the development of nephrotoxic serum (NTS) nephritis in mice. We also compared the relative amounts of saikosaponins a, c, and d in the two Bupleuri species, using high-performance liquid chromatography. Our hypothesis was that the administration of *Bupleurum scorsoneraefolium*, *Bupleurum falcatum*, or their individual saikosaponins might ameliorate NTS nephritis *in vivo*.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were fed on

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0378-8741/$ – see front matter © 2008 Published by Elsevier Ireland Ltd.
doi:10.1016/j.jep.2007.11.026

standard mouse chow and given free access to water throughout the experiment. New Zealand White rabbits were purchased from the Takasugi Laboratory Animal Center (Nagano, Japan).

2.2. Drugs

Bupleurum falcatum came from Mishima, Shizuoka, Japan, and *Bupleurum scorzoneraefolium* from Hubei, China. Concentrated extracts of the rough powders of the two plants were prepared (by Kotaro Pharmaceutical Company, Ltd., Osaka, Japan) by placing the rough powder of each in a flask, adding distilled water, heating in a water incubator for 1 h and filtering. The filtrate was saved, distilled water added to the residue and the mixture heated again for an hour, filtered, and the filtrate saved. After a third repetition of this process, the three filtrates were combined, centrifuged, concentrated with pressure-decreased vacuum concentrators, and then frozen and dried, thus producing a concentrated extract of each plant species. Saikosaponins a, c, and d were also prepared by Kotaro Pharmaceutical, and were dissolved in saline.

2.3. Purification of glomerular basement membrane (GBM) antigen (GBM-Ag)

The process of producing NTS followed a previously published procedure (Chen et al., 2002) and is briefly described in the following three sections. Glomeruli-rich material was obtained from normal kidneys of C57BL/6 mice by a sieving technique using a rough mesh (no. 100 and 250 meshes). The glomerular fraction retained on the no. 250 mesh was disrupted by sonication to separate the cellular components from the GBM. An adequate amount of trypsin (25 mg trypsin/10 g GBM) was added to isolate the GBM-rich fraction in 0.1 M Tris–HCl buffer containing 0.02 M $CaCl_2$ (pH 8.2), and this mixture was incubated while stirring at 37 °C for 18 h. After centrifugation at 4 °C and 10,000 rpm for 60 min, a supernatant containing the crude solubilized GBM antigen (GBM-Ag) was obtained (Wakashin et al., 1980).

2.4. Preparation of NTS

New Zealand White rabbits were immunized subcutaneously with 5 mg of GBM-Ag in complete Freund's adjuvant five times at 1-week intervals. Rabbits were injected with the GBM-Ag without complete Freund's adjuvant 7 days after the fifth immunization. Antisera from the three animals was collected 7 days after the final injection of antigen. All sera were pooled together and used as the NTS throughout this study.

2.5. Preparation of immunoglobulin G (IgG)

Normal rabbit sera were obtained from New Zealand White rabbits. Normal rabbit IgG (NRIgG) was purified from this sera using an affinity chromatography kit (Mab Trap® GII: Pheracia Biotech).

2.6. Experimental design

Mice were divided into 7 groups of 10 mice each: 2 control groups without herbal treatment, 1 of these without (normal group) and the other with (control group) NTS administration; and 5 groups with both NTS administration and 1 of the 5 herbal treatments. To produce NTS nephritis in the six NTS groups, C57BL/6 mice were first immunized with 250 μg of normal rabbit IgG and 0.05 ml of complete Freund's adjuvant in the rear footpad. After 5 days, the mice were then given 150 μl of NTS intravenously. After the NTS injection, mice in the five treatment groups were also treated daily for 14 days with i.p. injections of one of the following compounds: 5 mg/kg of *Bupleurum falcatum* extract, 5 mg/kg of *Bupleurum scorzoneraefolium* extract, or 0.2 mg/kg of saikosaponin a, c, or d. The mice were then sacrificed on day 15 for blood biochemistry and histological studies.

2.7. Assay of Bupleurum falcatum and Bupleurum scorzoneraefolium saikosaponin content

The crude powder and concentrates of these two species were assayed for saikosaponin content and type by high-performance liquid chromatography (HPLC) using a Mightysil RP-18 column (4.6 mm × 150 mm) from Kanto Chemistry (Tokyo, Japan). Column temperature was maintained at 40 °C, with a flow rate of 1.0 ml/min, and a detection wavelength of 203 nm. For each mobile phase, acetonitrile–water at a ratio of 6:4 or 7:3 was used to detect saikosaponins a, c, and d.

