

Full Length Research Paper

Effect of *Viola tricolor* on pentobarbital-induced sleep in mice

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Traditionally, *Viola tricolor* has been recommended for its sedative property. However, no pharmacological studies have yet evaluated the effect of this plant on sleep. The hydro-alcoholic extract (HAE) was prepared by the extraction of the aerial parts of *V. tricolor* in 70% ethanol using a Soxhlet apparatus. Also, with solvent-solvent extraction, the HAE was further fractionated to water fraction (WF), ethyl acetate fraction (EAF) and N-butanol fraction (NBF). The extract (50, 75, 100, 150, 200, 300 mg/kg, ip) and its fractions (200 mg/kg, ip) were administered to mice, 30 min before pentobarbital (30 mg/kg, ip) injection. Furthermore, the possible neurotoxicity of the plant was assessed using PC12 neuron cell line. The HAE, at 300 mg/kg, significantly prolonged (34%) duration of pentobarbital-induced sleep. Similarly, the EAF significantly increased (51%) the sleep duration. None of the HAE doses or the fractions could significantly change the sleep latency time. The sedative effect of *V. tricolor* accompanied with no neuron toxicity, except for very high concentrations of EAF. The results suggest that *V. tricolor* potentiates pentobarbital hypnosis and the main component(s) responsible for this effect is most likely found in EAF. Isolation and purification of the active compound(s) may yield novel sleep-prolonging agents.

Key words: *Viola*, sleep, pentobarbital, mice.

INTRODUCTION

Sleep dissatisfaction and insomnia have a relatively high prevalence in the world (Roth, 2005). Chronic sleep disorders give rise to some health problems such as emotional disturbances, slower reactions and poor memorizing (Orzel-Gryglewska, 2010; Zaharna and Guilleminault, 2010). Despite its high prevalence, insomnia has not received sufficient clinical attention. Currently, benzodiazepines are the most widely used medications. However, the clinical uses of benzodiazepines are accompanied with unpleasant side effects such as drug dependence, tolerance, rebound insomnia, amnesia, psychomotor impairment and

potentiating of other central depressant drugs (Uzun et al., 2010). Therefore, the search for new hypnotic agents with lesser side effects remains an attractive subject.

Over the last years, the reputation of medicinal plants has increased due to their therapeutic efficacy and lesser side effects and many traditional medicinal plants have been tested for their hypnotic potential in the experimental animals (Hossain et al., 2009; Ngo Bum et al., 2008). *Viola tricolor*, the subject of this study, is a common horticultural plant in Iran. It has been reported to have a number of medicinal attributes including anti-inflammatory (Toiu et al., 2007), antimicrobial (Witkowska-Banaszczak et al., 2005), antioxidant (Vukics et al., 2008) and diuretic (Toiu et al., 2009) activity. Traditionally, *V. tricolor* has been suggested to have sedative-hypnotic property (Duke et al., 2002). However, no pharmacological studies have been undertaken so far

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to determine its hypnotic effect. Therefore, the present study was designed to investigate the hypnotic effect of *V. tricolor* hydro-alcoholic extract (HAE) and its fractions. Also, the possible cytotoxicity of the plant was tested against a neuron cell line, PC12, to ensure that the effect accompanied with no negative impact on neurons.

MATERIALS AND METHODS

Drugs and chemicals

Dimethyl sulfoxide (DMSO), pentobarbital sodium, penicillin-streptomycin and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-Diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). Diazepam was obtained from Chemidarou Company (Iran). Tween 80 was from Merck (Germany). Dulbeccos Modified Eagles Medium (DMEM) and fetal bovine serum (FBS) were bought from GIBCO (USA).

Preparation of extracts

The *V. tricolor* aerial parts of the flowering plants were collected from Pardis Campus (Mashhad, Iran). The identity of the plant was confirmed and for future reference a voucher specimen (12568) was deposited at the herbarium of school of Pharmacy (Mashhad University of Medical Sciences, Iran). The plant materials were dried, powdered and subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h. The HAE was then dried on a water bath and the yield (30% w/w) dissolved in saline containing 1% (v/v) of tween 80.

For preparation of fractions, 10 g of HAE was suspended in distilled water and transferred to a separator funnel. With solvent-solvent extraction, it was fractionated using ethyl acetate and N-butanol. The ethyl acetate fraction (EAF) and N-butanol fraction (NBF) were separated to obtain water fraction (WF). The fractions were dried on a water bath and working solutions made up in saline, saline containing 1% tween, and 10% DMSO for WF, EAF and NBF, respectively.

