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Atractylodin, β-eudesmol, and (+)-hinesol in *Atractylodes chinensis* rhizomes improve glomerular injuries in high immunoglobulin A mice

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ABSTRACT

Background: The rhizome of *Atractylodes chinensis* (Asteraceae), a crude drug of Japanese Kampo medicines, has been administered to patients with edema, nephrotic syndrome, and gastrointestinal disorders. Essential oils, such as sesquiterpenoids (e.g., β-eudesmol and hinesol) and atractylodin, are rich in the rhizomes. Previously, we discovered that atractylodin, a polyacetylene compound, found in an ethyl acetate (EtOAc)-soluble fraction from a methanol extract of *A. chinensis* rhizomes, possessed marked anti-inflammatory activities. Oral administration of the EtOAc-soluble fraction reduced immunoglobulin A (IgA) deposition in the renal glomeruli of high immunoglobulin A (HIGA) mice, a model of human IgA nephropathy. An increased serum IgA forms immune complexes and causes the deposition on renal glomeruli, leading to inflammation by the complement-mediated pathway.

Objective: The aim of this study is to identify the compounds that reduce IgA deposition in an EtOAc-soluble fraction of *A. chinensis* rhizomes.

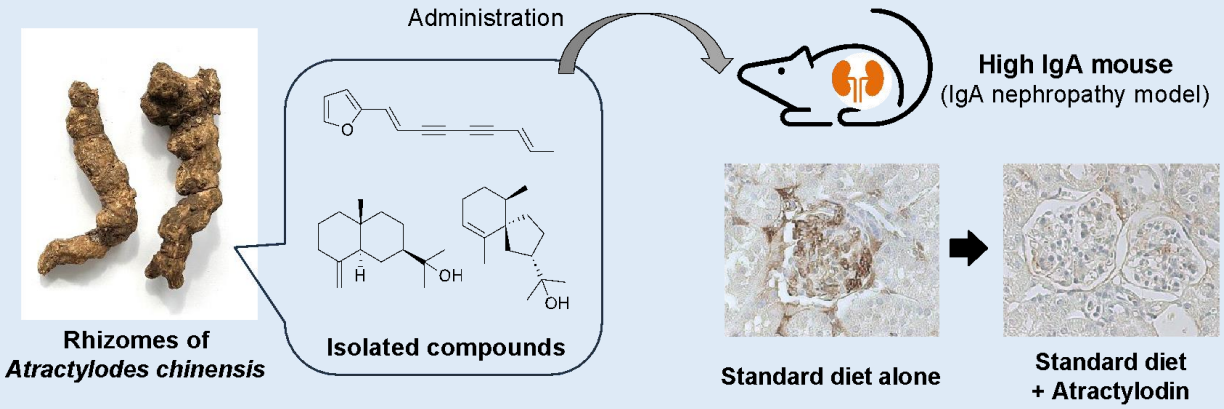
Methods: Metabolites in the serum of HIGA and control BALB/c mice were analyzed by gas chromatography–mass spectrometry. A standard diet including each compound was fed to HIGA and BALB/c mice for 20 weeks to evaluate the improvement of glomerular IgA deposition.

Results: Metabolomic analysis of serum suggested that the HIGA mice exhibit a state near the early stage of chronic kidney disease, compared with the BALB/c mice. When mice were orally administered each hydrophobic compound of

A. chinensis rhizomes, it was revealed that atractylodin, as well as β-eudesmol and (+)-hinesol, efficiently inhibited glomerular IgA deposition. Furthermore, the renal levels of complement component 3 (C3) and proinflammatory cytokine mRNAs were decreased, when the hydrophobic compounds were orally administered to HIGA mice.

Conclusion: Atractylodin, β-eudesmol, and (+)-hinesol may inhibit glomerular IgA deposition, probably by attenuating complement-mediated injuries and suppressing proliferation of mesangial cells in the renal glomeruli. These compounds might improve the pathological findings of human IgA nephropathy.

Keywords: *Atractylodes chinensis*; Crude drug; Immunoglobulin A nephropathy; Metabolomics; Complement



INTRODUCTION

Atractylodes chinensis Koidzumi and *A. lancea* De Candolle are perennial plants (Asteraceae) that are widely distributed in East Asia [1]. Their rhizomes (called *Sojutsu*) have been included in traditional Japanese medicines (Kampo medicines), such as the Kampo formula *Saireito*, which shows an anti-edematous effect due to its anti-inflammatory and water-modulation properties [2–3]. Pharmacological studies on *A. chinensis* rhizome extracts showed biological activities, such as hepatoprotective, immunomodulatory, anti-gastritis, anti-obesity, and antibacterial activities, and upregulated the expression of insulin-like growth factor-1 [4–8].

A phytochemical investigation of the *A. chinensis* rhizomes revealed the presence of hydrophobic compounds, including sesquiterpenoids, e.g., β-eudesmol and hinesol, and polyacetylene compounds, e.g., atractylodin [9]. In our previous paper, we confirmed that β-eudesmol, (+)-hinesol, and atractylodin are the major constituents of the ethyl acetate (EtOAc)-soluble fraction from the *A. chinensis* rhizome extract [10]. Other groups reported that β-eudesmol showed a variety of activities, such as anti-angiogenic, anti-inflammatory, anti-allergic, and gastroprotective activities [9,11], Hinesol suppressed the induced inflammatory cytokine expression in the mouse macrophage line RAW264 treated with lipopolysaccharide (LPS) [12].

