RESEARCH ARTICLE

Analysis of *GABRB3* gene mRNA expression and motor coordination after administration of valerian extracts (*Valeriana officinalis*) in BALB/c mice [version 1; peer review: 2 approved with reservations]

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Abstract

Background: *Valeriana officinalis* has often been used to treat sleep disorders as a traditional medicine for 2000 years. The sedative effect of valerian extract is facilitated by the GABA_A receptor $\beta 3$ subunit. The aim of this study is to determine the effect of valerian extract on *GABRB3* gene mRNA expression and sedative effect in BALB/c mice.

Methods: This is an experimental preclinical study using a posttest-only control group design. A total of 20 BALB/c mice were randomly allocated into four groups consisting of five mice each. Group I was given 5 ml of Aqua Dest (distilled water), group II was given 0.025 mg/10 g of diazepam, group III was given 2.5 mg/10 g of valerian extract, and group IV was given 5 mg/10 g of valerian extract. The drugs were administered for seven days through a gastric gavage. The rotarod test was performed on the seventh day. A blood sample was taken on the first day before drug administration and after the rotarod test on the seventh day to be analyzed using quantitative real-time PCR.

Results: *GABRB3* gene mRNA expression showed a significant increase in groups II, III, and IV ($p < 0.0001$). There was significant difference between group III and IV. The examination of motor coordination (rotarod test) showed a significant difference ($p < 0.05$) between group I and group II, between group I and group III, and between group I and group IV. There was no significant difference between group II and both groups III and IV.

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2. **Margot Ernst** , Medical University of Vienna, Vienna, Austria

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Conclusions: *GABRB3* gene mRNA expression was significantly increased after the administration of valerian extract. Based on the rotarod test, valerian extract and diazepam had a clinically similar sedation effect. A higher dose of valerian extract does not yield a higher level of *GABRB3* gene mRNA expression nor sedative effects.

Keywords

Valeriana officinalis, *GABRB3* genes, sedative, real-time polymerase chain reaction, rotarod, BALB/c

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Introduction

GABRB3 is the gene that encodes the GABA_A receptor β_3 subunit, which is one of the chloride sub-channels of the gamma aminobutyric acid (GABA) receptor in humans. The protein encoded by the *GABRB3* gene is one of 13 sub-units of the multiple chloride canal sub-units that act as GABA receptors. *GABRB3* is a member of the GABA_A receptor family genes and has a heterometric structure of pentameric ion channel ligand channels^{1–3}. GABA is a very important inhibitory neurotransmitter of the central nervous system. GABA has an important role in reducing the excitation of neurons by inhibiting the transmission of nerve impulses in the brain and also plays a role in the regulation of muscle tone. GABA is classified as an amino acid because it has an amine group⁴. GABA receptors are the target workplace of GABA. GABA_A is one of the GABA receptors that works in 40% of the mammalian central nervous system as a major inhibitory neurotransmitter. GABA_A receptors are the site of action of a variety pharmacological drugs including barbiturates, benzodiazepines and ethanol. The majority of gene coding in the GABA_A receptor sub-units is arranged into four clusters on chromosomes 4, 5, 15 and X in the human genome. The cluster of genes is assumed to contribute to the expression of genes. There are two clusters that have three genes, namely $\alpha 5$, $\beta 3$ and $\gamma 3$ on chromosome 15. The similarity of all clusters is that there is a β subunit that shows the orientation of the reverse transcription process^{5–7}.

Sleep is a vital physiological phenomenon, which maintains the balance of human body regulation. Insomnia is a temporary or persistent difficulty in starting or maintaining sleep. Insomnia should be treated optimally, for it decreases the quality of life, stamina, productivity, and even triggers mental illnesses. The impact of insomnia cannot be underestimated, because it can lead to more serious conditions and endanger health and safety. Therefore, every insomniac needs to find the right solution^{8–12}. Diazepam is one of the oldest and most commonly prescribed drugs for treating sleep disorders and belongs to the benzodiazepines class of drugs. Benzodiazepines exert their sedative-hypnotic properties by increasing or potentiating the binding of major inhibitory neurotransmitter GABA at the GABA_A receptor¹³. These receptors are found in the central nervous system and consist of five subunits of proteins: two alpha sub-units, two beta sub-units, and one gamma sub-unit. Under normal conditions, GABA binds to alpha subunits at the GABA_A receptor and facilitates the entry of negatively charged chloride ions into neurons. Benzodiazepines bind allosterically to the gamma subunit of the GABA_A receptor and increase GABA binding to alpha subunits. This results in more chloride ions being diffused into the neurons and hyperpolarization ensues, thus making neurons less responsive to excitatory postsynaptic potential stimulation. In other words, benzodiazepines suppress the central nervous system, hence the sedative effects. The use of benzodiazepines is countered by several adverse effects, such as daytime drowsiness and dizziness or lightheadedness^{14–17}.