Animals

Male BALB/c mice weighting 25-35 g were used in this study. The animals were maintained under controlled conditions including 12 h light and dark cycle, 22-24°C temperature and 50% relative humidity with laboratory chow and water provided *ad libitum*. The study protocol using the laboratory rats complied with the general guidelines of the animal care of Mashhad University of Medical Sciences, Iran. The animals were randomly divided into 12 groups. In the first experiment, to determine if HAE has sleep-prolonging effect, the following solutions were injected (i.p.) to seven groups: saline (as vehicle) and HAE (50, 75, 100, 150, 200, 300 mg/kg). In the second experiment, to determine the most effective fraction, five groups of mice were treated (i.p.) with the following agents (200 mg/kg): WF, EAF, NBF, 10% DMSO (vehicle for NBF) and diazepam (3 mg/kg) as positive control.

Effect of extract and fractions on pentobarbital-induced sleep onset and duration

The sleep evaluation method was based on potentiation of

pentobarbital-induced sleeping time. Briefly, the animals were given (i.p.) a single dose of the vehicles, diazepam, or the extracts. Thirty minutes later, pentobarbital (30 mg/kg, i.p.) was injected to induce sleep. The mice were considered asleep if stayed immobile and lost its righting reflex when positioned on its back. The animal was judged to be awake if it could return to upright position. The time interval between injection of pentobarbital and onset of sleep (lost righting reflex) was recorded as the latency time.

Neurotoxicity assessment

The possible neurotoxicity of the plant was tested against PC12 cells, a rat pheochromocytoma-derived cell line. The cells were seeded in 96-well plates and cultured for 24 h in DMEM containing 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C in an atmosphere of 5% CO₂. The culture medium was then changed to fresh one containing vehicle (1% DMSO) or different concentrations (100, 200, 400, 800 and 1600 µg/ml) of HAE or EAF. These concentrations were used based on the levels applied *in vivo* and on the fact that extracellular volume of the rodent is approximately 25% of body weight (Barratt and Walser, 1969). The cells were further incubated for 24 h. Then, the effect of extracts on cell proliferation was measured using MTT colorimetric assay as previously described (Hadjzadeh et al., 2006; Tavakkol-Afshari et al., 2006). The assay was carried out in triplicate and repeated three times for confirmation.

Statistics

The results are presented as the mean ± standard error (SEM). Normality and homogeneity of variance were tested by the Kolmogorov-Smirnov test. The values were compared using the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. The p-values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of *V. tricolor* on pentobarbital-induced sleeping duration

The sleep duration induced by 30 mg/kg pentobarbital in the animals receiving saline was 35 ± 3.6 min (Figure 1). HAE at doses of 300 mg/kg significantly increased the sleeping time (47 ± 2.7 min, $P < 0.05$ vs. saline). With the lower doses, however, the effect of HAE was not significant and the sleep duration reached to 40 ± 3.8, 37 ± 3.4, 37 ± 4.2, 36 ± 6.2 and 48 ± 7.6 min for 50, 75, 100, 150 and 200 mg/kg, respectively.

As shown in Figure 2, among the three fractions of HAE, EAF and NBF significantly prolonged the sleep duration to 53 ± 7.6 ($P < 0.05$ vs saline) and 46 ± 1.6 min ($P < 0.05$ vs. its related vehicle, DMSO), respectively. On the contrary, WF failed to change pentobarbital-induced sleeping time (38 ± 4.4 min). As expected, duration of sleep in the animals receiving diazepam, as positive control, was significantly greater (78 ± 12.7 min, $P < 0.001$) than that of control animals.

