Journal of Ethnopharmacology 278 (2021) 114262

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

# Binding activity of *Valeriana fauriei* root extract on $GABA_A$ receptor flunitrazepam sites and distribution of its active ingredients in the brain of mice – A comparison with that of *V. officinalis* root

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ARTICLE INFO

*Keywords:*
*Valeriana fauriei* root
γ-aminobutyric acid type A receptor
Kessyl glycol diacetate

*Chemical compounds studied in this article:*
CI
Coniferyl isovalerate
α-KA
α-Kessyl acetate
KG
Kessyl glycol
KG2
Kessyl glycol 2-acetate
KG8
Kessyl glycol 8-acetate
KGD
Kessyl glycol diacetate
PHB
*p*-hydroxybenzoic acid butyl ester
SDS
Sodium lauryl sulfate
Umbelliferone
Valerenic acid

ABSTRACT

*Ethnopharmacological relevance:* *Valeriana fauriei* root (VF) is a crude drug registered in the Japanese Pharmacopeia 17th Edition and a known substitute for *V. officinalis* (VO). Although VO has been pharmacologically evaluated for its sedative effects and mechanism of action, data regarding VF remain scarce.

*Aim of the study:* We compared the binding affinity of VF and VO extracts, as well as examined the active ingredients in the VF extract, on flunitrazepam sites of γ-aminobutyric acid receptor type A ($GABA_A$ receptor). Furthermore, we confirmed whether these active ingredients were distributed in the brain of mice orally administered the VF extract.

*Materials and methods:* We prepared the assay system to evaluate the binding activity of flunitrazepam sites of $GABA_A$ receptor using a 96-well plate and assessed the activities of VF and VO extracts. We then analyzed their constituents using HPLC with principal component analysis (PCA) and evaluated active ingredients correlated with their activities. The distribution of active ingredients in the plasma and brain of mice orally administered the VF extract prepared with different emulsifiers were analyzed by LC-MS/MS.

*Results:* The ethanol extract of VF exhibited significantly higher activity on flunitrazepam sites of $GABA_A$ receptor than VO. For the VF extract, kessyl glycol diacetate (KGD) was markedly associated with the binding activities; however, active ingredients included KGD, kessyl glycol 8-acetate (KG8), α-kessyl acetate (α-KA), and coniferyl isovalerate (CI). For VO, valerenic acid and five other compounds were associated with the binding affinity on flunitrazepam sites of $GABA_A$ receptor. On emulsifying the VF extract with a fat-soluble glycerin fatty acid ester, the plasma and brain distributions of KGD tended to be higher, those of KG8 were significantly more than 10-times higher, and those of α-KA was lower than those of the VF extract emulsified with water-soluble gum arabic, after oral administration in mice.

*Conclusions:* Based on the binding activity on flunitrazepam sites of $GABA_A$ receptor and brain distribution, KGD, KG8, and α-KA can be considered active ingredients of VF. The addition of a fat-soluble emulsifier promoted the absorption of KGD, the main active ingredient, and KGD was metabolized to KG8 in the body. The present results suggest a possible mechanism underlying the sedative effect for VF, and these three compounds can be used as marker compounds to evaluate the quality of VF products.

## 1. Introduction

Dried root of *Valeriana fauriei* Briq. (VF) is a crude drug registered in the Japanese Pharmacopeia 17th Edition (Pharmaceutical and Medical Device Regulatory Science Society of Japan, 2016) and is used to treat diseases in women. The root of European valerian *V. officinalis* L. (VO) is a crude drug registered in the 10th Edition of the European Pharmacopeia (European Directorate for the Quality of Medicines & Health Care, 2020), frequently used to treat depression and insomnia or to improve

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https://doi.org/10.1016/j.jep.2021.114262
Received 4 March 2021; Received in revised form 21 May 2021; Accepted 27 May 2021
Available online 8 June 2021

**A list of abbreviations**

| | |
|---|---|
| ANOVA | one-way analysis of variance |
| $C_{brain}$ | brain concentration |
| $C_{plasma}$ | plasma concentration |
| CI | coniferyl isovalerate |
| DPM | disintegrations per minute |
| $EC_{50}$ | half maximal effective concentrations |
| GA | gum arabic |
| GABA | γ-aminobutyric acid receptor type A, $GABA_A$ receptor |
| GFE | glycerin fatty acid ester |
| α-KA | α-kessyl acetate |
| KG | kessyl glycol |
| KG2 | kessyl glycol 2-acetate |
| KG8 | kessyl glycol 8-acetate |
| KGD | kessyl glycol diacetate |
| NSB | nonspecific binding |
| PBS | phosphate buffered saline (0.15 M, pH 7.2) |
| PC | principal component |
| PCA | PC analysis |
| PHB | *p*-hydroxybenzoic acid butyl ester |
| SDS | sodium lauryl sulfate |
| TB | total binding |
| TLC | thin layer chromatography |
| T-PBS | 0.05% Tween 20 in PBS |
| VF | *Valeriana fauriei* Briq. root |
| VO | *Valeriana officinalis* L. root |

sleep conditions as a sedative and tranquilizing drug (Weiss et al., 2002). In 1886, VO was registered in the Japanese Pharmacopeia 1st Edition; however, it disappeared after the 4th edition published in 1920. Conversely, VF has been registered in the Japanese Pharmacopeia since the 2nd edition, published in 1891 until the current edition (Yanagisawa et al., 1993; Yanagisawa, 2013; Ota et al., in press); this indicates the history of VF as a substitute for VO in Japan.

For VF and VO, the chemical composition of essential oils differs markedly, with kessyl alcohols and their acetates identified as characteristic ingredients of VF; *for example*, kessyl glycol diacetate (KGD) (Fig. 1) (Houghton, 1988; Suzuki et al., 1993). Reportedly, there are two types of VF: one mainly contains KGD, and the other contains less KGD but high amounts of α-kessyl acetate (α-KA) in the essential oil (Suzuki et al., 1993). In contrast, VO contains large amounts of bornyl acetate and isovaleric acid, although its sedative activity can be attributed to the valerenic acid composition (Houghton, 1988; Hendriks et al., 1981; Safaralie et al., 2008). As only a few ingredients in VF and VO are similar, their pharmacological activities should be distinct.

Previous animal studies have revealed that a VF extract prolonged the hexobarbital-induced sleeping time in mice and revealed active ingredients such as KGD, kessyl glycol 8-acetate (KG8), and kessyl glycol 2-acetate (KG2) (Takamura et al., 1973, 1975a, 1975b; Hikino et al., 1980; Sakamoto et al., 1992). Both KGD-rich and α-KA-rich VF extract reportedly prolong the pentobarbital-induced sleep time in mice (Yoshitomi et al., 2000). Furthermore, it has been reported that the VF extract possesses antidepressant-like effects in mice following restraint stress by inhibiting brain-derived neurotrophic factor; the active ingredient was identified as α-kessyl alcohol (Oshima et al., 1995; Choi et al., 2020). In addition, the VF extract revealed anxiolytic effects in mice by evoking physiological stresses using an elevated plus-maze test (Kim et al., 2015). However, the relationship between the mechanism underlying its pharmacological effects and active ingredients remains elusive.

Reportedly, inhalation of VO essential oil significantly prolonged the pentobarbital-induced sleeping time in rats (Komori et al., 2006). A VO extract significantly shortened sleep latency without altering the sleep-wakefulness cycle in rats (Tokunaga et al., 2007; Han et al., 2018). Valerenic acid, a major component of VO, has exhibited anxiolytic effects by modulating γ-aminobutyric acid receptor type A ($GABA_A$ receptor), and those effect was antagonized by bicuculline (Yuan et al., 2004). Moreover, valerenic acid had binding affinity of the flunitrazepam and flumazenil sites of $GABA_A$ receptor, and neurons