There are few reports focusing on the effects of *Sojutsu* on kidney disorders. Isohama reported that a water-soluble fraction of a *Sojutsu* extract inhibited the water permeability of MLE-12 cell membranes, a mouse lung epithelial cell line [13]. Additionally, manganese

ions, which were included in *Sojutsu*, markedly inhibited aquaporin 4, a member of the aquaporin water-channel family [14]. In contrast, we used an EtOAc-soluble fraction derived from the *A. chinensis* rhizomes, which included hydrophobic, anti-inflammatory compounds, i.e., β-eudesmol, (+)-hinesol, and atractylodin [10]. Oral administration of the EtOAc-soluble fraction decreased immunoglobulin A (IgA) deposition and proliferative changes in mesangial cells in the renal glomeruli of high immunoglobulin A (HIGA) mice. Because these mice have been used as an animal model of human IgA nephropathy [15], hydrophobic compounds may help improve IgA nephropathy.

IgA nephropathy is a chronic glomerulonephritis that is characterized by IgA deposition in renal glomeruli and proliferative mesangial changes [16]. Patients suffering from IgA nephropathy are often Asian, and approximately 40% of patients develop chronic renal failure (i.e., chronic kidney disease) within 20 years [16]. Although the pathogenesis of IgA nephropathy is not well understood, a multi-hit model has been proposed [17–18]. An abnormally modified IgA, i.e., galactose-deficient IgA1 (Gd-IgA1), observed in IgA nephropathy patients forms immune complexes, which accumulate in the mesangium of glomeruli. The complexes activate mesangial cells to induce secretion of extracellular matrix and production of proinflammatory cytokines and chemokines, leading to glomerular lesions. Immune cells, including macrophages, migrate to activate the pathway mediated by complements, especially complement component 3 (C3) [19]. As a result, inflammatory and tissue-destructive processes start, and symptoms of IgA nephropathy appear.

In the present study, metabolites in the serum were analyzed to investigate whether adult HIGA mice show a uremic state. Next, the constituents found within the EtOAc-soluble fraction of the *A. chinensis* rhizome extract were administered to HIGA mice. Improvements in glomerular lesions were examined by immunostaining IgA in the glomeruli to locate the principal compound in an EtOAc-soluble fraction. Finally, we discussed the contribution of atractylodin, β-eudesmol, and (+)-hinesol to the reduced pathological observations in the glomeruli of HIGA mice.

MATERIALS AND METHODS

Plant materials: *Atractylodes* rhizomes collected from Shaanxi Province, China were acquired from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan) and verified as *Sojutsu* by Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co. Ltd.). We identified their species as *A. chinensis* by a previously published method [10]. The voucher specimen was stored in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University under code No. RIN-AC-051.

Preparation of an EtOAc-soluble fraction and isolation of the compounds: As previously described, dried *A. chinensis* rhizomes (2004.5 g) underwent methanol extraction under reflux (Figure 1) [10]. Briefly, the filtrate was evaporated and yielded 404.83 g (20.20%) of an extract, which was then resuspended in water and extracted with EtOAc [10]. The EtOAc-soluble fraction (Fraction A) was concentrated to yield 69.65 g. Subsequently, it was subjected to further purification through Silica gel 60 (Nacalai Tesque Inc., Kyoto, Japan) column chromatography, involving elution with *n*-hexane to EtOAc (100:0 → 0:100) to provide seven subfractions (A1 to A7).

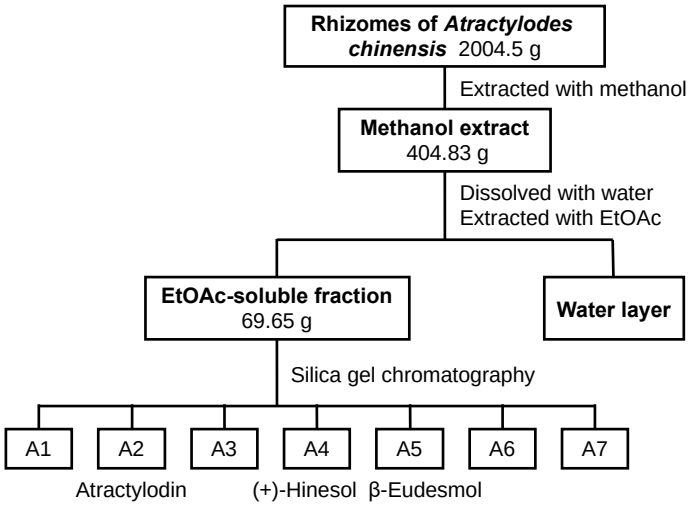


Figure 1. Purification of the compounds from *A. chinensis* rhizomes. Flowchart depicting the procedures employed for compound fractionation. The plant material was, first, extracted, followed by resuspentsion of the extract in water and subsequent extraction with ethyl acetate (EtOAc). The weight of each fraction is provided. The compounds are illustrated under the appropriate subfraction.