2.8. Urinalysis

Urine was collected from the mice in a metabolic cage once a week after the injection of NTS. The Pyrogallol Red method (Fujita et al., 1983) was used to assess the amount of the protein that had been excreted in the urine in 15 h.

2.9. Blood biochemistry

Blood urea nitrogen and creatinine in the serum samples were determined by the urease-GLDH and CRTNase-POD methods, respectively.

2.10. Light-microscopic histological findings

Blocks of renal tissue were fixed in 10% buffered formalin for routine histological examination. The severity of the glomerular lesions was graded (histological score) as the sum of glomerular change in the following three categories: cell proliferation, thrombus, and crescent formation, each category being graded on a scale of 0 (little of no change from normal) to 5 (extensive change seen), etc.

2.11. Immunofluorescence study

Blocks of kidney tissue obtained at autopsy were quickly frozen in acetone dry ice and sliced into 4-μm sections. The

cryostat sections were directly stained with FITC-conjugated anti-rabbit IgG, FITC-conjugated anti-mouse IgG, and FITC-conjugated anti-mouse C3.

2.12. Statistical analysis

Data were analyzed by the Bartlett test and one-factor analysis of variance (ANOVA).

3. Results

3.1. Proteinuria

Nephrotoxic serum-induced excretion of urinary protein (Fig. 1) was significantly suppressed by *Bupleurum scorzoneraefolium* (*$p < 0.05$), saikosaponin c ($p < 0.01$), and saikosaponin d ($p < 0.05$). *Bupleurum falcatum* and saikosaponin a showed no significant effect on protein excretion.

3.2. Blood biochemistry

Levels of serum blood urea nitrogen and creatinine in mice treated with nephrotoxic serum alone were significantly ($p < 0.001$) higher than those of normal, untreated animals (Table 1). Treatment with *Bupleurum scorzoneraefolium* extract, saikosaponin c, or saikosaponin d significantly lowered the levels of these indicators toward normal. These results show that treatment with nephrotoxic serum in the doses used here resulted in an observable deterioration in renal function that was partly or completely prevented by *Bupleurum scorzoneraefolium* or two of its components, saikosaponins c and d. As in prevention of NTS nephritic proteinuria, saikosaponin c was the most effective in preventing NTS nephritis-induced increases in blood urea nitrogen and creatinine. Saikosaponin a and *Bupleurum falcatum* had no such effect.

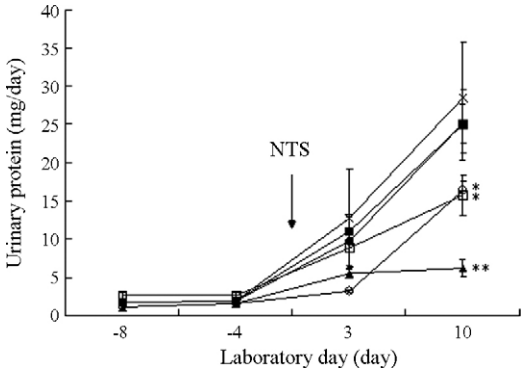

Fig. 1. Changes in urinary protein in nephrotoxic serum-treated mice. Control group (+); *Bupleurum scorzoneraefolium* (BSW) treatment group (×); *Bupleurum falcatum* (BFL) treatment group (■); saikosaponin a (sp-a) treatment group (●); saikosaponin c (sp-c) treatment group (▲); saikosaponin d (sp-d) treatment group (◯). *Bupleurum scorzoneraefolium* saikosaponin c, and saikosaponin d significantly suppressed the excretion of urinary protein compared to the control group. *Significantly different from control $p < 0.05$. **Significantly different from control $p < 0.01$. Data is shown as mean ± S.D.

Table 1
Serum biochemical determinations in experiment

Treatment ($n = 10$)	Blood urea nitrogen (mg/dL)	Creatinine (mg/dL)
Normal	19.9 ± 2.7	0.11 ± 0.05
Control	58.7 ± 28.2*	0.34 ± 0.16*
BSW	26.2 ± 3.0**	0.17 ± 0.02**
BFL	74.3 ± 5.1	0.40 ± 0.08
sp-a	59.9 ± 4.7	0.28 ± 0.04
sp-c	19.9 ± 2.3***	0.13 ± 0.03***
sp-d	30.6 ± 4.7**	0.24 ± 0.06

*$p < 0.001$ significantly different from normal group. **$p < 0.05$; ***$p < 0.001$ significantly different from control group.