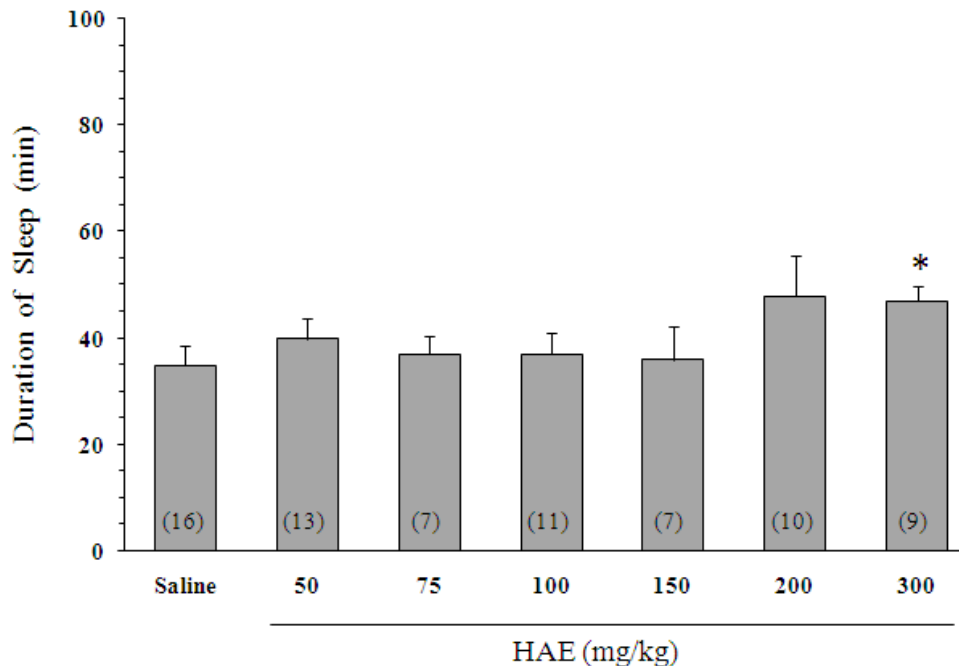


Figure 1. Effect of *Viola tricolor* hydro-alcoholic extract (HAE) on sleep duration. The extract was administrated (ip) to mice, 30 min before pentobarbital (30 mg/kg, ip) injection. * $P < 0.05$ vs saline. Data represent mean \pm SEM of the numbers shown in parentheses.

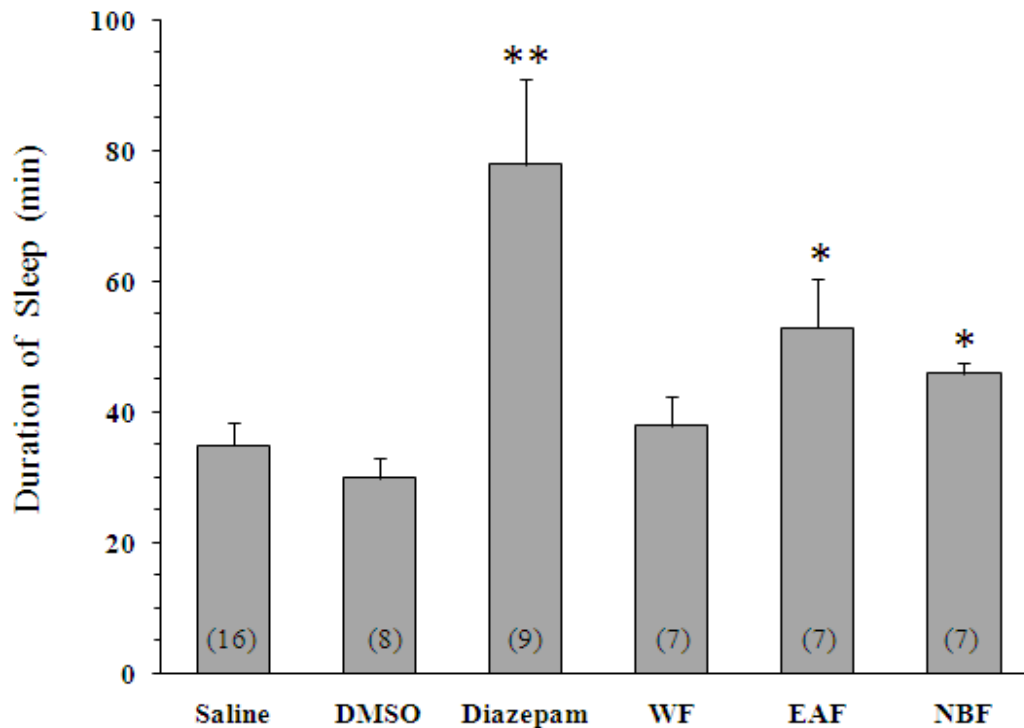


Figure 2. Effect of *Viola tricolor* fractions on sleep duration. The water fraction (WF), ethyl acetate fraction (EAF) and N-butanol fraction (NBF) of *V. tricolor* hydro-alcoholic extract was administrated (200 mg/kg, ip) to mice, 30 min before pentobarbital (30 mg/kg, ip) injection. * $P < 0.05$ vs saline or DMSO, ** $P < 0.001$ vs saline. Data represent mean \pm SEM of the numbers shown in parentheses.