Nuclear magnetic resonance (NMR) spectra were calculated using a JNM-ECS400 NMR spectrometer (JEOL Ltd., Tokyo, Japan) with tetramethylsilane as an internal standard. The ^{1}H and ^{13}C signals were assigned on the basis of the 2D NMR spectra. Mass spectra were acquired utilizing an Electron Ionization-Mass Spectrometry (EI-MS) approach on a JMS-700 MStation mass spectrometer (JEOL Ltd.). Subfraction A2 was crystallized from methanol to afford pale brown needles (1.99 g). Based on mass spectrometry (MS) and ^{1}H NMR and ^{13}C NMR spectral analysis, along with comparisons to previously reported data, atractylodin was identified as the compound [10,20]. Subfraction A4 was purified by silica gel column chromatography using *n*-hexane:EtOAc (95:5) to afford colorless needles (8.62 g). The optical rotation, MS, and ^{1}H NMR and ^{13}C NMR spectra agreed with the reported data of (+)-hinesol [21–22]. This identification was confirmed previously reported [10]. Subfraction A5 underwent purification via silica gel column chromatography using *n*-hexane:EtOAc (95:5) to afford colorless needles (8.04 g). This compound was identified as β-eudesmol based on optical rotation, MS, and ^{1}H NMR and ^{13}C NMR spectral analysis and comparison with published data [10,23].

Animals: All animal care and experimental practices adhered strictly to the regulations and guidelines required by the Japanese government and were granted approval by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus (No. BKC2020-019). Female HIGA mice and BALB/c mice (specific pathogen-free, 4 weeks old; Japan SLC, Inc., Hamamatsu, Japan) were provided with a CRF-1 diet (Oriental Yeast Co., Ltd., Tokyo, Japan) daily and had access to water *ad libitum* in order to acclimatize to housing as groups (two mice per cage) at 21–23 ℃ under a 12-h light-dark cycle for a week. CRF-1 diet was certified, with the following nutrient composition: moisture, 8.5 g; crude protein, 22.1 g; crude fat, 5.4 g; crude ash, 6.2 g; crude fiber, 3.1 g; nitrogen free extract, 54.6 g; and calories, 355.8 kcal (mean values per 100 g diet) [24]. BALB/c mice served as the healthy control group in the study.

Administration of the compounds to mice: According to our previous report [10,25], HIGA mice were randomly assigned to four groups (four mice per group, two mice per cage) and then fed a CRF-1 diet (ground to powder) alone or a CRF-1 diet containing each compound daily to each group from 10 weeks until 30 weeks of age. We showed that EtOAc-soluble fraction (Fraction A), which contained 4.88% (w/w) atractylodin, 24.5% β-eudesmol, and 23.0% (+)-hinesol, improved glomerular lesions of

HIGA mice at 1% (w/w) in a diet [10]. Therefore, a CRF-1 diet containing 0.05% (w/w) atractylodin, 0.25% β-eudesmol, or 0.25% hinesol was fed to the mice of each group. Pellets of CRF-1 diet were ground into powder and then mixed with each compound by vigorous shaking in a bag. The diet was fed using feeders for powder diets (model KN-675-4; Natsume Seisakusho Co., Ltd., Tokyo, Japan) to measure daily food intake. Water was supplied using polycarbonate bottles. At 30 weeks of age, the mice were euthanized via cervical dislocation, and blood samples were collected from the heart to obtain serum. The kidneys' (cortex) was excised and immersed in an RNA*later* Solution (Thermo Fisher Scientific Inc., Carlsbad, CA, USA) for use in subsequent experiments.

Metabolomic analysis: Serum obtained from blood taken from the heart of 30-week-old mice was aliquoted in 50 µL portions, and 250 µL of a methanol/water/chloroform mixture was added to each aliquot. The solution was heated at 37 °C for 30 min and centrifuged at 4 °C for 5 min to remove protein. The supernatant (200 µL) was mixed with an equal volume of water and centrifuged to extract hydrophilic metabolites. The resultant extract, except for the precipitate, was transferred to another tube, lyophilized, and redissolved in pyridine containing 20 mg/mL methoxyamine hydrochloride. Methoximation was performed by heating at 30 °C for 90 min, and 40 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Supelco, Darmstadt, Germany) was added followed by trimethylsilyl derivatization by heating at 37 °C for 30 min. The solution underwent gas chromatography–mass spectrometry (GC–MS) under the analytical conditions according to the Shimadzu Smart Metabolite Database (Shimadzu Corporation, Kyoto, Japan). The serum was mixed with 5 µL of 0.5 mg/mL 2-isopropyl malate and used as an internal standard. GC–MS/MS analyses were conducted utilizing a triple quadrupole mass spectrometer model GCMS-TQ8050 NX (Shimadzu Corporation), equipped with a GC-2030 gas chromatography system and an AOC-20i autosampler.