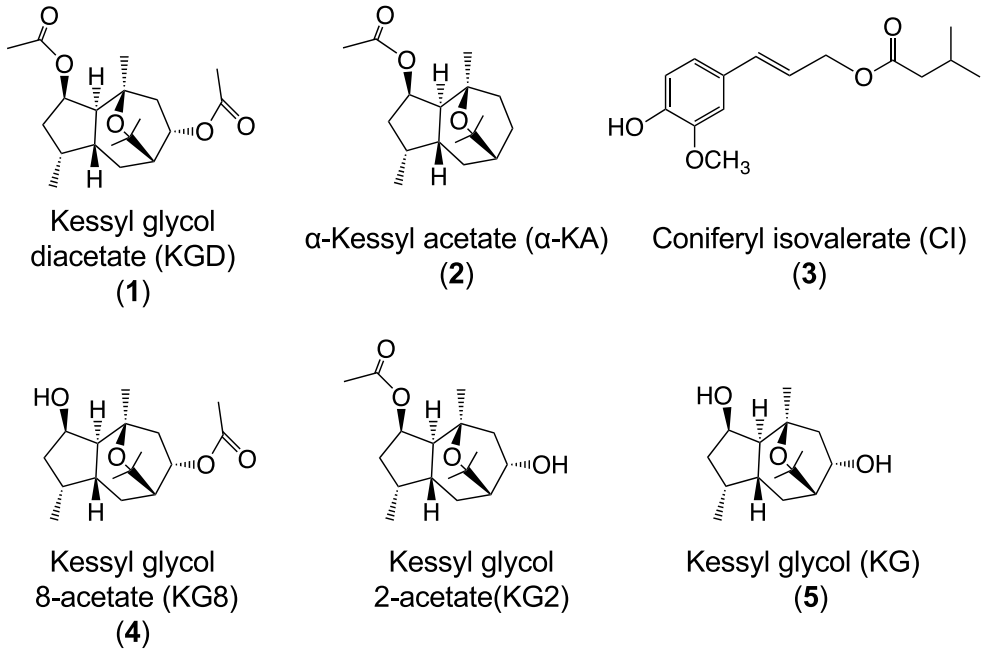


**Fig. 1.** Chemical structure of the ingredients in *Valeriana fauriei* root (VF).

expressing $\beta_3$-containing $GABA_A$ receptor were a major cellular substrate (Benke et al., 2009). $GABA_A$ receptors are ligand-gated ion channels expressed in the brain. Several psychological disorders, including epilepsy, anxiety, and sleep disorders, can be treated by activating the $GABA_A$ receptor response (Le et al., 2013). However, it remains unknown whether the essential oil components of VF modulate $GABA_A$ receptors and whether these highly lipophilic compounds are distributed in the brain. Although $GABA_A$ receptor has some subtypes (Sieghart and Savic, 2018), since valerenic acid had high affinity for the flunitrazepam binding sites (Benke et al., 2009), thus in this study also focused on the sites.

The objective of the present study was to elucidate the binding affinity on flunitrazepam sites of $GABA_A$ receptor of the VF extract in rats *in vitro*, to compare the activities with a VO extract, and to investigate the correlation between VF and VO extract components. We then examined the active ingredients in the VF extract using an *in vitro* assay, confirming their distribution in the brains of mice following oral administration.

## 2. Materials and methods

### 2.1. Materials

We used six batches of VF (Vf1–6) and four batches of VO (Vo1–4) samples, and their product information is presented in Table 1. Vf1–5 and Vo1–4 samples were used for *in vitro* and comparison studies, and Vf6 was used for the *in vivo* study. These samples were identified by anatomical studies with reference (Wang et al., 1998). Voucher specimens of these samples were deposited at Kuki Sangyo Co., Ltd.

### 2.2. Animals

Male Wistar/ST rats (7 weeks old) and male ddY mice (5 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The experimental procedures were conducted according to Nagoya City University Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the local Animal Ethics Committee of Nagoya City University.

### 2.3. Preparation of the extracts of VF

Vf2 (10 g) was extracted with 40 ml of ethanol or 70% ethanol at room temperature by standing for 48 h. The solution was filtered using a filter paper, and the residue was extracted in a similar manner. The filtrates were merged and evaporated, and the ratios of the extracts yielded were 3.4% and 18%, respectively. Vf6 (3.0 kg) was similarly extracted with ethanol (7.5 l), and the ratio of the extract yielded was 3.9%.

For the comparison study between VO and VF, each sample (0.50 g) was sonicated in 1 ml of ethanol and centrifuged at $1.5 \times 10^4$ rpm for 5 min. The supernatants were transferred to fresh tubes, evaporated under $N_2$ flow at room temperature, and finally lyophilized. The ratios of extracts yielded were 2.9%, 3.1%, 2.1%, 2.7%, and 2.7% for Vf1–Vf5, and 1.8%, 2.6%, 1.0%, and 1.2% for Vo1–Vo4, respectively. The extracts were dissolved in ethanol at 100 mg/ml and maintained at −20 °C until use.

### 2.4. Isolation of active ingredients from VF extract

Vf2 (1.0 kg) was extracted in 4 l of hexane by sonication for 1 h. The solution was filtered using a filter paper, with the residue extracted in a similar manner. The filtrate was evaporated at 40 °C under reduced pressure to obtain the extract (11 g). The extract was subjected to open silica gel chromatography eluted with ethyl acetate/hexane (2:8 → 5:5), and the fractions F1 (4.5 g), F2 (0.39 g), F3 (0.37 g), F4 (0.051 g), F5 (0.11 g), F6 (5.9 g), F7 (0.12 g), and F8 (0.14 g) were obtained by silica gel thin-layer chromatography (TLC) developed with hexane/ethyl acetate (8:2) or chloroform/methanol (98:2), and colored by anisaldehyde-sulfate solution. Fraction F6 exhibited the largest single spot with an $R_f$ value of 0.5, developed by chloroform/methanol (98:2), and compound **1** (1.9 g) was obtained by recrystallization with 2-propanol. Fraction F2 (0.39 g) was further applied to open silica gel chromatography eluted with chloroform/methanol (99:1), and then, compound **2** (84 mg) was isolated. Fraction F4 (51 mg) was further applied to open silica gel chromatography eluted with chloroform/methanol (99:1), and then, compound **3** (21 mg) was isolated. Fraction F8 (0.14 g) was further applied to open silica gel chromatography eluted with chloroform/methanol (95:5), and then, compounds **4** (28 mg) and **5** (18 mg) were isolated.

The chemical structures of compounds were analyzed using $^1$H and $^{13}$C-NMR and ESITOFMS. Compounds **1**, **2**, **4**, and **5** were identified as KGD, α-KA, KG8, and kessyl glycol (KG), respectively, according to a previous study (Nishiya et al., 1992). Compound **3** was identified as coniferyl isovalerate (CI), which was first isolated from the aerial part of *Eremantus incanus* (Bohlmann et al., 1980) (Fig. 1).

### 2.5. Synthesis of KGD derivatives by deacetylation of KGD

In brief, KGD (0.500 g) was dissolved in 100 ml of 2 M trifluoroacetic acid, and the solution was incubated at 30 °C for 24 h. After neutralization with sodium hydrogen carbonate, 100 ml of ethyl acetate was added, and the organic layer was collected. The same procedure was repeated twice for the aqueous layer, and the total organic layer was evaporated and dried using anhydrous sodium sulfate.

The organic layer was subjected to open silica gel chromatography and eluted with chloroform/isopropanol (95:5), KG8 (5.4 mg), KG 2-acetate (KG2) (0.125 mg), and KG (65.9 mg), and the compounds were identified by employing above listed methods.

**Table 1**
Sample list of the roots of *Valeriana fauriei* and *V. officinalis*.

| No. | Original plant species | Production area | Distributor | Lot No. | Obtained year | Voucher No. |
|---|---|---|---|---|---|---|
| Vf1 | *Valeriana fauriei* | Mie, Japan | Kuki Sangyo | KUK-00002 | 2017 | KUK-00002 |
| Vf2 | | Mie, Japan | Kuki Sangyo | A2018031901 | 2018 | A2018031901 |
| Vf3 | | Hokkaido, Japan | Uchida | EAG0110 | 2017 | KUK-00008 |
| Vf4 | | Hokkaido, Japan | Tochimoto | 1816001 | 2017 | KUK-00009 |
| Vf5 | | Hokkaido, Japan | Maechu | 20-SN2016A | 2017 | KUK-00016 |
| Vf6 | | Mie, Japan | Kuki Sangyo | A2019031401 | 2019 | A2019031401 |
| Vo1 | *V. officinalis* | Poland | Koujien | – | 2018 | KUK-00036 |
| Vo2 | | Poland | Café de Savon | – | 2018 | KUK-00037 |
| Vo3 | | United States | Sakurakougetu | – | 2018 | KUK-00038 |
| Vo4 | | Germany | Tree of Life | – | 2018 | KUK-00039 |

Voucher specimens were deposited at Kuki Sangyo Co., Ltd. Samples without lot numbers are written as "–". Kuki Sangyo, Mie, Japan; Uchida, Uchida Wakanyaku, Tokyo, Japan; Tochimoto, Tochimoto Tenkaido, Osaka, Japan; Maechu, Nara, Japan; Koujien, Osaka, Japan; Café de Savon, Yamanashi, Japan; Sakurakougetu, Shiga, Japan; Tree of Life, Tokyo, Japan.

