

Original Research Article

Hydroalcoholic extract of needles of *Pinus eldarica* enhances pentobarbital-induced sleep: possible involvement of GABAergic system

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Abstract

Objective: Insomnia is accompanied by several health complications and the currently used soporific drugs can induce several side effects such as psychomotor impairment, amnesia, and tolerance. The present study was planned to investigate the sleep prolonging effect of *Pinus eldarica*.

Materials and Methods: Hydroalcoholic extract (HAE) of *P. eldarica*, its water fraction (WF), ethyl acetate fraction (EAF) and n-butanol fraction (NBF) were injected (intraperitoneally) to mice 30 min before administration of pentobarbital. Then, the latent period and continuous sleeping time were recorded. Also, LD₅₀ of *P. eldarica* extract was determined and the possible neurotoxicity of the extract was tested on neural PC12 cells.

Results: The HAE and NBF decreased the latency of sleep ($p < 0.05$) and significantly increased duration of sleep ($p < 0.05$) induced by pentobarbital. These effects of *P. eldarica* were reversed by flumazenil. The LD₅₀ value for HAE was found to be 4.8 g/Kg. HAE and its fractions did not show neurotoxic effects in cultured PC12-cell line.

Conclusion: The present data indicate that *P. eldarica* potentiated pentobarbital hypnosis without major toxic effect. Most probably, the main components responsible for this effect are non-polar agents which are found in NBF of this plant.

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Introduction

Insomnia is a common complaint that is characterized by lack of healthy and relaxing sleep. Thirty to fifty percent of

people are reported to be affected by insomnia, whereas 10% of them have chronic insomnia (Vallièrès et al., 2005). Most patients use benzodiazepines to treat

insomnia. However, consumption of these drugs are accompanied by some adverse effects including impaired cognitive function, poor memorizing, disturbance of general daytime performance, dependence, and tolerance (Roth and Drake, 2004). Therefore, further investigation for finding new hypnotic drugs with lesser unwanted effects is of great interest. Natural products have always been good sources for development of new therapeutics for management of several diseases (Meletis and Zabriskie, 2008). It has been reported that some herbal extracts stimulate sleep through alternation of the level of some neurotransmitters such as gamma-aminobutyric acid (GABA) in the central nervous system (Currie and Wheat, 2007).

Pinus eldarica (family Pinaceae) is an evergreen tree that is found naturally in the transcaucasian area between Europe and Asia, and also grows in Iran, Afghanistan and Pakistan (Michelozzi *et al.*, 2008). Different parts of this tree (e.g. tar, buds, resin, and needles) have been broadly used in traditional medicine of Russia and the central Asian countries for treating bronchial asthma, skin wounds, skin irritations, allergic rashes and dermatitis (Mamedov and Craker 2001; Mamedov *et al.*, 2007). The needle parts of *P. eldarica* have several chemical compounds including tannins, terpenoids and polyphenols (Lee, 2008). Pharmacological studies have revealed that extracts of the needle parts have antioxidant (Guriet *al.*, 2006; Vuorela *et al.*, 2010), immunomodulatory (Li *et al.*, 2007; Rohdewald, 2002), anti-inflammatory (Rohdewald, 2002) and antineoplastic properties (Li *et al.*, 2007; Potta *et al.*, 2005). These properties are linked to the effects of *P. eldarica* extracts on cyclo-oxygenase activity (Potta *et al.*, 2005), production of prostaglandin E2 (Vuorela *et al.*, 2005; Karonen *et al.*, 2004), synthesis of nitric oxide (Karonen *et al.*, 2004; Virgiliet *al.*, 1998), and regulation of cancer-related proteins (Li *et al.*, 2007). In some traditional medicine books it was reported

that plants of Pinaceae family have sedative and hypnotic effects (Mirheydar, 1993). Therefore, the present work was designed to evaluate sleep-prolonging action of *P. eldarica* hydro-alcoholic extract (HAE) and its fractions. Also, the safety of this plant was evaluated by determination of LD₅₀ and testing its effect on the viability of neural cells.