3.3. Light-microscopic histological findings

Typical nephrotoxic serum nephritis was produced by preimmunization with normal rabbit IgG and injection with 150 μl of nephrotoxic serum. Histological specimens from the control (NTS) group showed cellular proliferation in the glomeruli, glomerular hypertrophy, formation of crescents, occlusion of glomerular loops, mononuclear cell infiltration into the interstitium, and interstitial fibrosis (Fig. 2B). Specimens from the *Bupleurum scorzoneraefolium* treatment group showed only mild cellular proliferation in glomeruli (Fig. 2C), and specimens from the saikosaponin c treatment group showed clear improvement in renal histology compared to the nephritic control (Fig. 2D). Specimens from the *Bupleurum falcatum* treatment group showed no visible difference from the nephritic control group (Fig. 2B) A specimen from normal kidney is shown in Fig. 2A for comparison.

Quantitative histology of the renal specimens showed the similar results (Fig. 3). The histological score of the nephritic, control group was 8.6 ± 2.9. Treatment with *Bupleurum scorzoneraefolium* or saikosaponin c significantly lowered this score (histological score 4.1 ± 1.2, $p < 0.01$ and histological score 3.4 ± 0.1, $p < 0.005$, respectively). Saikosaponins a and d and *Bupleurum falcatum* had no significant effect.

3.4. Direct immunofluorescence histological results

In the positive (NTS-treated) control group, specific linear and granular deposition of rabbit IgG (Fig. 4A) and mouse IgG (Fig. 4C) was clearly found along the glomerular basement membrane. In the saikosaponin c-treated group, specific staining of rabbit IgG (Fig. 4B) and mouse IgG (Fig. 4D) were also found to be as strong as in the control group.

3.5. Saponin content of Bupleurum scorzoneraefolium and Bupleurum falcatum

The quantitative determination of saikosaponins a, c, and d in the two Bupleuri species was performed by HPLC. *Bupleurum scorzoneraefolium* had higher levels of saikosaponins a, c, and d than did *Bupleurum falcatum*. Extracts of the two species were also analyzed by the same method. The extract of *Bupleurum scorzoneraefolium* also had higher levels of saikosaponins a and c than did the extract of *Bupleurum falcatum*. Saikosaponin d