### 2.6. Preparation of brain homogenate

The preparation of the brain homogenate was performed with modification according to methods described in previous studies (Risa et al., 2004; Choi et al., 2017). Rats were sacrificed by $CO_2$ inhalation, the heads were separated from the body, and the cerebral cortex was collected. Cerebral cortex samples from eight rats were homogenized in 40 ml of Tris-citrate buffer (50 mM, pH 7.1), and the suspension was centrifuged at 4 °C for 15 min at $2.7 \times 10^4 \times g$. The sediments were washed three times with Tris-citrate buffer. The washed sediment was resuspended in 40 ml Tris-citrate buffer. The suspension was incubated at 37 °C for 30 min and then centrifuged for 10 min at $2.7 \times 10^4 \times g$. The final sediment was resuspended in 60 ml of Tris-citrate buffer and stored in aliquots at −80 °C until use. The protein concentration of this suspension was 7.94 mg/ml, as measured using a BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

### 2.7. Binding assay for flunitrazepam sites of $GABA_A$ receptor

The $GABA_A$ receptor binding assay was performed as described previously (Benke et al., 2009; Choi et al., 2017; Risa et al., 2004), with the modification using a 96-well plate instead of a glass filter. The wells of a 96-well plate were coated with poly L-lysine solution (20 μg/ml, Sigma Aldrich, St. Louis, MO, USA) for 1 h at room temperature. After washing the wells with $H_2O$, 50 μl of rat brain homogenate (4 mg protein/ml) was added to each well and dried by standing on a hot plate (40 °C) overnight. After washing the wells with 200 μl of 0.05% Tween 20 in phosphate-buffered saline (T-PBS; 0.15 M, pH 7.2) three times, 200 μl of Block Ace® solution (blocking buffer; 40 mg/ml in Tris-citrate buffer, pH 7.1, Snow Brand Milk Products, Tokyo, Japan) was added to wells, and the plate was incubated at 37 °C for 1 h. The samples dissolved in ethanol was collected into the test tubes, and dried up under $N_2$ gas flow, and then, dissolved in blocking buffer directly by sonication at room temperature for more than 30 min. After discarding the blocking buffer in 96-well plate, 50 μl of each sample or blank solution in blocking buffer and 50 μl of [$^3$H]-flunitrazepam (1 nM in blocking buffer, American Radiolabeled Chemicals, St. Louis, MO, USA) were added to the wells; the plate was incubated at 4 °C overnight. Diazepam (Fujifilm Wako Pure Chemicals, Osaka, Japan) was used as the positive control. For the nonspecific binding (NSB) group, another excessive concentration of clonazepam (20 μM, Fujifilm Wako) was selected based on the previous literature (Benke et al., 2009; Risa et al., 2004). In addition, total binding (TB) was determined using a blank blocking buffer. After washing the wells with 200 μl of T-PBS five times, 100 μl of 10% sodium lauryl sulfate (SDS, Nacalai Tesque, Kyoto, Japan) solution was added to the wells, and the plate was incubated at room temperature for 1 h. The contents of each well were transferred into liquid scintillation vials containing 3 ml Clear-sol® (Nacalai Tesque), and the radioactivity (disintegration per minute, DPM) was measured using a liquid scintillation counter.

The following equation was employed to determine the displacement percentage of radioligand binding:

$$Binding\ displacement\ (\%) = \left(1 - \frac{DPM_{sample} - DPM_{NSB}}{DPM_{TB} - DPM_{NSB}}\right) \times 100$$

The half-maximal effective concentrations ($EC_{50}$) were calculated from the least square regression line plotted from 3 points that crossed 50% of the control logarithmic concentration values.

### 2.8. Qualitative analysis using HPLC

For each powdered sample (Vf1–5 and Vo1–4), 200 mg was sonicated in 10 ml of ethanol for 30 min. After centrifugation at 3500 rpm for 10 min, the supernatants were filtered through a 0.45 μm membrane filter and analyzed using an HPLC system (Alliance HPLC, Waters, Milford, MA, USA). The analytical column used was a Mightysil RP-18 GP II (4.6 i.d. × 250 mm, 5 μm, Kanto Chemical). For condition **1**, the mobile phase was passaged using a linear gradient elution system of 0.1% formic acid (A) and methanol (B), at a flow rate of 1.0 ml/min, with the following gradient profile: increasing from 10% B to 20% (0–7 min), increasing from 20% to 25% (7–13 min), 25% (13–28 min), increasing from 25% to 35% (28–29 min), increasing from 35% to 45% (29–43 min), increasing from 45% to 85% (43–48 min), increasing from 85% to 100% (48–53 min), and 100% (53–63 min). For condition **2**, the mobile phase used an isocratic system with 70% B. The column temperature was 40 °C for both conditions, and the detection wavelengths for conditions **1** and **2** were 210–340 nm and 198 nm, respectively. This method was based on previous studies assessing VO (Navarrete et al., 2006; Lucio-Gutiérrez et al., 2012). The peaks of valerenic acid and KGD were identified by comparison with standard compounds (valerenic acid, Extrasynthese, Lyon, France).

### 2.9. Quantitative analysis using LC-MS/MS for VF extracts

LC/MS/MS system (Quattro Premier XE, Waters) was used. Herein, the mass spectrometer used an electrospray ionization source in positive ion mode with multiple reaction monitoring. The analytical column used was an Inertsil ODS-4, 2.1 i.d. × 100 mm, 3 μm (GL Sciences Inc., Tokyo, Japan). The mobile phase was delivered using a linear gradient elution system with 0.5% acetic acid (A) and acetonitrile containing 0.5% acetic acid (B), at a flow rate of 0.25 ml/min, with the following gradient profile: increasing from 25% B to 30% (0–0.8 min), increasing from 30% to 60% (0.8–3.2 min), 60% (3.2–7.6 min), increasing from 60% to 80% (7.6–7.7 min), and 80% (7.7–9.0 min). The column temperature was maintained at room temperature. The transitions (precursor to daughter) were monitored, with retention times detected as 339.4 to 201.2 *m/z* for KGD (6.2 min), 297.4 to 291.2 *m/z* for KG2 and KG8 (4.4 and 5.1 min, respectively), 255.4 to 219.2 *m/z* for KG (3.0 min), 281.4 to 203.2 *m/z* for α-KA (7.4 min), 163.1 to 130.9 *m/z* for CI (6.2 min), and 195.2 to 139.0 *m/z* for *p*-hydroxybenzoic acid butyl ester (PHB, Nacalai Tesque, 5.7 min; used as an internal standard). Linear regressions over the concentration range 7.81 ng/ml– 2.00 μg/ml for KGD, KG2, KG8, α-KA, KG, and CI were examined using the peak-area ratios of the compounds to their internal standards and the least-squares method ($r^2 > 0.98$).

### 2.10. Animal experiments

The Vf6 ethanol extract was mixed with the same amount of dextrin (VIANDEX-BH®, Showa Sangyo, Tokyo, Japan), and the following samples were prepared: sample **A**, glycerin fatty acid ester (GFE; EXCEL S-95®, Kao, Tokyo, Japan) and sesame oil (Kuki Sangyo, Mie, Japan) mixed in a ratio of 0.5:14 (w/w). Then, 0.79 g of Vf6 extract/dextrin mixture was mixed with 10 ml of GFE/sesame oil mixture; sample **B**, 0.79 g of Vf6 extract/dextrin mixture was mixed with 10 ml of 5% gum arabic (GA; Kanto Chemical, Tokyo, Japan). The difference between these two emulsifiers is that GFE uses oil as the solvent, and GA uses water. The Vf6 extract/dextrin mixture (0.79 g) corresponded to 10 g of the Vf6 sample.

Mice were fasted overnight and then orally administered 10 ml/kg of sample **A** or **B**. Thirty minutes after administration, mice were sacrificed by $CO_2$ inhalation, and blood samples were collected from the abdominal vein into heparinized tubes; brain tissues were then separated. The blood samples were centrifuged at 4 °C for 7 min at $1.4 \times 10^4 \times g$, and the plasma samples were maintained at −20 °C until analysis. The preparation of brain samples was performed in accordance with previously described methods, with slight modifications (Hashiguchi et al., 2001; Fong et al., 2017). Individual brain tissue was washed with ice-cold PBS, gently wiped with paper to remove excess water, and then the weight of brain tissue was recorded. The brain tissue was dipped into 2-fold weight of ethanol, and homogenized using Pestle tissue grinder.

The brain homogenates were centrifuged at 4 °C for 7 min at $1.4 \times 10^4 \times g$. Then, plasma and brain samples (15 μl each) were mixed with 37.5 μl of ethanol containing 140 ng/ml of umbelliferone (Fujifilm Wako) used as an internal standard. The samples were centrifuged at 4 °C for 7 min at $1.4 \times 10^4 \times g$, and supernatants were analyzed using HPLC (DIONEX Ultimate 3000) with an MS/MS (TSQ QUANTUM ACCESS MAX) system (Thermo Fisher Scientific, MA, USA).