According to the World Health Organization (WHO), up to 80% of the world's population uses natural herbal medicines¹⁸. The valerian plant (*Valeriana officinalis*) is often used to treat

sleep disorders. The use of valerian to reduce tremors, depression, treat headaches, and as antispasmodics has also been reported. The three main chemical ingredients of this plant include essential oils (valerenic acid), valepotriates, and alkaloids. Valerian extract contains GABA, which works by depressing the central nervous system. Valerian's mechanism of action is similar to benzodiazepines, which increases the number of chloride ions that enter the neurons. One of the ingredients of valerian is glutamine, which has the ability to cross the blood-brain barrier and will then be converted into GABA. Valerenic acid inhibits the breakdown of enzymatic GABA, causing the amount of GABA in the synaptic space to remain high. Valerian works by inhibiting GABA reuptake, so that the concentration of GABA in the synaptic cleft remains high, which will induce sedation^{19–23}.

However, unlike benzodiazepines, valerian binds to the beta sub-unit of the GABA_A receptor. Valerian also inhibits GABA metabolism, which increases the abundance of GABA and prolongs the effects of hyperpolarization. The sedative effect of valerian extract is facilitated by GABA_A receptor sub-unit $\beta 3$. A previous study has found that consumption of 400–450 mg valerian extract before bedtime can improve sleep latency and sleep quality. Administration of 2000 mg/kg valerian liquid suspension daily for seven days in mice resulted in not only sedation, but also hyperthermia, defecation, and reflex disorder. The available evidence suggests that valerian might improve sleep quality without producing side effects^{24–29}.

To the best of the author's knowledge, there have been no published studies at the level of gene expression yet. We aimed to evaluate the level of *GABRB3* gene mRNA expression and sedative effect after administration of valerian extract. We hypothesized that the administration of valerian extract results in increased *GABRB3* gene mRNA expression and sedation in BALB/c mice. BALB/c mice have the characteristics of easy breeding and minimal weight variations between males and females. Therefore, it is the one of the most extensively used strains in experimental studies, particularly in neurobiology and neuroscience research. BALB/c mice were also used in our reference study regarding the use of valerian extract³⁰.

Methods

Ethical statement

Animals were treated according to the Ethical Principles and Guidelines for Scientific Experiments on Animals (2005) by the Swiss Academy of Medical Sciences and ARRIVE guidelines for animal studies. All experimental protocols employed in this study were approved by the Medical Research Ethics Committee of Hasanuddin University Makassar, Indonesia (903/H4.8.4.5.31/PP36-KOMTEK/2017). Principles for the ethical treatment of animals in research environments are described (Government Regulation of Republic of Indonesia No. 95/2012). Specific and detailed directions for the care and use of animals in biomedical research are available in the guidelines, developed in 2011 by Indonesia Health Research Ethics Committee of the Ministry of Health (Indonesia National Guidelines on Health Research Ethics 2011)³¹.

Study setting and design

This study was conducted at the Laboratory of Molecular Biology, Faculty of Medicine, Hasanuddin University, Makassar. This study was an experimental laboratory study with BALB/c mice as an animal model, using a posttest-only control group design.

Animals

This is an experimental study using 28 healthy adult male BALB/c mice that were eight weeks old, weighed approximately 25–35 grams, with no anatomical abnormality, that had never been used in experimental studies before. Mice were obtained from the maintenance and development unit of the experimental animal laboratory of Molecular Biology Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. Out of 28 mice, 20 mice that could last ≥ 180 seconds in at least two of the three trials were included. All mice were housed in standard polycarbonate cages (60 cm long $\times$ 30 cm wide $\times$ 30 cm high) with rice husks, with each cage consisting of five mice. The animal housing kept was at $25 \pm 2^\circ\text{C}$ under a 12-hour light/dark cycle. Mice were fed on standard diet in the form of mouse pellets (food made by Japfa Comfeed Indonesia Inc) and given tap water ad libitum for a period of seven days during this study. We assessed the clinical status of the mice every day during routine checks to prevent any clinical changes in the laboratory mice. No mice were found dead or having decreased appetite/growth during the study period. The mice were handled in accordance with the regulations set by the Animal Care and Use Committee at Indonesian National Guidelines on Health Research Ethics³¹.