Materials and Methods

Drugs and chemicals

Pentobarbital sodium, penicillin-streptomycin, flumazenil and 3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (USA). Diazepam was bought from Chemidarou Company (Iran). Tween 80 was bought from Merck (Germany). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO (USA).

Preparation of *P. eldarica* extract

The needles parts of *P. eldarica* were collected from Mashhad (Khorasan province, Iran). The voucher specimen was prepared and deposited (No. 11945) in the School of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran and dried in a dark place at room temperature. The plant material was powdered and the HAE was prepared using 70% ethanol in a Soxhlet apparatus for 48 hr (Mortazavian and Ghorbani, 2012; Shafiee-Nick *et al.*, 2012). Then, HAE was dried on a water bath and the dried remaining (32% w/w) was dissolved in saline that contains 1% (v/v) Tween 80. The HAE was stored at 4°C until use. Control group received saline containing 1% Tween 80. To prepare the fractions of HAE, a part of dried HAE was suspended in distilled water and transferred to a separator funnel. Using solvent-solvent extraction, it was fractionated with *n*-butanol or ethyl acetate to obtain ethyl acetate fraction (EAF) and *n*-butanol fraction (NBF). Then, to obtain water fraction (WF), the ethyl acetate

fraction (EAF) was further partitioned with *n*-butanol and the *n*-butanol soluble layer was removed. The lower water-soluble layer in separator funnel was considered as WF (Ghorbaniet al., 2015). The resulting fractions were dried on a water bath and working solutions were prepared in saline and saline containing 1% Tween 80 for WF and EAF or NBF, respectively. The yields obtained from the extract fractionation were 63.5%, 17.5% and 19% for WF, EAF and NBF, respectively.

Animals

Male mice weighing 20-30 g and male Wistar rats weighing 200-250 g were obtained from the animal house of Mashhad University of Medical Sciences, Mashhad, Iran. Animals were kept in a room with 12 hr/12 hr light/dark cycle at 21 ± 2 °C. Animals had free access to water and food. All animal experiments were done in accordance with ethical guidelines approved by the Animal Care Use Committee of Mashhad University of Medical Sciences.

Experimental groups

The mice were randomly divided into 14 groups of seven. Group 1 received normal saline containing 1% Tween as vehicle and served as a negative control for HAE extract. Animals of group 2 received 3 mg/kg diazepam as positive control. Mice in groups 3-7 received extracts at doses of 25, 50, 100, 150 and 200 mg/kg, respectively. Animals in group 8-11 received 50 mg/kg WF, 50 mg/kg EAF, 25 mg/kg of NBF and 50 mg/kg of NBF, respectively. Moreover, animals were treated with 2 mg/kg flumazenil as a diazepam antagonist before receiving diazepam (group 12), HAE (group 13) or NBF (group 14). All the test compounds were administered intraperitoneally (i.p.).

Sleep induction

The sleep evaluation technique was based on prolongation of sleep induced by pentobarbital (Rakhshandehet al., 2012).

Briefly, the test compounds were administered (i.p.) 30 min before injection of 30 mg/kg sodium pentobarbital. The mice were considered asleep if stayed immobile and lost its righting reflex when positioned on its back. The time interval between injection of pentobarbital and start of sleep was noted as sleep latency.

Rotarod test

The rotarod test was used to measure motor resistance and coordination. The experimental procedure for learning and adaptation was done for 3 consecutive days. On the next day, rats were placed on a rotating rod that accelerated smoothly from 4 to 40 rpm over a period of 5 min. The length of time they could maintain their balance on the turntable against the movement's strength was recorded. Then, the extract or vehicle was injected and after 30 min, the animals were placed on rotarod again (Pritchett and Mulder, 2003; Vafae et al., 2014).

Neurotoxicity assessment

The rat pheochromocytoma-derived (PC12) cells were seeded (5000 cell/well) in 96-well plates and cultured for 48 hr in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C with 5% CO₂. Then, the medium was changed to a fresh one containing saline, HAE (100, 200, 400 and 800 µg/ml) or the fractions (800 µg/ml). Then, the cells were further incubated for 24 hr. After that, cell proliferation was evaluated using MTT assay.