The mass spectrometer used an electrospray ionization source in positive ion mode with multiple reaction monitoring. A Mightysil RP-18 GP II, 3.0 i.d. × 150 mm, 3 μm (Kanto Chemical) analytical column was used. The mobile phase was delivered using a linear gradient elution system with 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.5 ml/min, with the following gradient profile: increasing from 25% B to 30% (0–1 min), increasing from 30% to 60% (14. min), 60% (4–11.8 min), increasing from 60% to 90% (11.8–11.9 min), and 90% (11.9–15.9 min). The column temperature was set at 40 °C. The transitions (precursor to daughter) were monitored, and retention times were as follows: 339.27 to 219.22 *m/z* for KGD (7.7 min), 237.20 to 219.22 *m/z* for KG2 and KG8 (5.3 and 6.1 min, respectively), 255.23 to 219.22 *m/z* for KG (3.7 min), 263.28 to 203.34 *m/z* for α-KA (11.2 min), 163.14 to 131.13 *m/z* for CI (7.6 min), and 204.18 to 163.17 *m/z* for umbelliferone (3.4 min). The standard solution was diluted with plasma or brain homogenates prepared from normal rats. In plasma samples, the calibration curve was found to be linear over the concentration range of 7.81 ng/ml–2.00 μg/ml for KGD, KG2, and KG, 1.96 ng/ml–2.00 μg/ml for KG8, and 31.3 ng/ml–2.00 μg/ml for α-KA, respectively. As CI demonstrates a high binding rate to albumin in plasma and could not be detected, the calibration curve was not plotted. In brain samples, the calibration curve was linear over the concentration range of 1.95 ng/ml–2.00 μg/ml for KGD, KG8, and α-KA, 0.488 ng/ml–2.00 μg/ml for CI, and 7.81 ng/ml–2.00 μg/ml for KG and KG2, respectively. These were examined using the peak-area ratios of compounds to their internal standards and the least-squares method ($r^2 > 0.99$).

### 2.11. Statistical analysis

Principal component analysis (PCA) was performed using the R software (Version 3.5.1, https://www.r-project.org). Statistical analyses of the data included were performed by one-way analysis of variance (ANOVA) and Dunnett's s multiple *t*-tests for comparison of multiple data, with two-way ANOVA employed for comparing dose-dependent activities between two groups. The analyses were conducted using Rstudio software (Version 1.1.447). A *P*-value of less than 0.05 ($p < 0.05$) was considered significant. All data are expressed as mean ± standard error (S.E.). In the animal study, samples with $p < 0.05$, determined by the Smirnov-Grubbs test, were removed as abnormal values.

## 3. Results

### 3.1. Confirmation for the binding assay system

To validate the modified binding assay system using a 96-well plate, we evaluated the competitive binding effects of diazepam, the representative benzodiazepine, and radiolabeled flunitrazepam (5 nM), a benzodiazepine agonist with specific activity on the $GABA_A$ receptor benzodiazepine-binding site, in the wells of 96-well plates coated with the rat brain homogenate. Diazepam exhibited significant competition with flunitrazepam in a concentration-dependent manner; 300 nM diazepam demonstrated nearly 100% binding capacity (Suppl. Fig. 1).

### 3.2. Binding effect of ethanol or 70% ethanol extract of VF on flunitrazepam sites of $GABA_A$ receptor

We prepared two different extracts from VF samples using ethanol and 70% ethanol and evaluated their binding activities on flunitrazepam sites of $GABA_A$ receptor. Both extracts exhibited similar binding activities in a concentration-dependent manner, and the concentration related to the crude drug weight (1.00 g/ml) exhibited approximately 100% binding activity. For ethanol and 70% ethanol extracts, $EC_{50}$ values were 161 crude drug mg/ml and 143 crude drug mg/ml, respectively (Fig. 2). These values were equivalent to 5.38 extract mg/ml and 25.9 mg/ml, respectively.

### 3.3. Comparison of binding activity between ethanol extracts of VF and VO

We prepared ethanol extracts of five batches of VF (Vf1–5) and four batches of VO (Vo1–4). The binding activities of VF extracts were significantly higher ($p = 0.002$) than those of VO extracts (Fig. 3).

### 3.4. Comparison of VF and VO constituents

Fig. 4 shows the chromatographs of Vf2 and Vo4 samples as representative data. Vf2 presented a smaller number of UV-detectable peaks when compared with Vo4 in the chromatograph using condition 1 (Fig. 4a and b); however, in the chromatograph using condition 2, KGD was detected in Vf2 (Fig. 4c). We compared the peak areas of VF and VO samples and observed characteristic peaks. To consider the extraction efficiencies of each sample, the value of the peak area divided by the extraction efficiency converted from the maximum value to 1 was adopted. In the VF samples, the peak areas of 5 peaks (peak nos. 7, 23, 35, 36, and 44) and 13 peaks (peak nos. 8, 12, 18, 21, 25, 29, 30, 32, 33, 34, 40, 41, and 43) were significantly higher and lower than those in the VO samples, respectively (Table 2).

Then, PCA was performed for the peak area of 44 peaks for VF and VO samples, as described in Table 2. Fig. 5 shows the PCA score plots of samples and loading plots of the peaks. The first two PCs accounted for 78% (PC1, 54%; PC2, 24%). The PC1–PC2 score plot clearly shows the separation between the VF and VO samples. The loading plots of PC1–PC2 score showed four peaks (peak nos. 44, 35, 23, and 36) and seven peaks (peak nos. 27, 29, 26, 32, 28, 41, and 34), which mainly contributed to VF and VO, respectively, and peaks 2 and 10 contributed to both VF and VO.

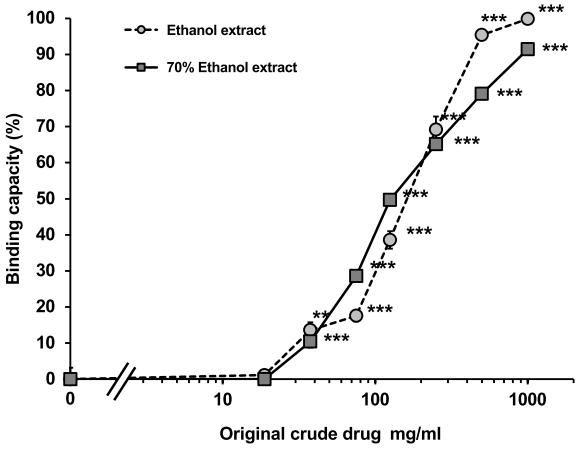


**Fig. 2.** Binding capacity of ethanol or 70% ethanol extract of *Valeriana fauriei* root (VF) to $GABA_A$ receptor in rat brain homogenate. Binding capacity was calculated as the ratio to the value of excessive clonazepam binding. Data were mean ± S.E. ($n = 4$).**$p < 0.01$, ***$p < 0.001$ *vs* control group (0 nM) by Dunnett's multiple *t*-test.

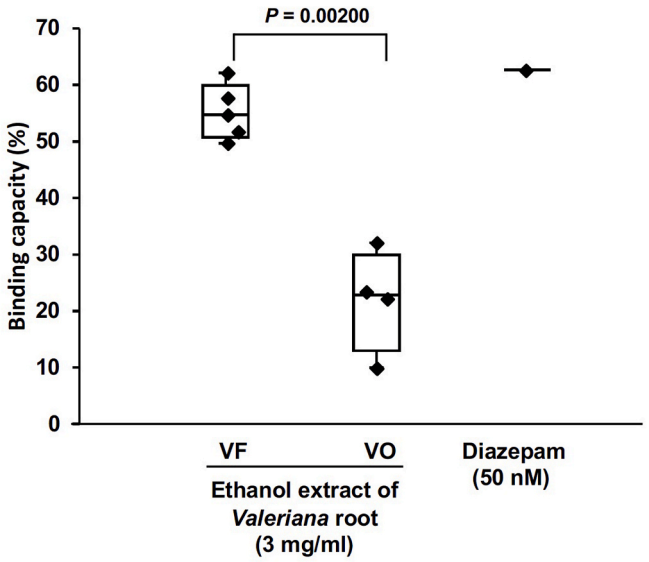


**Fig. 3.** Binding capacity of the ethanol extracts of *Valeriana fauriei* root (VF) and *V. officinalis* roots (VO) to $GABA_A$ receptor in rat brain homogenate. Binding capacity was calculated as the ratio to the value of excessive clonazepam binding. Each diamond symbol represents a data point of 1 batch of the sample ($n = 5$ for VF and $n = 4$ for VO). Diazepam used 1 batch, and data are shown as the mean of quadruple experiments. The *p*-value was calculated using the Student's *t*-test.