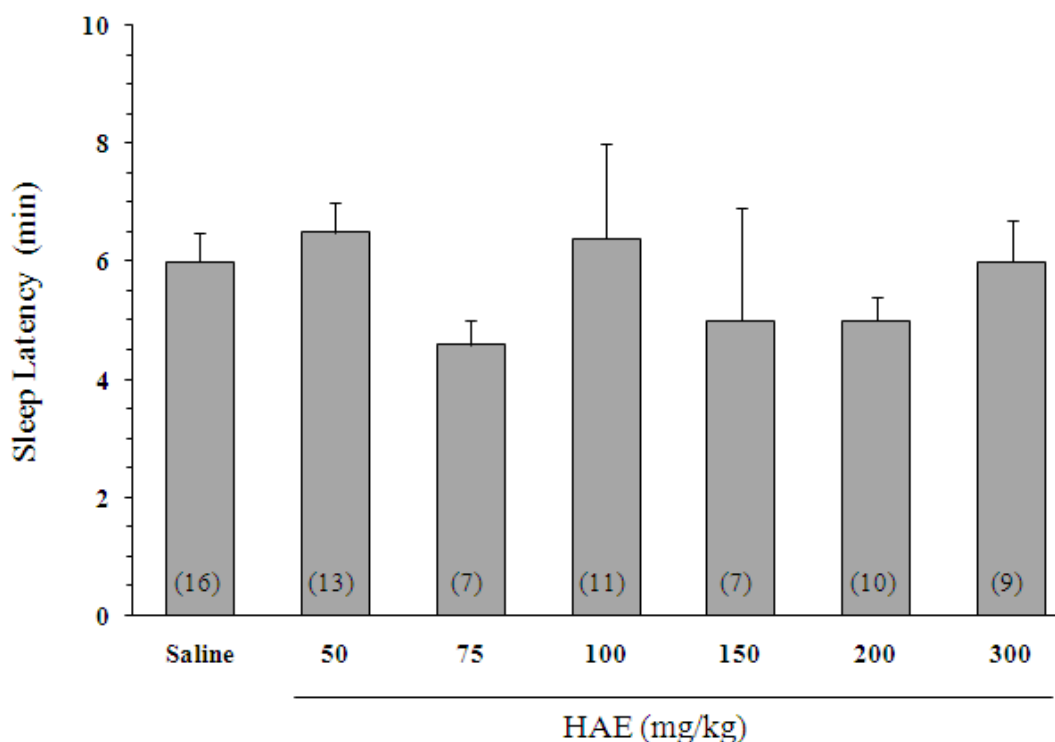


Figure 3. Effect of *Viola tricolor* hydro-alcoholic extract (HAE) on sleep latency. The extract was administered (ip) to mice, 30 min before pentobarbital (30 mg/kg, ip) injection. The time interval between injection of pentobarbital and onset of sleep was recorded as the latency time. Data represent mean \pm SEM of the numbers shown in parentheses.

Effects of *V. tricolor* on pentobarbital-induced sleeping onset

As shown in Figure 3, the sleeping onset in animals receiving pentobarbital was 6 ± 0.5 min. In groups treated with 50, 75, 100, 150, 200 and 300 mg/kg of HAE, the onset was 6.5 ± 0.5 , 4.6 ± 0.4 , 6.4 ± 1.6 , 5 ± 1.9 , 5 ± 0.4 and 6 ± 0.7 min, respectively. None of the onset was significantly different from that of pentobarbital group.

Similarly, no significant changes were observed after administration of the three fractions as compared with pentobarbital group. The onset of the animals receiving WF, EAF and NBF was 6.7 ± 0.5 , 5.1 ± 0.3 and 5 ± 1 min, respectively (Figure 4). On the other hand, diazepam could decrease the onset induced by pentobarbital (4.3 ± 0.5 min, $P < 0.05$).