Immunohistochemistry: In accordance with a previously published methodology, specimens obtained from the kidneys of 30-week-old mice (in RNA*later* Solution) were fixated in 4% formaldehyde, paraffin-embedded, and stained to detect IgA deposition [10]. Briefly, the deparaffinized sections were stained with hematoxylin and eosin or treated with hydrogen peroxide, blocked, and incubated with a goat anti-mouse IgA antibody conjugated to horseradish peroxidase (Bethyl Laboratories, Montgomery, TX, USA; 200:1 dilution). Immunoreactivity was detected with the substrate 3,3'-diaminobenzidine tetrachloride. The hematoxylin-counterstained sections were examined under a Research Biological Digital Microscope BA210EINT (Shimadzu Rika, Tokyo, Japan). Glomeruli exhibiting IgA immunoreactivity in ten low-power fields (LPFs; ×100) were counted in a blinded manner five times.

Quantitative reverse transcription–polymerase chain reaction (RT–PCR): The cortex was lysed using Sepasol I Super G solution (Nacalai Tesque, Inc.) followed by DNase treatment [26]. To detect mRNAs, cDNA was synthesized from the total RNA using oligo(dT) primers and PCR-amplification using primers shown in Table 1. mRNA levels were estimated in triplicate using SYBR Green I and a Thermal Cycler Dice Real Time System (Takara Bio Inc., Kusatsu, Japan), as described previously [10]. According to the ΔΔCt method, the relative mRNA level was calculated from the obtained threshold cycle (Ct) value, which was normalized to that of the internal control mRNA, i.e., eukaryotic elongation factor 1α (*Ef*) mRNA [26]. The level of mRNA of interest is indicated as a percentage relative to the *Ef* mRNA level.

Table 1. Primers used for quantitative RT–PCR to detect mRNA

mRNA	Direction	Sequence (5' to 3')
Complement component 3 (*C3*)	Forward	CATCAAGTCAGGCTCAGATGAGG
	Reverse	ATGTAGCTGGTGTTGGGCTTTTCTC
Tumor necrosis factor α (*Tnf*)	Forward	GTCTCAGCCTCTTCTCATTCCTG
	Reverse	CACTTGGTGGTTTGCTACGACGT
Lipocalin-2 (*Lcn2*)	Forward	GAAATATGCACAGGTATCCTCAGG
	Reverse	CCTTGGTTCTTCCATACAGGGTAA
Eukaryotic elongation factor 1α (*Ef*)	Forward	TCTGGTTGGAATGGTGACAACATGC
	Reverse	CCAGGAAGAGCTTCACTCAAAGCTT

Statistical analysis: Values are represented as the means ± standard deviations (SDs). Using IBM SPSS Statistics 29.0 (IBM Japan, Ltd., Tokyo, Japan), we performed a non-parametric analysis of covariance (ANCOVA) or one-way analysis of variance (ANOVA). Then, differences between values were analyzed using Student's *t* test (Microsoft Office Excel; Microsoft Japan Co., Ltd., Tokyo, Japan) followed by Bonferroni correction. Statistical significance was considered at $P < 0.05$ and $P < 0.01$.

RESULTS

Serum metabolomics reveals systematic changes in metabolites in HIGA mice: To examine whether uremic toxins are included in the serum of HIGA mice (IgA nephropathy model), metabolomics of the serum of 30-week-old HIGA and BALB/c (healthy control) mice was performed. We analyzed the serum samples by a triple quadrupole mass spectrometer, which had a high scan rate and high sensitivity; as a result, 253 metabolites were identified.

The concentrations of metabolites were significantly increased in the serum of HIGA mice compared with BALB/c mice (Table 2). Metabolites were classified by Duranton et al., and '*uremic toxins*' were specified [27] (Table 2, bold letters). The uremic toxins are solutes that retain in the blood of the patients suffering with chronic renal failure, i.e., chronic kidney disease (CKD). Some uremic toxins, such as spermine and noradrenaline, were significantly increased, suggesting that these toxins accumulated in HIGA mice.

Table 2. Metabolites that were increased in the serum of HIGA mice compared to that of BALB/c mice.[a]

Metabolite	HIGA-to-BALB/c ratio
2-Aminoadipic acid	$9.87 \pm 1.75^{\#\#}$
Uridine	$4.61 \pm 1.77^{\#}$
2'-Deoxyuridine	$4.28 \pm 1.63^{\#}$
Eicosapentaenoic acid	$4.17 \pm 0.56^{\#\#}$
Homogentisic acid	$4.15 \pm 0.96^{\#\#}$
Spermine	$3.03 \pm 0.85^{\#}$
Homoserine	$2.89 \pm 0.64^{\#\#}$
Inositol phosphate	$2.74 \pm 0.42^{\#\#}$
Ascorbic acid	$2.71 \pm 0.58^{\#\#}$
Noradrenaline	$2.32 \pm 0.64^{\#}$
Cystathionine	$2.26 \pm 0.50^{\#}$
Juniperic acid	$2.16 \pm 0.45^{\#}$
3-Hydroxybutyric acid	$1.95 \pm 0.20^{\#\#}$
N-Butyrylglycine	$1.90 \pm 0.40^{\#}$
Phenylacetic acid	1.41 ± 0.56
Uric acid	1.15 ± 0.24

The content of metabolites were measured using GC–MS, after methoximation-trimethylsilyl derivatization, as described in Materials and Methods. The value of HIGA mouse was divided by the average of BALB/c mice, and the average was set at 1. These ratios of metabolites (mean ± SD) are aligned in descending order. Metabolites classified as uremic toxins [27] are indicated in bold letters. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus BALB/c mice ($n = 4$).