Drugs and treatments

Valerian extracts used in this study were Blackmores Valerian Forte, a standardized herbal pill equivalent to 2 g (2000 mg) of *Valeriana officinalis* dry root or rhizome. The extract was diluted with Aqua Dest (distilled water) to 2 mg/0.1 mL. The drugs were administered via gastric gavage with 1 ml syringe. The dose was determined with the dose used in humans as a reference; the maximum tolerated dose in humans is 81.2 mg/kg as determined by Kakeshi (2014)³², or 51 mg/kg by Al-Majed (2006)²⁹. The Food and Drugs Administration (FDA) recommends converting the doses from human to mice by using 12.3 as a conversion factor, which led to the final dose of 2.5 mg/10 g and 5 mg/10 g as the dose for mice based on the average weight of 20 grams. Meanwhile, the dose of diazepam was adjusted from the human daily dose of 0.2 mg/kg, leading to the final dose of 2.5 mg/kg or 0.025 mg/10 g^{33,34}.

Motor coordination/sedation (rotarod test)

The rotarod test is one of the most commonly used methods to assess motor coordination and balance in mice. It has also been used to assess sedation level effectively in previous studies. The latency of falling from the rod at a constant speed is sensitive to the presence of a biological change (i.e. sedation) that affects motor coordination and balance^{35,36}. A rotarod consists of rods with multiple pointed edges along the seam, with a rod diameter of 3–7 cm, speed control, and a lever that halts the timer after the mice fell from the rod³⁷. The mice underwent a

“trial” using the rotarod machine for 300 seconds, at a speed of 20 rotations per minute (rpm), repeated three times with intervals of 10–15 minutes. The trial was conducted 24 hours before the study began. All mice underwent rotarod machine “training” for 60 seconds, at a speed of 20 rpm, repeated three times with five minute intervals, for seven days, 30 minutes before drug administration. On the seventh day, 30 minutes after drug administration, mice were placed on the rotarod machine for 300 seconds, speed of 20 rpm, repeated five times with 10–15 minute intervals. The timer starts when the rotary engine is running. The timer stops when the mouse falls from the rod or until 300 seconds is reached. The time taken to fall from the rod was recorded.

Study protocol

After two-weeks acclimatization the mice were randomly divided into four groups (group I Aqua Dest, group 2 diazepam, group 3 valerian 2.5 mg and group IV valerian 5 mg) consisting of five animals each using the GraphPad calculator tool to prevent any bias. The sample size in this study refers to the minimum sample size according to the WHO for research on traditional short-term treatments using animal models, which is five animals per group and Federer's formula³⁸. The intervention group, group I, was given 5 ml of Aqua Dest; group II was given 0.025 mg/10 g of diazepam diluted with distilled water to 0.5 mg/0.1 mL; group III was given 2.5 mg/10 g valerian extract diluted with distilled water to 2 mg/0.1 mL; and group IV was given 5 mg/10 g valerian extract diluted with distilled water to 2 mg/0.1 mL. All interventions were administered once daily every morning at 08:00 am for seven days through a gastric gavage in the animal laboratory. All mice were fasted for three hours before drug administration. The body weight of mice was also recorded before drug administration. On the seventh day, 30 minutes after drug administration, mice were placed on the rotarod machine. The time taken to fall from the rod was recorded. Mice were returned to the cage to rest during the intervals. A blood sample of 0.2 ml was taken on the first day before drug administration and after the rotarod test on the seventh day to be analyzed using quantitative real-time PCR (Bio-Rad CFX Connect, USA). The blood was taken from the tail vein without anesthesia. Efforts were made to minimize suffering, such as limiting the frequency of blood sampling, providing adequate space for living and performing no other experimentation or pain inducing procedures.

RNA nucleic acid extraction

Three important steps in this method are lysing, binding, and washing according to the Boom methods³⁹. A 100 $\mu\text{g}/\mu\text{l}$ of blood volume sample was dissolved into 900 μl of L6 solution, which consists of 120 g of guanidium thiocyanate (GuSCN) (Fluka Chemie AG, Buchs, Switzerland, cat no. 50990) in 100 ml of 0.1 M Tris HCl, pH 6.4, 22 ml 0.2 M ethylenediamine-tetraacetate (EDTA) pH 8.0 and 2.6 g Triton X-100 (Packard, Instrumens) with a final concentration of 50 mM Tris HCl, 5 M GuSCN, 20 mM EDTA, and 0.1% Triton X-100. The solution was centrifuged at 12,000 rpm. After the lysing process, 20 μl of diatom suspension consisting of 50 ml H_2O and 500 μl of 32% (w/v) Celite (diatom) (Jansen Chimica,