Cell proliferation assay

At the end of incubation with HAE or its fractions, 10 µl of MTT solution (5 mg/ml) was added to cell culture medium of the each well. Then, the culture medium was incubated for 2 hr at 37 °C with 5% CO₂. Then, the resulting formazan of each well was dissolved in DMSO. The optical density of formazan dye was read at 545 nm using microplate reader. Cell proliferation which reflects cell viability

was calculated as percentage of untreated cells (Ghorbani *et al.*, 2014; Mortazavian *et al.*, 2012).

Determination of LD₅₀

The median lethal dose of HAE was determined using the method of Akhila *et al.* (2007) as previously described (Ghorbani *et al.*, 2013; Hosseini *et al.*, 2014). Briefly, different doses (0.8-6.4 g/kg) of HAE extract were injected to mice (two mice were used for each dose). Then, the treated animals were observed for mortality for 24 hr. The highest dose which did not kill any animal and the lowest dose that killed one mouse were recorded. Mean of these two doses was taken as the LD₅₀ of *P. eldarica* HAE.

Statistical analysis

Data were expressed as mean±SEM. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tamhane's T2 *post-hoc* test. Values were considered significant if $p < 0.05$.

Results

Effect of *P. eldarica* on sleep duration

As shown in Figure 1, sleeping time in animals receiving saline was 22.8 ± 1.3 min. Diazepam significantly increased the pentobarbital-induced sleeping time to 47.7 ± 1.2 min ($p < 0.001$ vs saline). Similarly, duration of sleep in animals receiving HAE was increased to 35.2 ± 2.5 min ($p < 0.05$), 39 ± 4.4 min ($p < 0.001$), 54.8 ± 2 min ($p < 0.001$), 63 ± 3.4 min ($p < 0.001$) and 65.3 ± 2 min ($p < 0.001$) at doses of 25, 50, 100, 150 and 200 mg/Kg, respectively. The HAE at dose of 150 and 200 mg/kg increased sleeping time more than diazepam ($p < 0.01$). Treatment with flumazenil could restore the hypnotic effect of both diazepam (22.6 ± 1.3 , $p < 0.001$) and 200 mg/kg HAE (19.5 ± 0.9 , $p < 0.001$).

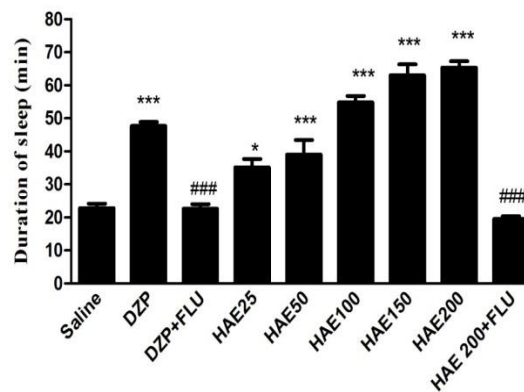


Figure 1. Effects of hydroalcoholic extract of *P. eldarica* on duration of sleep. The saline containing 1% Tween (vehicle), hydroalcoholic extract (HAE), or diazepam (DZP, 3 mg/kg) was administered (ip) to mice 30 min before injection of pentobarbital (30 mg/kg, ip). Data are expressed as mean± SEM of 7 animals in each group. * $p < 0.05$, *** $p < 0.001$ vs saline; ### $p < 0.001$ vs the same group plus flumazenil (FLU, 2mg/Kg).

Figure 2 shows that NBF was the only fraction that significantly increased the sleep duration from 23 ± 1 min (saline) to 37.3 ± 2.5 (25 mg/kg, $p < 0.01$) and 64.2 ± 3 (50 mg/kg, $p < 0.001$). In the presence of flumazenil, this effect of NBF was blocked (20.3 ± 1.7 min, $p < 0.001$).