### 3.5. Correlation between binding activities on flunitrazepam sites of $GABA_A$ receptor and the compounds in VF and VO

We evaluated the correlation between the binding activities on flunitrazepam sites of $GABA_A$ receptor and peak areas of VF and VO, as shown in Table 3. The peaks showing significant differences between VF and VO are described in Table 2, and characteristic peaks for PCA analysis shown in Fig. 5 were selected. In the PCA analysis, peaks 2 and 10 contributing to both VF and VO correlated with the activity of VO but not with that of VF. Among characteristic VF peaks, peak No. 44, identified as KGD, was highly correlated with activity ($r = 0.91$). Meanwhile, among the 16 characteristic peaks of VO, 9 peaks (peak nos. 8, 12, 18, 27, 28, 32, 34, 40, and 41) correlated with the activities ($r \geq 0.7$). Although three peaks (peak nos. 12, 28, and 40) were also detected for VF, these peaks did not correlate with the activities. Peak No. 32 was identified as valerenic acid.

### 3.6. Exploration of active ingredients in VF extract

To explore the binding activities of active ingredients on flunitrazepam sites of $GABA_A$ receptor in VF, we prepared a hexane extract of VF and conducted activity-guided fractionation. First, we prepared fractions F1–8 and observed that significant activity of KGD contained F6, as described below. Among other fractions, we observed that fractions F2, F4, F5, F7, and F8 had significant effects (Suppl. Fig. 2). On performing $^{1}H$ and $^{13}C$-NMR analysis, fractions F2 and F4 were found to contain α-KA and CI, respectively, and F8 contained KG8 and KG. Fractions F5 and F7 were composed of KGD and other unknown compounds, respectively.

We evaluated the binding activity of each constituent in the VF extract. Among the six compounds, CI exhibited the highest binding activity. KGD, KG8, and α-KA exhibited binding activities in a concentration-dependent manner, and the activity was nearly 30% at concentrations ranging between 3 and10 mM. KG and KG2 showed binding activities of approximately 15% at a concentration of 10 mM, and the effects of KG and KG2 were lower than those of KGD, KG8, and α-KA (Fig. 6).

### 3.7. Calculation of the binding activity of each compound contained in ethanol extract of VF on flunitrazepam sites of $GABA_A$ receptor

The content of KGD, KG2, KG8, α-KA, KG, and CI in each ethanol or 70% ethanol extract of VF was measured by LC-MS/MS. In both extracts, KGD was the major constituent detected compounds analyzed; the contents of other constituents were one-tenth or less than that of KGD. CI was present only in the ethanol extract, whereas the amount of KG8 and KG in the 70% ethanol extract was higher than those in the ethanol extract (Table 4).

We then calculated the binding titers of these compounds on flunitrazepam sites of $GABA_A$ receptor when ethanol and 70% ethanol extracts of VF presented $EC_{50}$ values. For the ethanol extract of VF, the $EC_{50}$ value (161 crude drug mg/ml) was equivalent to the concentrations of KG (0.18 mM), KG2 (0.01 mM), KG8 (0.08 mM), KGD (4.44 mM), α-KA (0.26 mM), and CI (0.37 mM), respectively. From the activity-concentration curve shown in Fig. 6, KG (0.18 mM) and KG2 (0.01 mM) did not exhibit any binding activity, whereas binding activities of KG8 (0.08 mM), KGD (4.44 mM), α-KA (0.26 mM), and CI (0.37 mM) were estimated to be 5%, 38%, 10%, and 14%, respectively.

For the 70% ethanol extract of VF, $EC_{50}$ values (143 crude drug mg/ml) were equivalent to the concentrations of KG (0.77 mM), KG2 (0.01 mM), KG8 (0.19 mM), KGD (3.93 mM), and α-KA (0.23 mM), respectively. Similar to the above calculation, KG (0.77 mM) and KG2 (0.01 mM) did not exhibit any binding activity, whereas binding activities of KG8 (0.19 mM), KGD (3.93 mM), and α-KA (0.23 mM) were estimated to be 5%, 38%, and 9%, respectively.

### 3.8. Distribution of active ingredients in the brain of mice orally administered VF extract

We added two types of emulsifiers, GFE and GA, to the ethanol extract of VF, and then examined differences in the constituent concentrations, both in the plasma and brain, 30 min after oral administration in mice. The brain ($C_{brain}$) and plasma ($C_{plasma}$) concentrations of constituent compounds are shown in Table 5. KGD, KG8, KG2, and α-KA were detected in the plasma and brain of mice treated with both preparations. CI was only marginally detected in the brain. On using either GFE or GA for emulsification, KG in the plasma and brain were below the quantification limit of determination because of large impurities.

Although KGD was the main component detected in the VF extract, the brain distribution of KGD was the lowest among those of other compounds, except for CI, on using GFE as the emulsifier. The brain distributions of KG2 and KG8 were more than 10 times higher than those of the other compounds. On using GA for emulsification, the brain distribution of KGD was the lowest among those of other compounds, except for CI. On comparing the two preparations, the brain distributions of KG2, KG8, and KGD were higher when GFE was used as an emulsifier than when GA was employed. However, the brain distribution of α-KA tended to be higher when GA was used rather than when GFE was employed for emulsification. $C_{brain}/C_{plasma}$ values were calculated by dividing $C_{brain}$ by the corresponding $C_{plasma}$. The values of KG8 and KGD on utilizing GFE were similar to those obtained with GA, whereas the values of α-KA with GA were higher than when GFE was used.

## 4. Discussion

In the present study, we first identified the active ingredients of VF demonstrating sedative effects using a binding assay on flunitrazepam sites of $GABA_A$ receptor, which focuses on the pharmacological target for insomnia treatment (Le et al., 2013). VO, which belongs to the same genus as VF, is typically employed as a botanical sedative drug in European countries (Weiss et al., 2002; European Directorate for the Quality of Medicines & Health Care, 2020; U.S. Pharmacopeial United States Pharmacopeial Convention, 2020) and had the binding activity on $GABA_A$ receptor (Yuan et al., 2004; Benke et al., 2009).

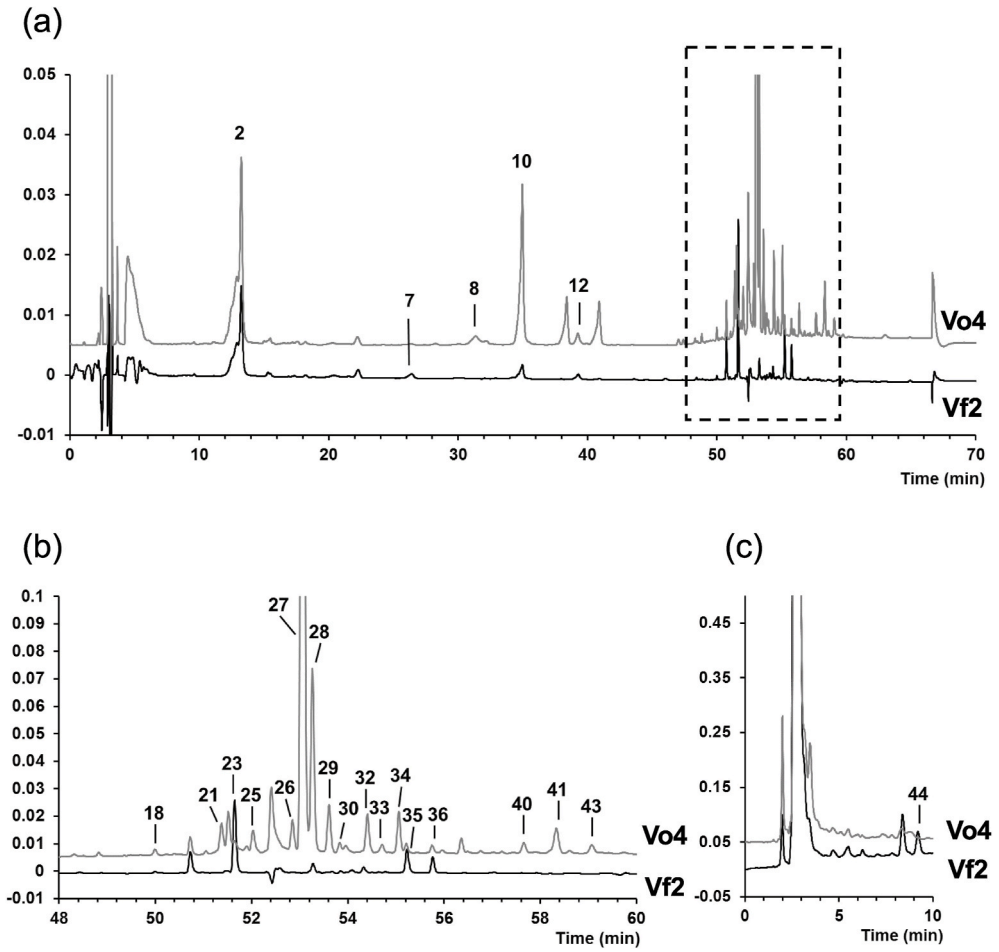


**Fig. 4.** Comparison of the HPLC chromatographs of *Valeriana fauriei* root (Vf2) and *V. officinalis* root (Vo4). (a) Maximum chromatograph plot of 210–340 nm analyzed by condition **1**. (b) Enlarged view of the dotted line in Fig. 4a. (c) Chromatograph detected at 198 nm analyzed by condition **2**. HPLC conditions are described in the Materials and Methods section. Peak numbers are listed in Table 2.