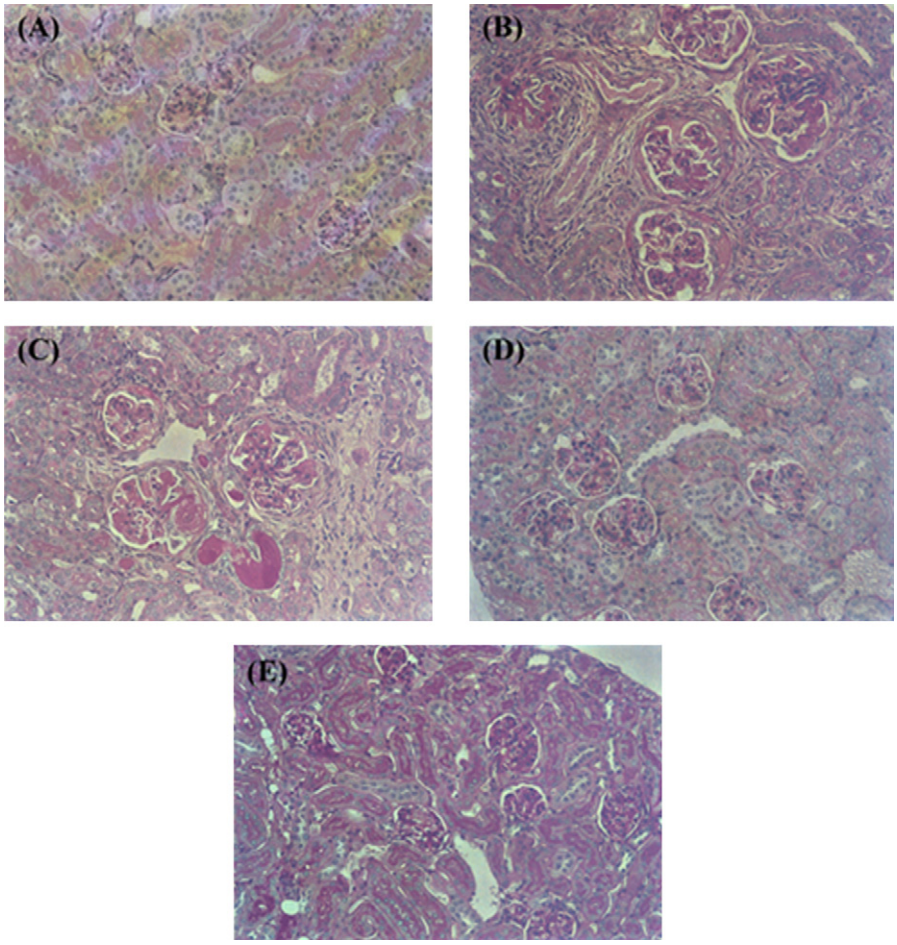

Fig. 2. Light microscopic findings of renal tissue. Normal kidney is shown in panel A. In panel B, the control (NTS alone) group demonstrates cellular proliferation in glomeruli, glomerular hypertrophy, occlusion of glomerular loops, formation of crescents, and tubulointerstitial damage, such as mononuclear cell infiltration into the interstitium, tubular cell atrophy, and interstitial fibrosis. The *Bupleurum falcatum* (BFL) treatment group (panel C) shows no significant difference in renal histology from the group treated with NTS alone (panel B). The *Bupleurum scorzoneraefolium* (BSW) (panel D) and saikosaponin c treatment groups (sp-c) (panel E) showed only minor damage in the kidney (2 weeks after the injection, PAS stain, 200×).

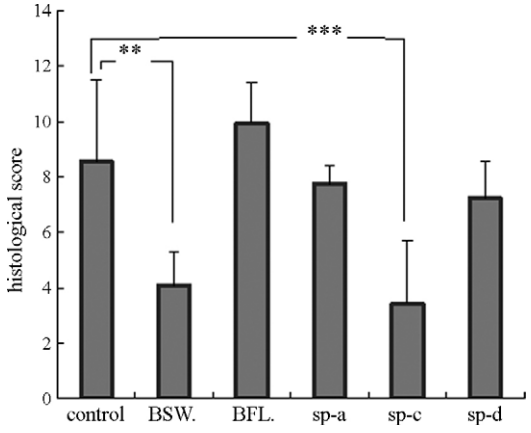

Fig. 3. Histological scores (mean ± S.D.) of renal tissue in Bupleuri radix-treated groups. Control group, *Bupleurum scorzoneraefolium* (BSW) treatment group, *Bupleurum falcatum* (BFL) treatment group. Saikosaponin a (sp-a) treatment group, saikosaponin c (sp-c) treatment group, and saikosaponin d (sp-d) treatment group (**$p < 0.01$ and ***$p < 0.005$ compared with the control).

levels were extremely low in the *Bupleurum falcatum* extract and were undetectable in the extract of *Bupleurum scorzoneraefolium* (Table 2).

4. Discussion

4.1. Saikosaponin c

Our results show that administration of saikosaponin c almost totally prevents development of the immune-complex nephritis that occurs after the administration of nephrotoxic serum. *Bupleurum scorzoneraefolium* and saikosaponin d have significant, but lesser, effects in decreasing nephritis, and *Bupleurum falcatum* and saikosaponin a have no detectable effect at all.

Saikosaponin c acts on some event subsequent to immune-complex deposition, for it does not prevent the formation or deposition of these complexes. In immune-complex nephritis, the deposition of immune complexes on the glomeruli attracts neutrophils and macrophages to the site. These cells secrete proteolytic enzymes that digest and clear the immune complexes,