Beerse, Belgium, 10.846.79) was added. Around 20 µl of this diatom suspension can bind to 10 µg RNA/tissue/blood DNA. Then, a vortex was performed, followed by centrifugation in a 1.5 ml Eppendorf tube with a speed of 12,000 rpm for 15 minutes. The supernatant was removed and the sediment was washed with 1 ml L2 solution, consisting of 120 g GuSCN in 100 ml of 0.1 M Tris-HCl, pH 6.4. The mixture was vortexed and centrifuged at 12,000 rpm for 15 minutes, and washed two times with an L2 solution, and washed again with 1 ml of 70% ethanol twice and with 1 ml of acetone. The solution was then heated in water at a temperature of 56°C for 10 minutes and 60 µl of TE solution, consisting of 1 mM EDTA in 10 mM Tris HCL pH 8.0, was added. The solution was then vortexed and centrifuged at 12,000 rpm for 30 seconds and incubated in the oven for 10 minutes at a temperature of 56°C. Following the incubation, the sample was vortexed and centrifuged again at 12,000 rpm for 30 seconds. The supernatant was obtained by nucleotide extraction and stored at -80°C before PCR analysis.

qRT-PCR analysis

Samples for *GABRB3* gene mRNA measurements were collected from the tail vein (0.2 ml for each sample). Total RNA was isolated from venous blood using the Qiagen RNeasy Micro Kit (DNA Genotek, Qiagen, Cat No 74004) according to the manufacturer's instructions. Complementary DNA synthesis was performed by using iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA, USA, Cat No 172503) according to the manufacturer's instructions. The synthesized cDNA was diluted 10 times and used to determine GABAAR subunit mRNA levels. Following reverse transcription, quantitative RT-PCR (qRT-PCR) was performed in triplicate using the iQ SYBR Green Supermix (Bio-Rad, Cat No 170-888) PCR mix containing 100 mM KCl, 40 mM Tris-HCl pH 8.4, 0.4 mM of each dNTP, 50 U/mL DNA Polymerase (iTaQ), 6 mM MgCl₂, SYBR Green I, 20 nM fluorescein, and stabilizers. The reaction was performed using a thermocycler (Real-Time PCR detection system C1000/CFX96, Bio-Rad, Hercules, CA, USA) in a final volume of 25 µL (5 µL RNase-free H₂O, 5 µL cDNA template, 2.5 µL of each primer, and 12.5 µL of 2x iQ SYBR™ Green Supermix) under the following PCR conditions: initial heating at 95°C for three minutes to denature the cDNA and activate the Taq DNA Polymerase, followed by 40 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for two minutes. The reaction was then stopped with a final step at 72°C for 15 minutes. Qiagen QuantiTect Primer Assay systems with the following 10X primers were used: *GABRB3* (product number 249900, NM_021912, final concentration 1X), forward (5' GCTTCTTGGCTTCTCTGGTG -3') and reverse (5'- AACGAGATGCCATTCACCTCC -3'). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (product number 249900, NM_002046, final conc.1X) was used as an internal control for normalization, *GAPDH* primer, forward (5' TCCAGGGTTGGTCTTAGTGG -3') and reverse (5'- TTCCACCAGGCTCTCTGTCT -3'). Analysis was performed using CFX Manager software (Bio-Rad) for calculating gene expression in the analysis mode $\Delta\Delta C(t)$ according to the manufacturer's instructions. All measurements were conducted as per manufacturer's instructions and repeated three times to

ensure the validity. Target mRNA expression was read as fold change^{40,41}.

Statistical analysis

Statistical analysis was performed using the IBM® SPSS® Statistics version 26.0 for Windows (SPSS Inc., Chicago, IL, U.S.A.). Data normality tests were performed using the Shapiro-Wilks statistical test. ANOVA and Kruskal Wallis tests were used to analyze numerical differences between the four groups. The paired t-test was used to analyze mean values before and after treatment between each group, while the independent t-test was used to analyze mean values before and after treatment between two different groups. The analyzed variables were mouse weight, *GABRB3* gene mRNA expression, and rotarod test results. A *p*-value of <0.05 was considered significant.