In a general receptor binding assay system, the radiolabeled ligand and receptor are separated using glass microfiber filters. As VF contains a large amount of essential oil components, nonspecific binding on the glass fiber was observed, and the analysis could not be performed. Thus, we prepared a new assay system to evaluate the $GABA_A$ receptor binding activity using [$^3$H]-flunitrazepam and a rat cerebral cortex homogenate in a 96-well plate. In this system, the representative benzodiazepine, diazepam, exhibited an $EC_{50}$ value of approximately 10–30 nM. In a previous study using a general receptor binding assay (Watanabe et al., 1986), the $EC_{50}$ values of diazepam in competition with [$^3$H]-flunitrazepam binding, using rat brain cerebellum and spinal cord, were approximately 10 nM, which is similar to that observed in the present study. This indicates that the tested system can be applied not only for VF but also for various plant materials rich in essential oil components.

By employing this system, we compared the binding activities on flunitrazepam sites of $GABA_A$ receptor between VF and VO extracts; the latter has been previously reported (Yuan et al., 2004; Benke et al., 2009). We observed that the binding activities of VF extracts were significantly higher than those of VO extracts. Reportedly, mice treated with the VO extract show a longer hexobarbital-induced sleeping time than mice treated with the VF extract (Takamura et al., 1973). However, it remains debatable whether these two plant species could be accurately compared using only one individual of each species. The present study is the first to compare the activities of VO and VF using several individual constituents, and the VF extract exhibited significantly higher binding activity on flunitrazepam sites of $GABA_A$ receptor than the VO extract. It was reported that the molecular target of valerenic acid, the main compound of VO, on $GABA_A$ receptor was $\beta_3$-subunit among its flunitrazepam sites (Benke et al., 2009). However, we did not elucidate the interactions between the major substrates for VF and any allosteric sites of $GABA_A$ receptor. This is the limit of the present study, and the further investigation is needed.

In Japan, VF was used as a folk medicine in the middle of the Edo era (1603–1868). It was then used for hysteria like VO in European countries after the late Edo era when European culture had been imported from the Netherlands to Japan. As hysteria was associated with female medical disorders during the Meiji era (1868–1912), VF began to be used as a women's home medicine in Japan (Ota et al., in press). Furthermore, as VF contains a higher amount of essential oil than VO (Houghton, 1988), VF has been exported from Japan to Germany at a high price between the Taisho (1912–1925) and early Showa era (1926–1988) (Yanagisawa et al., 1993; Yanagisawa, 2014). The present results suggest that VF is superior to VO not only in essential oil content and but also sedative and anxiolytic activities.

Furthermore, we observed that VF constituents significantly differed from those in VO, and PCA analysis revealed that they were located in different clusters. Among these compounds, the KGD content highly correlated with the binding activity of the VF extract on flunitrazepam sites of $GABA_A$ receptor. Meanwhile, valerenic acid (peak No. 32) and the other five compounds (peak nos. 8, 18, 27, 34, and 41) correlated with the VO extract binding activity. Valerenic acid was also slightly associated with the VF extract activity; however, the other compounds contained in both VF and VO were not correlated with the activity. These results suggest that the KGD content may primarily contribute to

**Table 2**
HPLC peak areas of *Valeriana fauriei* root (VF) and *V. officinalis* root (VO).

| Peak No. | $t_R$ (min) | Peak areas of VF converted to extract concentration | Peak areas of VO converted to extract concentration | *P* value |
|---|---|---|---|---|
| 1 | 12.9 | $(5.50 \pm 2.31) \times 10^3$ | $(9.70 \pm 9.70) \times 10^3$ | |
| 2 | 13.2 | $(8.93 \pm 1.75) \times 10^4$ | $(2.13 \pm 0.91) \times 10^5$ | |
| 3 | 15.5 | $(5.87 \pm 5.87) \times 10^3$ | $(2.06 \pm 1.55) \times 10^4$ | |
| 4 | 18.2 | $(1.32 \pm 1.32) \times 10^2$ | n.d. | |
| 5 | 20.4 | $(3.43 \pm 3.43) \times 10^3$ | n.d. | |
| 6 | 22.2 | $(2.09 \pm 0.60) \times 10^4$ | $(1.94 \pm 1.18) \times 10^4$ | |
| 7 | 26.4 | $(2.81 \pm 0.31) \times 10^4$ | n.d. | *** |
| 8 | 31.3 | n.d. | $(6.04 \pm 1.16) \times 10^4$ | *** |
| 9 | 34.2 | $(1.51 \pm 1.51) \times 10^3$ | n.d. | |
| 10 | 35.0 | $(3.46 \pm 0.73) \times 10^4$ | $(2.71 \pm 1.82) \times 10^5$ | |
| 11 | 38.4 | n.d. | $(6.95 \pm 5.94) \times 10^4$ | |
| 12 | 39.3 | $(3.60 \pm 3.60) \times 10^3$ | $(3.74 \pm 0.80) \times 10^4$ | ** |
| 13 | 40.9 | n.d. | $(7.67 \pm 5.75) \times 10^4$ | |
| 14 | 43.6 | $(4.60 \pm 2.82) \times 10^3$ | $(1.31 \pm 0.84) \times 10^4$ | |
| 15 | 47.0 | n.d. | $(8.02 \pm 4.64) \times 10^3$ | |
| 16 | 47.4 | n.d. | $(2.82 \pm 2.82) \times 10^3$ | |
| 17 | 48.8 | n.d. | $(8.87 \pm 5.48) \times 10^3$ | |
| 18 | 50.0 | n.d. | $(1.72 \pm 0.21) \times 10^4$ | *** |
| 19 | 50.7 | $(2.32 \pm 0.48) \times 10^4$ | $(2.10 \pm 1.26) \times 10^4$ | |
| 20 | 51.1 | n.d. | $(3.44 \pm 2.04) \times 10^3$ | |
| 21 | 51.4 | n.d. | $(6.73 \pm 3.03) \times 10^4$ | * |
| 22 | 51.5 | n.d. | $(2.57 \pm 1.89) \times 10^4$ | |
| 23 | 51.6 | $(8.47 \pm 1.37) \times 10^4$ | $(6.99 \pm 4.36) \times 10^3$ | ** |
| 24 | 51.9 | n.d. | $(5.46 \pm 3.19) \times 10^3$ | |
| 25 | 52.0 | n.d. | $(6.98 \pm 2.62) \times 10^4$ | * |
| 26 | 52.8 | n.d. | $(1.47 \pm 0.76) \times 10^4$ | |
| 27 | 53.1 | n.d. | $(1.60 \pm 0.89) \times 10^6$ | |
| 28 | 53.3 | $(2.53 \pm 2.53) \times 10^3$ | $(2.12 \pm 1.07) \times 10^5$ | |
| 29 | 53.6 | $(1.02 \pm 1.02) \times 10^3$ | $(2.06 \pm 0.51) \times 10^5$ | ** |
| 30 | 53.8 | $(7.31 \pm 2.56) \times 10^3$ | $(3.80 \pm 1.33) \times 10^4$ | * |
| 31 | 54.0 | $(2.36 \pm 1.45) \times 10^3$ | $(4.48 \pm 3.00) \times 10^3$ | |
| 32 | 54.4 | $(9.45 \pm 0.57) \times 10^3$ | $(9.86 \pm 2.10) \times 10^4$ | ** |
| 33 | 54.7 | n.d. | $(3.32 \pm 1.21) \times 10^4$ | * |
| 34 | 55.1 | n.d. | $(9.56 \pm 2.80) \times 10^4$ | ** |
| 35 | 55.2 | $(9.20 \pm 2.24) \times 10^4$ | $(6.14 \pm 4.51) \times 10^3$ | * |
| 36 | 55.8 | $(5.70 \pm 1.31) \times 10^4$ | $(8.13 \pm 5.85) \times 10^3$ | * |
| 37 | 56.0 | n.d. | $(2.17 \pm 2.17) \times 10^3$ | |
| 38 | 56.4 | n.d. | $(5.58 \pm 2.94) \times 10^4$ | |
| 39 | 56.8 | n.d. | $(1.81 \pm 1.81) \times 10^3$ | |
| 40 | 57.7 | $(2.02 \pm 1.24) \times 10^3$ | $(2.62 \pm 0.68) \times 10^4$ | ** |
| 41 | 58.3 | n.d. | $(8.57 \pm 2.50) \times 10^4$ | ** |
| 42 | 58.6 | n.d. | $(1.13 \pm 0.68) \times 10^4$ | |
| 43 | 59.1 | n.d. | $(3.32 \pm 1.02) \times 10^4$ | ** |
| 44 | 9.1 | $(4.66 \pm 0.57) \times 10^5$ | n.d. | *** |

Data are expressed as the mean ± S.E. ($n = 5$ for VF and $n = 4$ for VO). The conversion of the peak area was calculated by dividing by the extraction efficiency with a maximum value of 1.
*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ between VF and VO by Student's *t*-test. n.d., not detected. $t_R$, retention time.