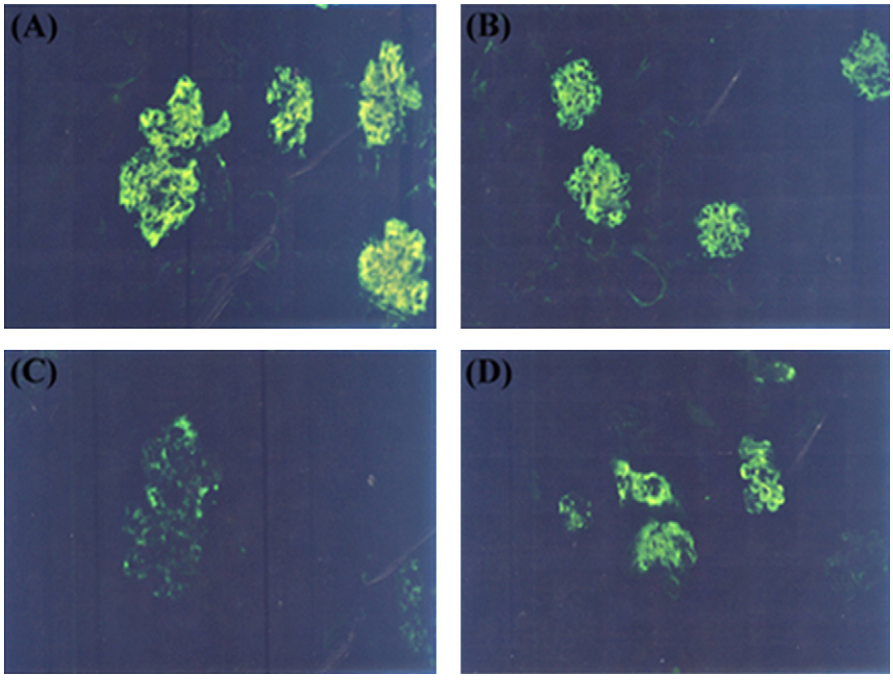

Fig. 4. Direct immunofluorescence staining of renal tissue from a nephrotoxic serum (NTS) nephritis mouse (A, C) and that from a saikosaponin c-treated mouse (B, D) 14 days after an injection of NTS. There was no difference in the strength of specific staining of anti-rabbit immunoglobulin (IgG) (A, B: FITC-anti-rabbit IgG, 200×) and anti-mouse IgG (C, D: FITC-anti-mouse IgG, 200×).

but also damage tissue and cause inflammation. Saikosaponins have a structure similar to prednisolone. Yamamoto-Shuda et al. (1999) have reported that prednisolone significantly suppressed the infiltration of CD8+ cells, monocytes, and cellular crescents and reduced proteinuria, BUN, and creatinine levels in NTS nephritis in rats, and saikosaponin c might act in a similar manner. Our experimental design did not address this question.

4.2. Saikosaponin d

Other researchers have reported that saikosaponin d lessens the development of pathological changes in rat models of mesangioproliferative glomerulitis (Li et al., 1997) and nephrotic syndrome (Abe et al., 1986). Our finding was that saikosaponin d also lessened pathology in immune-complex nephritis in mice, but was not as effective in this regard as saikosaponin c.

Saikosaponin c differs from saikosaponin d in that in some respects. In endothelial cell culture, unlike saikosaponin, it induces cell growth (Shyu et al., 2004), and in rat glioma cells, it lacks the ability of saikosaponin d to inhibit proliferation and alter morphology (Tsai et al., 2002). Our findings are consistent with those of others as to the effect of saponin d in nephritis, but a further comparison of the two saponins needs to be done.

4.2.1. Comparison of the effects and constituents of *Bupleurum scorzoneraefolium* and *Bupleurum falcatum*

In our results, *Bupleurum scorzoneraefolium* extract significantly lessened the immune-complex nephritis caused by nephrotoxic serum, while *Bupleurum falcatum* showed no such activity. But levels of the most active saikosaponin, saikosaponin c, in the *Bupleurum falcatum* extract were 80% of those found in the *Bupleurum scorzoneraefolium* extract, and this minor difference seems hardly enough to explain the profound difference in their activities. Saikosaponin d recovery from both extracts was almost negligible, so differences between the two extracts in this saikosaponin cannot explain the pronounced difference in activity. It has been reported that saikosaponin d is easily converted to saikosaponin b2, when extraction takes place over a long time period or in an acidic solvent. Since C-13 and C-28

Table 2
Quantitative determination components of saikosaponins a, c and d by HPLC method

	Saikosaponin a	Saikosaponin c	Saikosaponin d
BSW	1.05% (CV 0.96%)	0.25% (CV 0.33%)	1.07% (CV 0.48%)
BFL	0.46% (CV 0.61%)	0.18% (CV 0.43%)	0.55% (CV 0.15%)
Extract of BSW	0.73% (CV 0.76%)	0.21% (CV 1.5%)	N.D. (CV 0.76%)
Extract of BFL	0.5% (CV 2.5%)	0.17% (CV 1.9%)	0.03% (CV 4.5%)

The column is Mightysil RP-18 (4.6 mm × 150 mm), temperature was maintained 40 °C, with a flow rate of 1.0 ml/min, and detection wavelength was 203 nm. Bupleuri radixes were used in each mobile phase to detect the saikosaponins a, c and d. N.D.: no detect; CV: coefficient variation.

of the arylether ring are easily cleaved in organic acids (Kubota and Hinoh, 1968a,b; Kubota et al., 1968; Akihori et al., 1975), it seems that saikosaponin d may be more labile and difficult to extract than the other saikosaponins.

As far as the difference in activity between the two bupleuri species is concerned, since differences in saikosaponin content do not seem to explain our results, one is left with the speculation that the water extract of *Bupleurum scorzoneraefolium* contained some unknown active ingredient not found in the *Bupleurum falcatum*. Further research would be needed, however, to examine this speculation.

It is evident that *Bupleurum scorzoneraefolium* and saikosaponin c provide beneficial results in the treatment of NTS nephritis mice, therefore the mechanisms of *Bupleurum scorzoneraefolium* and saikosaponin c should be further studied.

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