In contrast, the concentrations of sugars, including glucose, arabinose, galactose, lyxose, xylose, and fructose, were significantly decreased in HIGA mice (Table 3). Lower levels of glucose have also been observed in IgA patients, although the mechanism that leads to the decreases in sugars is unknown [28]. This accordance shows that an HIGA mouse is a model that exhibits similar findings of human IgA nephropathy.

Table 3. Metabolites that were decreased in the serum of HIGA mice.[a]

Metabolite	HIGA-to-BALB/c ratio
Hypoxanthine	0.31 ± 0.26
Niacinamide	0.41 ± 0.05[#]
Allose	0.45 ± 0.07[##]
N-Acetylaspartic acid	0.45 ± 0.27[#]
Homovanillic acid	0.46 ± 0.26[#]
Arabitol	0.49 ± 0.10[##]
Ribitol	0.50 ± 0.10[##]
Fructose	0.52 ± 0.03[#]
Xylose	0.53 ± 0.13[##]
Lyxose	0.54 ± 0.14[##]
Galactose	0.56 ± 0.06[#]
Phosphoric acid	0.56 ± 0.09[##]
Adipic acid	0.57 ± 0.07[#]
4-Hydroxphenylpyruvic acid	0.60 ± 0.07[#]
Arabinose	0.65 ± 0.16[#]
Glucose	0.70 ± 0.02[##]
5-Dehydroquinic acid	0.74 ± 0.03[#]
Oxalic acid	0.75 ± 0.13
Mannose	0.76 ± 0.13[#]
Anthranilic acid	0.79 ± 0.13
Hippuric acid	0.85 ± 0.20

[a] The content of metabolites were measured using GC–MS after methoximation-trimethylsilyl derivatization. The value of HIGA mouse was divided by the average of BALB/c mice, and the average was set at 1. These ratios of metabolites (mean ± SD) are aligned in ascending order. Metabolites classified as uremic toxins [27] are indicated in bold letters. [#] $p < 0.05$ and [##] $p < 0.01$ versus BALB/c mice ($n = 4$).

The concentrations of urea and creatinine, markers that reversely correlate to the renal function, were not high, and significant differences of the concentrations were not observed between BALB/c and HIGA mice (Table 4). Our previous report of biochemical analysis of the serum of 30-week-old HIGA and BALB/c mice gave the HIGA-to-BALB/c ratios of 0.62 (blood urea nitrogen, BUN) and 0.84 (creatinine) [10], which are comparable to the data of this study.

Table 4. Urea and creatinine in the serum of BALB/c and HIGA mice.[a]

	HIGA-to-BALB/c ratio
Urea	0.83 ± 0.05
Creatinine	0.53 ± 0.30
Urea/creatinine ratio	1.41 ± 0.54

[a] Serum urea and creatinine concentrations were measured using GC–MS after methoximation-trimethylsilyl derivatizations. The value of HIGA mouse was divided by the average of BALB/c mice, and the average was set at 1. Each value is represented as a mean ± SD ($n = 4$).

The hydrophobic compounds of A. chinensis rhizomes do not affect the body weight of HIGA mice: The extract of *A. chinensis* rhizomes was extracted with EtOAc to yield the EtOAc-soluble fraction, which was purified to obtain atractylodin, (+)-hinesol, and β-eudesmol (Figure 2). To identify the principal constituents in the EtOAc-

soluble fraction derived from *A. chinensis* rhizomes, 0.25% (w/w) β-eudesmol, 0.25% (w/w) (+)-hinesol, or 0.05% (w/w) atractylodin in a standard diet (CRF-1) was orally administered to HIGA mice. The dosage of each compound was determined based on knowledge that the 1% (w/w) EtOAc-soluble fraction in a diet reduced renal IgA deposition and that this crude fraction contained 4.88% atractylodin, 24.5% β-eudesmol, and 23.0% (+)-hinesol [10].

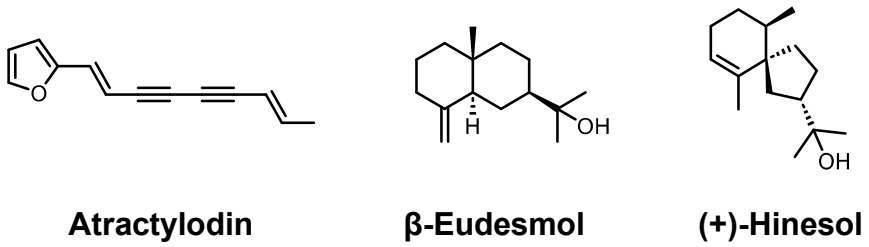


Figure 2. Chemical structures of the hydrophobic compounds in the *A. chinensis* rhizomes. Atractylodin, β-eudesmol, and (+)-hinesol are shown.