Results

Normality test

GABRB3 mRNA expression before and after treatment showed normal data distribution, with a *p*-value of >0.05 within each group (Table 1)⁴². The Shapiro-Wilks normality test was also performed on motor coordination functions (Table 2). In this study, motor coordination functions were tested with the rotarod test, which showed a mean of 183.71 seconds with a median of 215.9 seconds⁴². The normality test showed normal data distribution with *p*-value of >0.05 in each group.

Mice body weight

The Kruskal Wallis test (Table 3) showed that there was no significant difference between the weight of each mouse (*p*>0.05)⁴².

GABRB3 gene mRNA expression

Table 4 shows the mean value of *GABRB3* mRNA expression before and after treatment. In group I, there was no significant difference (*p* = 0.653). In group II, there was a significant increase (*p* <0.0001) from 5.88 to 8.064. The increase was most significant in group III (*p* <0.0001), from 6.321 to 12.796. In group IV, there was an increase from 6.028 to 9.778 (*p* <0.0001). The ANOVA test (Table 5) did not show any significant difference between each group before treatment (*p* = 0.562). However, there was a significant difference after treatment (*p* <0.0001). The independent t-test (Table 6) did not show any significant difference (*p* >0.05) between two groups before treatment. However, there were significant differences between two groups after treatment (*p* <0.0001) (Table 7).

Rotarod test

Table 8 shows the comparison of rotarod test results after treatment between two groups using the independent t-test. There were significant differences (*p*<0.05) between group I (Aqua Dest) and group II (diazepam), between group I (Aqua Dest) and the first treatment group (valerian 2.5 mg/10 g), and between group I (Aqua Dest) and the second treatment group (valerian 5 mg/10 g). There were no significant differences between the positive control group and either treatment group (*p* >0.05).

Table 1. Shapiro-Wilks normality test on *GABRB3* mRNA expression.

No.	Variable	N	Mean	SD	Min	Max	Median	<i>p</i> -value
1.	<i>GABRB3</i> mRNA before treatment	20	6.027	0.539	5.059	6.889	6.151	
	Aqua Dest 5 ml	5	5.88	0.469	5.448	6.409	5.617	0.069
	Diazepam 0.025 mg/10 g	5	5.88	0.693	5.059	6.654	5.913	0.577
	Valerian 2.5 mg/10 g	5	6.321	0.548	5.509	6.889	6.256	0.598
	Valerian 5 mg/10 g	5	6.028	0.467	5.379	6.585	6.115	0.951
2.	<i>GABRB3</i> mRNA after treatment	20	9.171	2.57	5.622	13.193	8.897	
	Aqua Dest 5 ml	5	6.046	0.377	5.622	6.603	5.916	0.769
	Diazepam 0.025 mg/10 g	5	8.064	0.451	7.529	8.746	8.019	0.885
	Valerian 2.5 mg/10 g	5	12.796	0.416	12.158	13.193	12.892	0.563
	Valerian 5 mg/10 g	5	9.778	0.486	9.048	10.347	9.809	0.898

Data were analyzed with the Shapiro-Wilks normality test; a *p*-value of >0.05 was considered as normal data distribution. The unit used to measure the expression was fold change.

Table 2. Shapiro-Wilks normality test on rotarod test.

Variable	N	Mean	SD	Min	Max	Median	<i>p</i> -value
Rotarod test	20	183.71	95.946	7.4	300	215.9	
Aqua Dest 5 ml	5	274.88	29.332	243	300	288	0.05
Diazepam 0.025 mg/10 g	5	181.68	86.214	71.2	282.2	212	0.678
Valerian 2.5 mg/10 g	5	121.44	109.921	7.4	251	99	0.371
Valerian 5 mg/10 g	5	156.84	84.893	15.2	221.8	200.4	0.1

Data were analyzed with the Shapiro-Wilks normality test; a *p*-value of >0.05 was considered as normal data distribution. The values indicate seconds before falling.

Table 3. Comparison of BALB/C mouse weight within seven days of observation.

Observation	Aqua Dest 5 ml	Diazepam 0.025 mg/10 g	Valerian 2.5 mg/10 g	Valerian 5 mg/10 g	<i>p</i> -value
Day 1	31.96	31.86	32.38	31,66	0.963
Day 2	32.22	31.66	32.08	31,62	0.955
Day 3	31.98	31.62	32.04	30,92	0.841
Day 4	32.22	31.92	32.2	30,98	0.943
Day 5	32.3	32.18	32.04	30,94	0.757
Day 6	32.8	32.16	32	30,8	0.635
Day 7	32.8	32.02	32.3	30,44	0.28

Data were analyzed by the Kruskal Wallis test; a *p*-value >0.05 was considered significant.