Effect of *V. tricolor* on neuron surviving

The possible neurotoxicity of HAE and its most effective fraction, EAF, was assessed using MTT method. It was found that up to 24 h none of HAE concentrations decreased proliferation of PC12 cells. In the presence of 100, 200, 400, 800 and 1600 $\mu\text{g/ml}$ of the extract, cell

surviving was 100 ± 5 , 106 ± 5 , 88 ± 4 , 97 ± 4 and $90 \pm 3\%$, respectively, as compared to vehicle group ($100 \pm 3.5\%$). Regarding EAF, the cell viability was 94 ± 5 , 98 ± 4 , 96 ± 3 , 80 ± 4 and $33 \pm 2\%$ for concentrations of 100, 200, 400, 800 and 1600 $\mu\text{g/ml}$, respectively, which only at 800 ($P < 0.01$) and 1600 $\mu\text{g/ml}$ ($P < 0.001$) showed significant decrease in comparison to saline group (Figure 5).

DISCUSSION

The present data demonstrate for the first time that *V. tricolor* potentiates pentobarbital-induced sleeping behaviors in mice. *V. tricolor* extracts increased sleep duration but did not significantly modify the sleep latency. In agreement with the previously published reports and as expected, diazepam significantly prolonged pentobarbital-induced sleeping time, indicating that our study procedures were optimized. Our results also showed that the CNS-depressant effect of *V. tricolor* HAE accompanied with no neuron toxicity.

Several neurotransmitters and endogenous molecules are involved in regulation of sleep and wakefulness. The sleep-promoting neurons located in the anterior

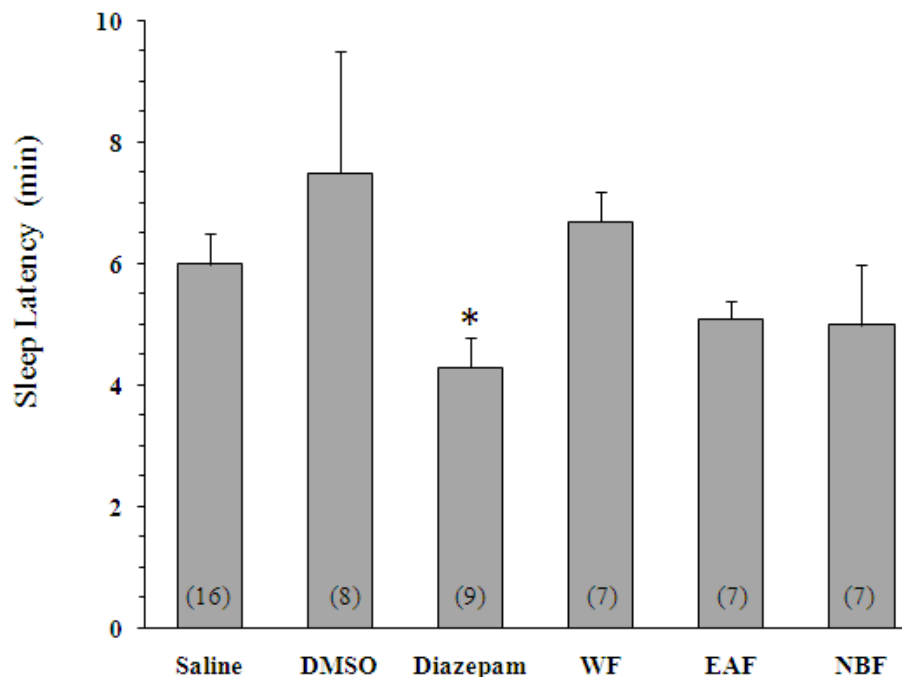


Figure 4. Effect of *Viola tricolor* fractions on sleep latency. The water fraction (WF), ethyl acetate fraction (EAF) and N-butanol fraction (NBF) of *V. tricolor* hydro-alcoholic extract was administrated (200 mg/kg, ip) to mice, 30 min before pentobarbital (30 mg/kg, ip) injection. The time interval between injection of pentobarbital and onset of sleep was recorded as the latency time. * $P < 0.05$ vs saline. Data represent mean \pm SEM of the numbers shown in parentheses.