HIGA mice were administered each hydrophobic compound in a standard diet daily, starting from the age of 10 to 30 weeks (for 20 weeks in total) because pathological alterations in renal glomeruli typically become evident around the age of 25 weeks [14,29]. We performed a non-parametric ANCOVA followed by Student's *t* test of body weight and the age in weeks (Figure 3). As previously reported, the HIGA mice that fed a standard diet alone [HIGA (–) mice; negative controls] were significantly heavier than BALB/c mice that fed a standard diet [BALB/c (–) mice; healthy controls] [10]. In addition, a significant difference between HIGA (–) and HIGA+Atractylodin mice was observed.

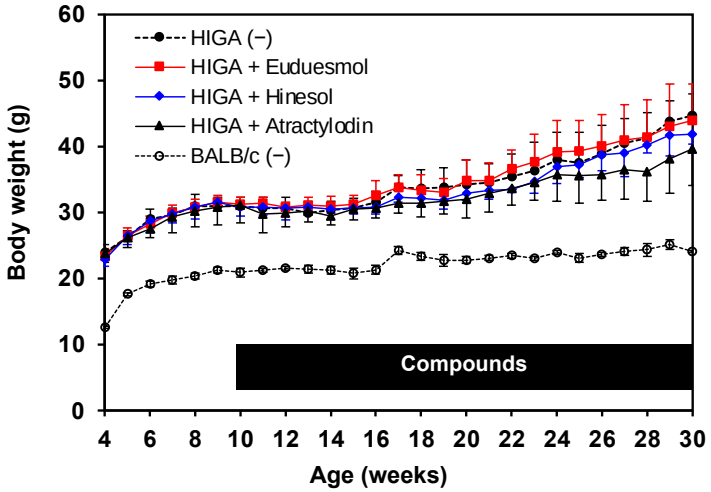


Figure 3. Body weight change in mice from each group. HIGA mice were assigned to four groups (four mice per group) and then fed a standard diet alone [HIGA (–) mice] or a standard diet containing β-eudesmol (HIGA+ Eudesmol), (+)-hinesol (HIGA+ Hinesol), and atractylodin (HIGA+ Atractylodin) daily to each group from 10 weeks until 30 weeks of age. BALB/c mice were fed a standard diet alone as a control group [BALB/c (–)]. Body weight is shown in the mean ± SD (*n* =4 mice).

Three hydrophobic compounds in A. chinensis rhizomes reduce glomerular lesions in HIGA mice: The kidneys of 30-week-old mice were histologically examined. The tissues stained by hematoxylin and eosin (Figure 4A) shows that there were multinucleated mesangial cells and enlarged matrix in the glomeruli of HIGA mice,

whereas proliferation of mesangial cells and expansion of the mesangial region were suppressed in those of HIGA+ Atractylodin mice. Strong IgA-immunoreactivity was located in the mesangial region of glomeruli in HIGA (–) mice (Figure 4B). The percentage of IgA-positive glomeruli was found to be 37.4% of the total number of glomeruli. In contrast, in HIGA+Atractylodin mice, weaker immunoreactivity to IgA was detected in the glomerular mesangial matrix, with the number of IgA-positive glomeruli reduced to 9.6% (Figure 4C). Furthermore, the number of IgA-positive glomeruli in HIGA+Eudesmol and HIGA+Hinesol mice was substantially lower than that in HIGA (−) mice.