Table 4. Comparison of *GABRB3* gene mRNA expression before and after treatment.

No	Group	N	Mean	SD	Min	Max	Median	p-value
1	Aqua Dest 5ml							
	Before treatment	5	5.88	0.469	5.448	6.409	5.617	0.653
	After treatment	5	6.046	0.377	5.622	6.603	5.916	
2	Diazepam 0.025 mg/10 g							
	Before treatment	5	5.88	0.693	5.059	6.654	5.913	<0.0001
	After treatment	5	8.064	0.451	7.529	8.746	8.019	
3	Valerian 2.5 mg/10 g							
	Before treatment	5	6.321	0.548	5.509	6.889	6.256	<0.0001
	After treatment	5	12.796	0.416	12.158	13.193	12.892	
4	Valerian 5 mg/10 g							
	Before treatment	5	6.028	0.467	5.379	6.585	6.115	<0.0001
	After treatment	5	9.778	0.486	9.048	10.347	9.809	

Data were analyzed by the paired t-test; a p-value of <0.05 was considered significant. The unit used to measure the expression was fold change.

Table 5. Comparison of *GABRB3* gene mRNA expression before and after treatment between four groups.

No	Variable	N	Mean	SD	Median	p-value	F-value
1.	<i>GABRB3</i> mRNA before						
	Aqua Dest 5 ml	5	5.88	0.469	5.617	0.562	0.71
	Diazepam 0.025 mg/10 g	5	5.88	0.693	5.913		
	Valerian 2.5 mg/10 g	5	6.321	0.548	6.256		
	Valerian 5 mg/10 g	5	6.028	0.467	6.115		
2.	<i>GABRB3</i> mRNA after						
	Aqua Dest 5 ml	5	6.046	0.377	5.916	<0.0001	216.61
	Diazepam 0.025 mg/10 g	5	8.064	0.451	8.019		
	Valerian 2.5 mg/10 g	5	12.796	0.416	12.892		
	Valerian 5 mg/10 g	5	9.778	0.486	9.809		

Data were analyzed with the ANOVA test; a p-value of <0.05 was considered significant. The unit used to measure the expression was fold change.

Discussion

Sedation is defined as a condition of decreased awareness of the surrounding environment and reaction to external stimuli. The sedative effect of several active substances in valerian extract work synergistically. Valerenic acid inhibits the breakdown of GABA, which results in an abundance of GABA in the synaptic space. Valerian extract also contains GABA, which suppresses the central nervous system. Furthermore, glutamine, one of the valerian extract substances, can cross the blood-brain barrier and will be converted into GABA. Lastly, valerian also acts by blocking GABA reuptake^{28,43}.

GABRB3 is a heteromeric gene that encodes the GABA_A receptor $\beta 3$ -subunit, a member of the ligand-gated ion channel family in humans. GABA_A receptors are the major inhibitory transmitter receptors in the brain and the site of action of a variety of pharmacologically and clinically important drugs¹⁻⁴. Meguro *et al.* (1997) have identified *GABRB3* expression in mouse A9 hybrids that contained either a paternal or maternal human chromosome 15⁴⁴.

Administration of diazepam and valerian extract in this study resulted in a significant increase in *GABRB3* gene mRNA

Table 6. Comparison of *GABRB3* gene mRNA expression before treatment between two groups.

No	Variable	Mean	SD	Min	Max	Median	p-value
1.	Aqua Dest 5ml	5.88	0.47	5.448	6.409	5.616	0.999
	Diazepam 0.025 mg/10g	5.88	0.693	5.058	6.653	5.913	
2.	Aqua Dest 5 ml	5.88	0.470	5.448	6.409	5.616	0.209
	Valerian 2.5 mg/10 g	6.321	0.548	5.509	6.889	6.256	
3.	Aqua Dest 5 ml	5.88	0.470	5.448	6.409	5.616	0.631
	Valerian 5 mg/10 g	6.028	0.467	5.379	6.585	6.115	
4.	Diazepam 0.025 mg/10 g	5.88	0.693	5.058	6.653	5.913	0.298
	Valerian 2.5 mg/10 g	6.321	0.548	5.509	6.889	6.256	
5.	Diazepam 0.025 mg/10 gr	5.88	0.693	5.058	6.653	5.913	0.703
	Valerian 5 mg/10 g	6.028	0.467	5.379	6.585	6.115	
6.	Valerian 2.5 mg/10 g	6.321	0.548	5.509	6.889	6.256	0.39
	Valerian 5 mg/10 g	6.028	0.467	5.379	6.585	6.115	

Data were analyzed with the independent t-test; a p-value of <0.05 was considered significant. The unit used to measure the expression was fold change.