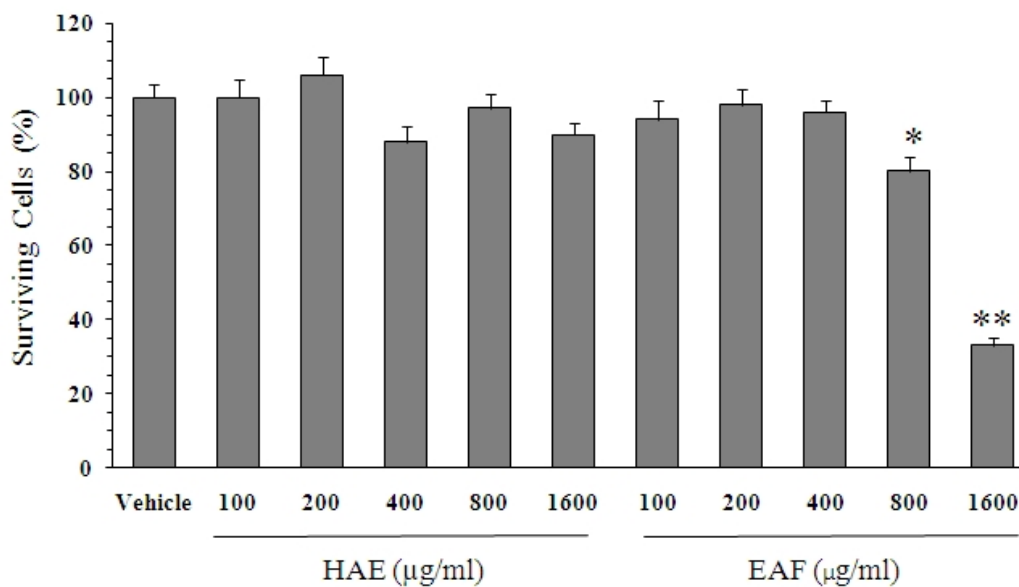


Figure 5. Effect of *Viola tricolor* hydro-alcoholic extract (HAE) and ethyl acetate fraction (EAF) on surviving of PC12 neuron-like cells. The cells were cultivated for 24h in DMEM supplemented with 10% FBS and containing vehicle (1% DMSO) or varying concentrations of the extracts. Data are mean \pm SEM of three independent experiments performed in triplicate. * $P < 0.01$ versus saline, ** $P < 0.001$ versus saline. HAE: hydro-alcoholic extract; WF: water fraction; EAF: ethyl acetate fraction; NBF: N-butanol fraction.

hypothalamus release gamma-aminobutyric acid (GABA) to suppress activity of wake-inducing areas of the brain (Datta, 2010). Pentobarbital is known to act at GABA receptors ionophore complex and favor the binding of GABA. Also, benzodiazepine agonists such as diazepam enhance the affinity of GABA for its receptor and hence prolong pentobarbital-induced sleep duration (Gottesmann, 2002). Similarly, some medicinal plants interact with GABAergic system to induce their hypnotic effect (Chu et al., 2007; Nogueira and Vassilieff, 2000). The hypnotic activity of medicinal plants has been attributed to different phytochemical compounds such as flavonoids, terpenes and saponins (Jiang et al., 2007; Linck et al., 2009). In the present study, to obtain better insight into the nature of compounds responsible for sleep-prolonging effect of *V. tricolor*, three fractions were prepared from its HAE: (1) The WF which extract water-soluble plant constituents (e.g. glycosides, quaternary alkaloids, tannins); (2) The EAF which extract compounds of intermediate polarity such as some flavonoids; (3) The NBF which bearing low polar agents like sterols, alkanes and some terpenoids (Seidel, 2006; Tian, 2011). Our data showed that EAF and NBF, but not WF, exert a sleep enhancing effect, suggesting that intermediate to low polar agents are responsible for the hypnotic effect of *V. tricolor*. Recent studies have characterized high amount of saponins, mucilages, cyclotides and flavonoids in this plants (Toiu et al., 2009; Vukics et al., 2008; Svargard et al., 2004). It has been reported that flavonoids bind with high affinity to benzodiazepine site of the GABA_A receptor (Wasowski and Marder, 2012). In addition, Nassiri-Asl et al. (2008) showed that rutin, a major flavonoid component in *V. tricolor* (Vukics et al., 2008), has sedative effects in the brain, possibly through positive allosteric modulation of the GABA_A receptor complex. In accordance with these reports, our finding that EAF has the best sleep-prolonging effect among the three fractions of HAE favors a role of flavonoids in the sleep-prolonging effect of *Viola tricolor*. In addition, the present study also showed that high concentration of EAF ($\geq 800 \mu\text{g/ml}$) may be neurotoxic.

In summary, the present study showed that *V. tricolor* extracts increase the sleep duration induced by pentobarbital in mice without neuronal toxicity unless at very high doses. The main component(s) responsible for this effect of *V. tricolor* may be found in EAF. Isolation and purification of the active compound(s) may yield novel sleep-prolonging agents.

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conflict of interest.

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