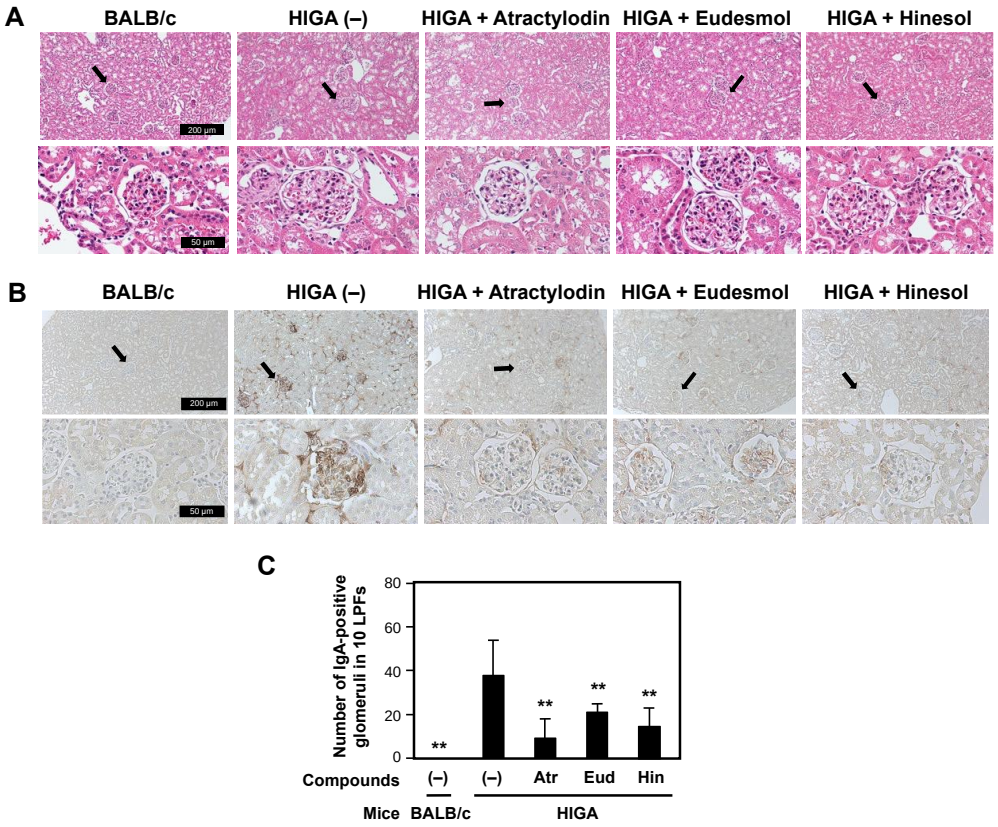


Figure 4. Atractylodin, β-eudesmol, and (+)-hinesol reduce glomerular IgA deposition in the kidneys. **(A)** Hematoxylin-eosin stain of kidney tissues. HIGA mice were orally administered atractylodin (HIGA +Atractylodin), β-eudesmol (HIGA + Eudesmol), or (+)-hinesol (HIGA +Hinesol) for 20 weeks. HIGA and BALB/c mice were fed a standard diet alone [HIGA (−) and BALB/c (−), respectively] as negative controls. Histological examination was performed on the kidneys of the 30-week-old mice. Glomeruli are indicated by arrows. (Upper) Original magnification, ×100. Scale bar, 200 μm. (Lower) Original magnification, ×400. Scale bar, 50 μm. **(B)** IgA deposition in the glomeruli. The kidneys of the mice in (A) were used. Tissue sections were immunostained with an anti-IgA antibody. Glomeruli are indicated by arrows. Original magnification, ×100 (upper) and ×400 (lower). **(C)** Effects of the compounds on the number of IgA-positive glomeruli. The number of glomeruli that showed IgA-immunoreactivity was counted in ten low-power fields (LPFs; ×100). The number of these IgA-immunostained glomeruli with respect to the total number of glomeruli in ten LPFs represents the mean ± SD. ** $P < 0.01$ versus the HIGA (−) group.

The hydrophobic compounds of A. chinensis rhizomes affect mRNA expression in the kidney: The pathway mediated by complements plays a crucial role with proinflammatory cytokines in the pathogenesis of IgA nephropathy [17–19]. The relative levels of *C3* mRNA were measured in the mouse kidney (renal cortex) by quantitative RT–PCR (Figure 5A). The *C3* mRNA levels in the kidneys of HIGA (−) mice slightly increased compared to those of BALB/c (–) mice. The *C3* mRNA levels of HIGA+Hinesol mice significantly decreased compared to those of HIGA (−) mice, whereas those of HIGA+Atractylodin and HIGA+β-Eudesmol mice were slightly decreased than those of HIGA (−) mice.

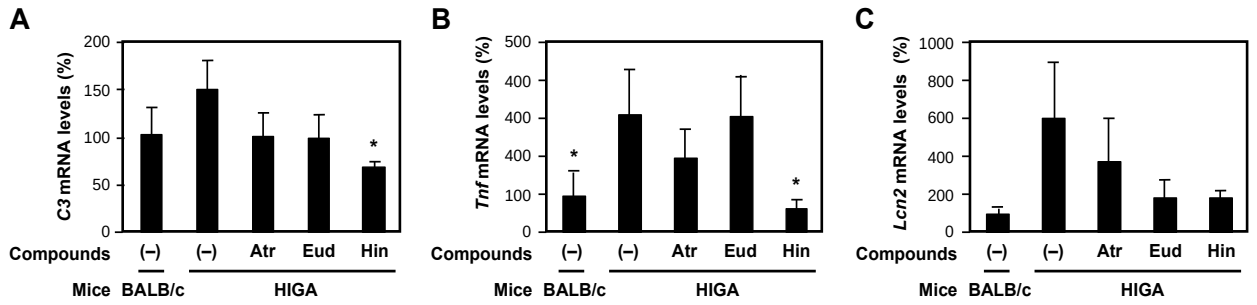


Figure 5. Effects of the compounds on renal mRNA expressions in the kidney. **(A)** Effects on the *C3* mRNA levels. **(B)** Effects on the *Tnf* mRNA levels. **(C)** Effects on the *Lcn2* mRNA levels. Total RNA was extracted from the kidney of each mouse administered atractylodin (Atr), β-eudesmol (Eud), or (+)-hinesol (Hin) and analyzed by quantitative RT–PCR. Each mRNA level was normalized against the *Ef* mRNA levels (internal control), and the relative levels are represented as percentages (means ± SD). Values were analyzed by one-way ANOVA and then Student's *t* test followed by Bonferroni correction. * $P < 0.05$ versus the HIGA (−) group ($n = 3$–4 mice).

The levels of *Tnf* mRNA of HIGA (−) mice significantly increased compared with those of BALB/c (–) mice (Figure 5B). The *Tnf* mRNA levels of HIGA+Hinesol mice significantly decreased than those of HIGA (−) mice, whereas those of HIGA+Atractylodin mice were slightly decreased. As shown in Figure 5C, similar trends were observed in the levels of *Lcn2* mRNA, although there were not statistical significances among the groups. Taken together, the administration of (+)-hinesol seems to be effective to reduce the levels of these mRNAs.