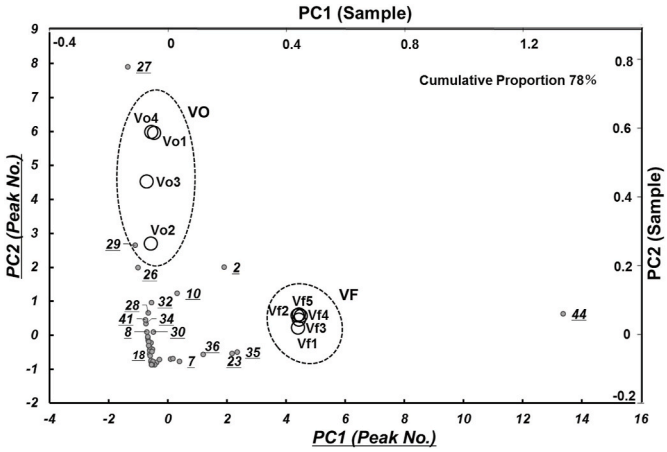


**Fig. 5.** Principal component analysis (PCA) score plots for 5 batches of *Valeriana fauriei* root (VF) and 4 batches of *V. officinalis* root (VO) and loading plots of peaks. The PCA was performed for the peak area of 44 peaks described in Table 2. The top and right axes indicate the score plots of *Valeriana* samples, and the bottom and left axes indicate the loading plots of peaks.

**Table 3**
Correlation between the $GABA_A$ receptor binding activity of *Valeriana fauriei* root (VF) or *V. officinalis* root (VO) and its peak area converted to extract concentration.

| Characteristic species | Peak No. | $t_R$ (min) | Correlation between the activity of either VF or VO and its peak area converted to extract concentration (*r*) VF | VO |
|---|---|---|---|---|
| VF, VO | 2 | 13.2 | − 0.21 | 0.90 |
| | 10 | 35.0 | − 0.20 | 0.81 |
| VF | 7 | 26.4 | − 0.56 | unreckonable |
| | 23 | 51.6 | − 0.45 | 0.54 |
| | 35 | 55.2 | 0.06 | 0.52 |
| | 36 | 55.8 | 0.16 | 0.50 |
| | 44 | 9.1 | 0.91 | unreckonable |
| VO | 8 | 31.3 | unreckonable | 0.81 |
| | 12 | 39.3 | 0.18 | 0.93 |
| | 18 | 50.0 | unreckonable | 0.85 |
| | 21 | 51.4 | unreckonable | 0.48 |
| | 25 | 52.0 | unreckonable | 0.50 |
| | 26 | 52.8 | unreckonable | 0.17 |
| | 27 | 53.1 | unreckonable | 0.82 |
| | 28 | 53.3 | 0.18 | 0.90 |
| | 29 | 53.6 | 0.21 | 0.30 |
| | 30 | 53.8 | 0.40 | − 0.15 |
| | 32 | 54.4 | 0.51 | 0.70 |
| | 33 | 54.7 | unreckonable | 0.42 |
| | 34 | 55.1 | unreckonable | 0.73 |
| | 40 | 57.7 | − 0.83 | 0.91 |
| | 41 | 58.3 | unreckonable | 0.78 |
| | 43 | 59.1 | unreckonable | 0.53 |

The peaks were selected significantly higher peaks of VF or VO, as described in Table 2 and characteristic peaks for the principal component analysis (PCA) shown in Fig. 5, are shown in Fig. 4. Peak nos. 32 and 44 were identified as valerenic acid and kessyl glycol diacetate, respectively, by comparison with standard compounds. The conversion of the peak area was calculated by dividing by the extraction efficiency with a maximum value of 1. When the peaks were not detected, *r* values were shown as "unreckonable". $t_R$, retention time.

the binding activity of VF on flunitrazepam sites of $GABA_A$ receptors. In contrast, valerenic acid might not be the only contributor to the activity of VO. In present study, although we used the VF and VO samples derived from different origins, we could only compare the total components of those two species because the number of samples was not enough to mention the difference of active ingredients due to the origin.

Further investigation revealed that ethanol and 70% ethanol extracts of VF exhibited similar binding activities on flunitrazepam sites of $GABA_A$ receptor converted as the dosage related to the original crude drug weight, suggesting that the active ingredients in VF would barely dissolve in water. We isolated and identified KGD, KG8, KG2, α-KA, KG, and CI as active ingredients of VF. Among the activity-concentration curves of these compounds, CI had the highest activity, followed by KGD, α-KA, KG8, KG2, and KG. Among these compounds, KGD had the highest concentration, followed by KG, α-KA, KG8, and KG2 in the 70% ethanol extract, and by CI, α-KA, KG, KG8, and KG2 in the ethanol extract.

In both VF extracts, as KG and KG2 did not exhibit any binding activity at the concentration of $EC_{50}$ values in VF extracts, it can be suggested that these two compounds did not contribute to the binding activity. As for the 70% ethanol extract of VF, CI did not contribute to the activity as it was absent in this extract. The sum of the activity values of other compounds was 52%, i.e., nearly 50%; hence, it can be suggested that KGD contributed to the majority of binding activity on

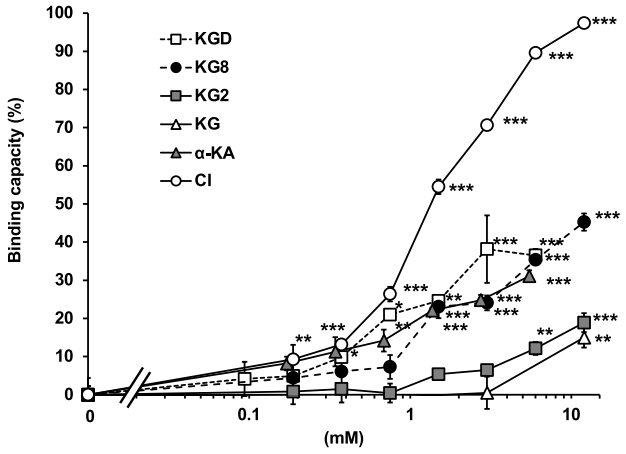


**Fig. 6.** Binding capacity of constituents in *Valeriana fauriei* root extract to $GABA_A$ receptor in rat brain homogenate. Binding capacity was calculated as the ratio to the value of excessive clonazepam binding. Data were mean ± S.E. ($n = 4$). $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* control group (0 nM) by Dunnett's multiple *t*-test. KGD, kessyl 2 glycol diacetate; KG2, kessyl glycol 2-acetate, KG8, kessyl glycol 8-acetate; α-KA, α-kessyl acetate; KG, kessyl glycol; CI, coniferyl isovalerate.