Table 7. Comparison of *GABRB3* gene mRNA expression after treatment between two groups.

No	Variable	Mean	SD	Min	Max	Median	p-value
1.	Aqua Dest 5ml	6.046	0.377	5.622	6.603	5.915	<0.0001
	Diazepam 0.025 mg/10g	8.064	0.451	7.529	8.745	8.019	
2.	Aqua Dest 5 ml	6.046	0.377	5.622	6.603	5.915	<0.0001
	Valerian 2.5 mg/10 g	12.796	0.416	12.158	13.193	12.892	
3.	Aqua Dest 5 ml	6.046	0.377	5.622	6.603	5.915	<0.0001
	Valerian 5 mg/10 g	9.778	0.486	9.048	10.347	9.809	
4.	Diazepam 0.025 mg/10 g	8.064	0.451	7.529	8.745	8.019	<0.0001
	Valerian 2.5 mg/10 g	12.796	0.416	12.158	13.193	12.892	
5.	Diazepam 0.025 mg/10 g	8.064	0.451	7.529	8.745	8.019	<0.0001
	Valerian 5 mg/10 g	9.778	0.486	9.048	10.347	9.809	
6.	Valerian 2.5 mg/10 g	12.796	0.416	12.158	13.193	12.892	<0.0001
	Valerian 5 mg/10 g	9.778	0.486	39.048	10.347	9.81	

Data were analyzed with the independent t-test; a p-value of <0.05 was considered significant. The unit used to measure the expression was fold change.

expression. The first treatment group and second treatment group had the highest motor coordination function impairment compared to other groups, although not significant. Thus, it can be interpreted that valerian extract exhibited a sedation effect on both the clinical and molecular levels. Based on the result of the rotarod test, there were significant differences between group I and the rest of the groups, while there were no significant differences between groups II, III, and IV. This result indicated

that valerian extract and diazepam have clinically comparable sedation effects, as shown in reports by Khom *et al.*⁵ and Budzinski *et al.*⁴⁵ Valerian has been shown to have a sedative effect and prolong sleep time in mice, as reported by Hendrik *et al.*⁴⁶ and Fernandez *et al.*²⁰ Regarding motoric coordination function, Khom *et al.* also reported that the administration of ≥ 10 mg/kg of valerian extract caused a significant reduction in locomotion in mice⁵. The mice in this study have

Table 8. Comparison of rotarod test results after treatment between two groups.

No	Variable (n=5)	Mean	SD	Min	Max	Median	p-value
1	Aqua Dest 5 ml	274.88	29.332	243	300	288	0.04
	Diazepam 0.025mg/10 g	181.68	86.214	71.2	282.2	212	
2	Aqua Dest 5 ml	274.88	29.332	243	300	288	0.017
	Valerian 2.5mg/10 g	121.44	109.921	7.4	251	99	
3	Aqua Dest 5 ml	274.88	29.332	243	300	288	0.019
	Valerian 5mg/10 g	156.84	84.893	15.2	221.8	200.4	
4	Diazepam 0.025mg/10 g	181.68	86.214	71.2	282.2	212	0.363
	Valerian 2.5mg/10 g	121.44	109.921	7.4	251	99	
5	Diazepam 0.025mg/10 g	181.68	86.214	71.2	282.2	212	0.658
	Valerian 5mg/10 g	156.84	84.893	15.2	221.8	200.4	
6	Valerian 2.5mg/10 g	121.44	109.921	7.4	251	99	0.584
	Valerian 5mg/10 g	156.84	84.893	15.2	221.8	200.4	

Data were analyzed with the independent t-test; a p-value of <0.05 was considered significant. The values indicate seconds before falling.

homogeneous characteristics (no difference in mean weight), thus not presenting as a confounding factor or bias that might affect the study's results.

Interestingly, the mice in first treatment group (valerian 2.5 mg/10 g) had the most significant increase in the mean value of *GABRB3* gene mRNA expression after treatment (up to two-fold), exceeding that of second group treatment (valerian 5 mg/10 g), which had a higher dose of valerian extract. Furthermore, the mice in first treatment group also demonstrated the shortest mean duration in the rotarod test. These results indicate that increasing the dose of valerian extract (2.5 mg to 5 mg) was not directly proportional to the increase in both *GABRB3* gene mRNA expression and sedation.