DISCUSSION

In this study, serum metabolome analysis of HIGA and BALB/c mice was performed by a triple quadrupole mass spectrometer, which is a latest model having higher scan rate and sensitivity. We identified many metabolites, i.e., 253 metabolites. Up to date, few reports on metabolomic analysis have focused on the metabolic profile of HIGA mice. Kurano and Yatomi reported that 206 metabolites were detected in the serum of 32-week-old HIGA mice using a single quadrupole mass spectrometer [31]. Metabolome analysis may be also applied to tissues or organs; for example, metabolites in the mouse liver were analyzed [32]. Such an approach may be useful to elucidate pathophysiology of IgA nephropathy.

The concentrations of uremic toxins found in CKD patients seem to be different from those in HIGA mice. A review regarding uremic retention solutes described the mean uremic concentrations (M) in the CKD patients and the normal concentrations (N) [27]. The concentrations of the uremic toxins, phenylacetic acid (M/N = 334) and uric acid (M/N = 1.59), increased in CKD patients, which are similar to those in HIGA mice (Table 2). However, the concentrations of hypoxanthine (M/N = 1.72), oxalic acid (M/N = 13.0), and hippuric acid (M/N = 23.8) that increased in CKD patients decreased in HIGA mice (Table 3). The concentrations of urea and creatinine in CKD patients increase according to the progression of renal failure. In HIGA mice, serum urea and creatinine concentrations were slightly decreased (Table 4) and did not change until the age of 30 weeks, although the glomerular IgA deposition and mesangial matrix increase were marked [10,15,29]. Therefore, the HIGA mouse may show less severe symptoms than that of uremic patients suffering with CKD, although it is a model that resembles human IgA nephropathy.

Oral administration of each hydrophobic compound in the EtOAc-soluble fraction from *A. chinensis* rhizomes to HIGA mice demonstrated that atractylodin, β-eudesmol, and (+)-hinesol improved IgA deposition in the glomeruli of HIGA mice (Figure 4). β-Eudesmol and (+)-hinesol were major constituents in the EtOAc-soluble fraction, but did not suppress NO production in hepatocytes, whereas atractylodin showed very high activity [10]. The other constituents in the EtOAc-soluble fraction, which may be sesquiterpenes, such as β-

selinene and elemol [30], seem to possess low or no activity in the suppression of NO production. Our previous data showed that the administration of a 1% EtOAc-soluble fraction, which included these compounds, significantly decreased the IgA deposition, as well as proliferation of mesangial cells, in HIGA mice [10]. Collectively, these results imply that the effects of the three compounds may generate the effects of the EtOAc-soluble fraction [10]. It is suggested that these compounds are responsible to reduction of IgA deposition and proliferation of mesangial cells may additively affect them. The finding that several compounds contribute together to the pharmacological activity of the *A. chinensis* rhizome emphasizes the characteristic feature of the crude drugs of Kampo medicines as multiple-component drugs.

Proinflammatory cytokines, such as TNFα, are detected at high concentrations in CKD patients [27], and complement-mediated pathway plays a crucial role in the pathogenesis of IgA nephropathy [17–19,33–34]. Glomerular inflammation occurs in the kidneys of IgA nephropathy patients [34–35]. These findings might be in accordance with the decreases of the *Tnf* and *Lcn2* mRNA levels in the kidney of HIGA (–) mice by the administration of the compounds (Figure 5).

C3 plays a central role in the activation pathways of the complement system [33,35]. Because C3 is produced in several tissues, such as the liver, local expression of C3 in the kidney may be slightly affected. In this study, an increase of the *C3* mRNA levels of HIGA (–) mice and their decreases of HIGA mice administered atractylodin or β-eudesmol were not prominent (Figure 5). It is plausible that all the hydrophobic compounds in *A. chinensis* rhizomes additively suppress inflammation and complement-mediated injury of renal glomeruli, leading to the protection of the kidney.

Collectively, an EtOAc fraction from *A. chinensis* rhizomes, as well as its bioactive compounds, i.e., atractylodin, β-eudesmol, and (+)-hinesol, suppressed IgA deposition and mesangial cell proliferation in HIGA mice. It seems rational that Kampo formulas that include *A. chinensis* rhizomes have been used to treat the patients suffering with renal diseases. There are no disease-specific treatments for human IgA nephropathy up to date, but many therapeutic approaches are currently applied [35]. Although pathogenesis and pathophysiology of HIGA mice may be not identical to those of human IgA nephropathy, these compounds in *A. chinensis* rhizomes might be candidates for the drugs specific to human IgA nephropathy.

CONCLUSION

Three principal constituents were identified in the EtOAc-soluble fraction derived from *A. chinensis* rhizomes. When atractylodin, β-eudesmol, and (+)-hinesol exerted effects on the kidneys of HIGA mice, all the compounds inhibited glomerular IgA deposition. These compounds might possess protective effects on the kidney in human IgA nephropathy by suppression of the complement-mediated mechanism.

List of abbreviations: EtOAc, ethyl acetate, IgA, immunoglobulin A, HIGA, high immunoglobulin A, NMR: nuclear magnetic resonance, MS: mass spectrometry, GC–MS: gas chromatography–mass spectrometry, CKD: chronic kidney disease.

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