**Table 4**
$GABA_A$ receptor binding activity of each compound when activities of ethanol and 70% ethanol extract of *Valeriana fauriei* root show 50% effective concentration ($EC_{50}$) values.

| Compound | Ethanol extract[a] | | 70% ethanol extract[b] | |
|---|---|---|---|---|
| | Concentration (mM)[c] | Activity (%)[d] | Concentration (mM)[c] | Activity (%)[d] |
| KG | 0.18 | 0 | 0.77 | 0 |
| KG2 | 0.01 | 0 | 0.01 | 0 |
| KG8 | 0.08 | 5 | 0.19 | 5 |
| KGD | 4.44 | 38 | 3.93 | 38 |
| α-KA | 0.26 | 10 | 0.23 | 9 |
| CI | 0.37 | 14 | n.d. | 0 |

$EC_{50}$ values were calculated from the least square regression line plotted from 3 points that crossed 50% of the control logarithmic concentration values (Fig. 2).
a) $EC_{50}$ value of ethanol extract is 161 mg (original crude drug)/ml which is related to 5.38 mg (extract)/ml.
b) $EC_{50}$ value of 70% ethanol extract is 143 mg (original crude drug)/ml which is related to 25.9 mg (extract)/ml.
c) Estimated concentration of each compound in the sample at a concentration equal to the $EC_{50}$ value.
d) $GABA_A$ receptor binding activity of each compound at that concentration was calculated from the activity-concentration curve (Fig. 6).
KG, kessyl glycol; KG2, kessyl glycol 2-acetate; KG8, kessyl glycol 8-acetate; KGD, kessyl glycol diacetate; α-KA, α-kessyl acetate; CI, coniferyl isovalerate.

flunitrazepam sites of $GABA_A$ receptor, whereas KG8 and α-KA demonstrated minor contributions, in the 70% ethanol extract of VF. Moreover, our findings indicate that KG8 and α-KA would interact additively and that other compounds in the extract might not contribute to the total effects.

For the ethanol extract of VF, the sum of these values was 67%; hence, it can be suggested that KGD contributed to the majority of the binding activity on flunitrazepam sites of $GABA_A$ receptor, whereas KG8, α-KA, and CI presented a minor contribution, and these compounds or other unknown compounds in the ethanol extract interact counteractively among the compounds. CI exhibited the highest *in vitro* binding activity on flunitrazepam sites of $GABA_A$ receptor. However, CI was scarcely distributed in the plasma and brain owing to the high binding rate to albumin, revealing that CI did not contribute to the actual activity. Therefore, the three compounds, KGD, KG8, and α-KA, would contribute to the binding activity of the ethanol extract of VF, as well as 70% ethanol extract, on flunitrazepam sites of $GABA_A$ receptor.

We elucidated that the three compounds, KGD, KG8, and α-KA, were distributed in the mouse brain following oral administration of the VF extract. Although KGD was the main component of the VF extract, only 0.144 ± 0.107% of KGD was distributed in the brain when GA, a water-soluble emulsifier, was used; however, brain distribution was approximately doubled with GFE, a fat-soluble emulsifier. Moreover, the brain distributions of KG8 and KG2 in mice treated with the GFE-emulsified formulation were more than 10-times higher than that of mice treated with the GA-emulsified preparation. Based on these findings, it can be postulated that incorporating a fat-soluble emulsifier could promote the absorption of KGD and that KGD might undergo de-esterification to KG8 and KG2 in the body. Although the brain distributions of KG8 and KGD were higher in mice treated with the GFE-emulsified preparation, the $C_{brain}/C_{plasma}$ values of these two compounds did not differ between the two formulations. As KG8 and KGD distribution depends on their plasma concentrations, the transport of these two compounds from the blood to the brain might be saturated. For α-KA, the $C_{brain}/C_{plasma}$ value was higher in mice treated with the GA-emulsified preparation than the GFE-added preparation; this indicated that the transfer of α-KA from the blood to the brain might be affected by drug additives.

Takamura et al. (1975b) and Hikino et al. (1980) have reported that the enhancing effect of KG8 emulsified with GA on hexobarbital-induced sleeping time was greater than that of KGD. In the present study, the distribution of KG8 in the plasma and brain of mice treated with the VF extract emulsified using GFE was significantly higher than that emulsified using GA, and the distribution of KGD was much less than that of KG8. On employing GFE as the emulsifier for KG8, the sleep time induced by KG8 might be considerably longer than that induced by KGD. Furthermore, both KGD-rich and α-KA-rich types of VF prolonged pentobarbital-induced sleeping time in mice (Yoshitomi et al., 2000). Reportedly, KGD prolongs hexobarbital-induced sleep time in mice (Takamura et al., 1973, 1975a, 1975b). However, there are no reports available regarding the effects of α-KA. Our present study revealed that both KGD and α-KA demonstrated the binding activities on flunitrazepam sites of $GABA_A$ receptor *in vitro*. Following oral administration, these compounds were distributed in the brains of mice. Although previous *in vivo* studies used excessively high dosages (approximately 20–40 g of VF/kg), our present results suggest a possible underlying mechanism and active ingredients of VF as folk medicine.

## 5. Conclusion

The extract of *Valeriana fauriei* root (VF) exhibited the binding activity on flunitrazepam sites of $GABA_A$ receptor, which was significantly higher than that of *Valeriana officinalis* root (VO). Kessyl glycol diacetate (KGD), kessyl glycol 8-acetate (KG8), and α-kessyl acetate (α-KA) are active ingredients present in VF that exhibit binding activity on flunitrazepam sites of $GABA_A$ receptor, and these three compounds can be distributed to the brain. For KGD, which is the main active ingredient, the addition of a fat-soluble emulsifier promoted absorption, and it was metabolized to KG8 in the body. Thus, these compounds can be used as marker compounds to evaluate the quality of VF products in terms of their sedative effects.

## A list of author's contributions

M.O., H.N., Y.M., D.K., S.M., and S.N. conducted the experiments. M. O., T.M, and Y.O. taught and advised the experiments to H.N., Y.M., D. K., S.M., and S.N. Y.O. performed statistical analysis. K.I. performed NMR analysis. M.O. wrote the draft article, T.M. and M.O. are presiding over the study.

**Table 5**
The brain ($C_{brain}$) and plasma ($C_{plasma}$) concentrations of the compounds 30 min after the oral administration of two VF extract preparations, emulsified with glycerin fatty acid ester (GFE) and dissolved with oil, or emulsified with gum arabic (GA) and dissolved with water, at the crude drug dosage of 10 g/kg to mice.

| Compound | The kinds of emulsifier | Dose in VF extract (mg/kg)[a)] | $C_{brain}$ (ng/g) | $C_{plasma}$ (ng/ml) | Distribution in brain (%)[b)] | $C_{brain}/C_{plasma}$[c)] |
|---|---|---|---|---|---|---|
| KG2 | GFE | 0.179 | 137 ± 26 ** | n.d. | 76.5 ± 14.3 ** | unreckonable |
| | GA | | 12.2 ± 7.5 | 2.19 ± 2.19 | 6.80 ± 4.21 | 1.78 ± 0.00 |
| KG8 | GFE | 1.55 | 506 ± 130 * | 378 ± 74.0 ** | 32.6 ± 8.41 * | 1.26 ± 0.11 |
| | GA | | 36.0 ± 8.2 | 33.1 ± 5.8 | 2.32 ± 0.53 | 1.17 ± 0.22 |
| KGD | GFE | 93.4 | 206 ± 48 | 219 ± 81.6 | 0.221 ± 0.051 | 1.31 ± 0.34 |
| | GA | | 135 ± 100 | 60.3 ± 17 | 0.144 ± 0.107 | 1.43 ± 0.56 |
| α-KA | GFE | 4.51 | 51.7 ± 23.7 | 114 ± 47 | 1.15 ± 0.53 | 0.822 ± 0.619 |
| | GA | | 290 ± 161 | 188 ± 46 | 6.43 ± 3.57 | 4.70 ± 2.58 |
| CI | GFE | 6.09 | 0.409 ± 0.379 | n.d. | 0.00672 ± 0.00622 | unreckonable |
| | GA | | 0.973 ± 0.522 | n.d. | 0.0160 ± 0.0086 | unreckonable |

Data were expressed as mean ± S.E. ($n$ = 6–7). *$p < 0.05$ and **$p < 0.01$ *vs* treated with GA-added preparation group by Student's *t*-test.
a) The dose in the VF extract was calculated based on the individual compound content in the VF extract.
b) Distribution in the brain (%) was calculated by dividing the measured $C_{brain}$ of the compound by its dose in the VF extract.
c) $C_{brain}/C_{plasma}$ was calculated by dividing the measured $C_{brain}$ of the compound by its corresponding $C_{plasma}$.
When the compounds were not detected, $C_{brain}/C_{plasma}$ values shown as "unreckonable".
KG, kessyl glycol; KG2, kessyl glycol 2-acetate; KG8, kessyl glycol 8-acetate; KGD, kessyl glycol diacetate; α-KA, α-kessyl acetate; CI, coniferyl isovalerate; trace, less than 1 μg/g for KG in $C_{brain}$ and 1.5 μg/ml for KG in $C_{plasma}$; n.d., not detected.

## Declaration of competing interest

T.M. received grant support from Tsumura & Co., Kracie Pharmaceuticals, and JPS Pharmaceuticals. M.O., Y.M., D.K., S.M., S.N., and Y. O. are employee of Kuki Sangyo.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114262.

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