This study has several limitations. First, there are various available formulations of valerian extract without a clear standard of processing method. Second, there are no previous studies capable of proving that valerian extract exerts its sedative effects by increasing the level of *GABRB3* gene mRNA gene expression. Finally, this study does not perform a brain biopsy for determining the level of *GABRB3* gene mRNA gene expression through immunohistochemistry tests. Further research is needed to establish the standardized dosage of valerian extract. More definitive studies to determine the correlation between the level of *GABRB3* gene mRNA expression and sedation effect are also warranted.

Conclusion

Valerian extract exerts its sedative properties by binding to the GABA_A receptor β 3 subunit. As the gene that encodes the

GABA_A receptor β 3 sub-unit, *GABRB3* gene mRNA expression was increased after the administration of valerian extract. Valerian extract had a clinically similar sedation effect to diazepam. However, a higher dose of valerian extract did not yield a higher level of *GABRB3* gene mRNA expression or sedative effects.

Data availability

Underlying data

Open Science Framework: Effect of Valerian Extracts on *GABRB3* Gene mRNA Expression and Motor Coordination in BALB/c mice. <https://doi.org/10.17605/OSF.IO/CT52W42>.

This project contains the following underlying data:

- Mice_Body_Weight.xlsx
- mRNA_expression_of_GABRB3.xlsx
- Rotarod.xlsx

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

Acknowledgments

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Margot Ernst

Department of Biochemistry and Molecular Biology, Center for Brain Research, Medical University of Vienna, Vienna, Austria

The authors describe changes in blood mRNA levels of GABRB3 in mice after treatment with valerian extracts or diazepam, alongside reduced rotarod performance. The results are presented well and worthy of publication. The text requires some work in several respects:

- Language issues: The first sentence of the introduction needs to be reworded: the term "chloride sub-channels" is inappropriate. It can alternatively be stated that b3 is a subunit that contributes to pentamer formation.
- IUPHAR terminology violations: The use of subscripts to denote isoforms as in " β_3 " is inconsistent. All subscripts should be removed as per IUPHAR (e.g. line 1 of the introduction).
- Contents: Abstract, second line: "The sedative effect of valerian extract is facilitated by the GABA-A R beta3 subunit" - this is certainly not the case. A single subunit rarely mediates a pharmacological effect. In the case of valerenic acid, a binding site has been proposed that overlaps with the site used by etomidate at the interface between beta and alpha subunits¹.
- In the same vein, in the introduction, the sentence "valerian binds to the beta subunit" needs to be corrected accordingly to reflect the contribution of alpha subunits to the binding site.
- The authors equate reduced rotarod performance with sedation – it can reflect ataxia and/or muscle relaxation as well. Without an open field test, sedation cannot be assessed. The term "reduced rotarod performance" would thus be strongly preferred over "sedation".
- Study design and methods: Why do the authors assume that GABRB3 will be upregulated? According to the literature, the application of compounds that cause increased function often induces compensatory down-regulation. I suggest rewording the hypothesis. Why

were blood mRNA levels examined rather than brain levels?

- Data presentation: I agree with reviewer 1 that some graphical data presentation would be helpful.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Neuropharmacology, GABA-A receptors

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 06 April 2022

<https://doi.org/10.5256/f1000research.26903.r128838>

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Boyang Jason Wu

Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences,
Washington State University, Spokane, WA, USA

In this manuscript, Mulyawan *et al.* determined the effect of valerian extracts on GABRB3 gene mRNA expression and motor coordination in mice. Their results showed that valerian extracts exert a comparable effect to diazepam by increased GABRB3 gene mRNA levels while decreased motor coordination in mice, suggesting a previously unrecognized sedation effect of valerian extracts. Overall, this manuscript is clearly written with interesting results presented and appropriate statistical analysis used. There are a few concerns for the authors to consider to improve the manuscript.

1. GABRB3 expression was analyzed only at the mRNA level, so how would this change correlate to the protein-level or receptor activity change if any? Has such correlation been established or implicated for any genes encoding GABA receptor subunits in the literature? This should be at least discussed to complement the mRNA-level analysis.
2. Valerian extracts did not show a dose-dependent effect on both GABRB3 gene expression and motor coordination. The possible underlying explanations should be discussed. Also, have valerian extracts with the two doses used in this study showed any dose-dependent effect on other measures from the literature? How about time-dependent responses?
3. It would be clearer and more reader-friendly to present the data as bar charts including the distribution of all measurement points in individual groups.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cancer Biology, Neuropharmacology, and Endocrinology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have

significant reservations, as outlined above.

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