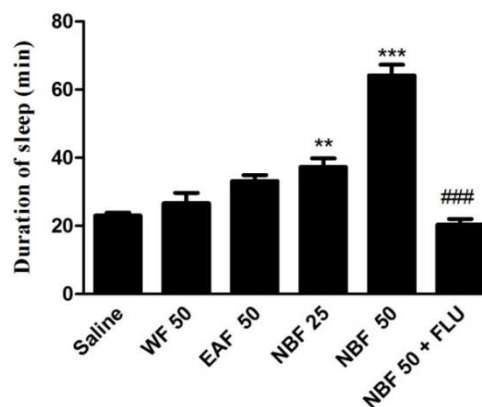


Figure 2. Effects of different fractions of *P. eldarica* extract on duration of sleep. The saline containing 1% Tween (vehicle), water fraction (WF), ethyl acetate fraction (EAF), or *n*-butanol fraction (NBF), was administered (ip) to mice 30 min before injection of pentobarbital (30 mg/kg, ip). Data are expressed as mean± SEM of 7 animals in each group. ** $p < 0.01$, *** $p < 0.001$ vs saline; ### $p < 0.001$ vs the same group plus flumazenil (FLU, 2mg/Kg).

Effect of *P. eldarica* on sleep latency

Data on sleep latency, time interval between the injection of pentobarbital and onset of sleep, is shown in Figure 3. HAE at doses of 50, 100, 150 and 200 mg/kg significantly decreased the sleep latency from 7.7 ± 0.5 min (saline) to 5.1 ± 0.5 ($p < 0.05$), 5 ± 0.4 min ($p < 0.01$), 3.7 ± 0.4 min ($p < 0.001$) and 4.1 ± 0.4 min ($p < 0.001$), respectively. Flumazenil completely reversed the effects of diazepam (7.1 ± 0.5 , $p < 0.001$) and 200 mg/kg HAE (7.8 ± 0.5 min, $p < 0.001$) on sleep latency.

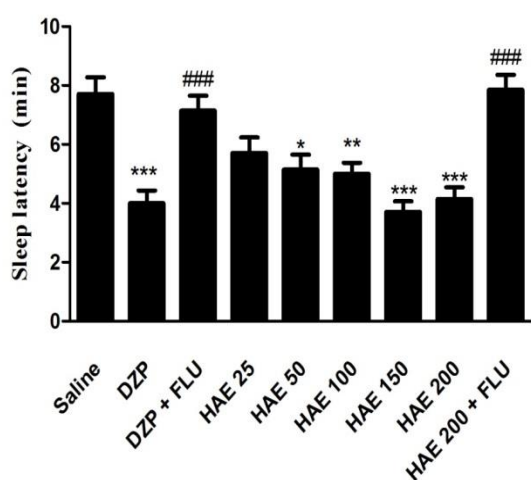


Figure 3. Effects of hydroalcoholic extract of *P. eldarica* on sleep latency. The saline containing 1% Tween (vehicle), hydroalcoholic extract (HAE), or diazepam (DZP, 3 mg/kg) was administered (ip) to mice 30 min before injection of pentobarbital (30 mg/kg, ip). Data are expressed as mean ± SEM of 7 animals in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs saline; ### $p < 0.001$ vs the same group plus flumazenil (FLU, 2mg/kg).

Among the three fractions, NBF significantly reduced the sleep latency from 8.4 ± 0.4 min (saline) to 5.7 ± 0.5 min (25 mg/kg, $p < 0.05$), 5 ± 0.5 min (50 mg/kg, $p < 0.01$). As shown in Figure 4, flumazenil could reverse this effect of NBF (10.5 ± 0.4 min, $p < 0.001$, Figure 4).

Effect of *P. eldarica* on motor coordination

Considering the results from the rotarod test, there were no significant differences between the groups when the animals were examined 30 min after injection of the

HAE of *P. eldarica* (Figure 5). The results showed that injection of diazepam (3 mg/kg) significantly shortened the length of time that the rats maintained their balance on rotarod apparatus compared to the control and all three doses of the extract ($p < 0.001$, Figure 5).

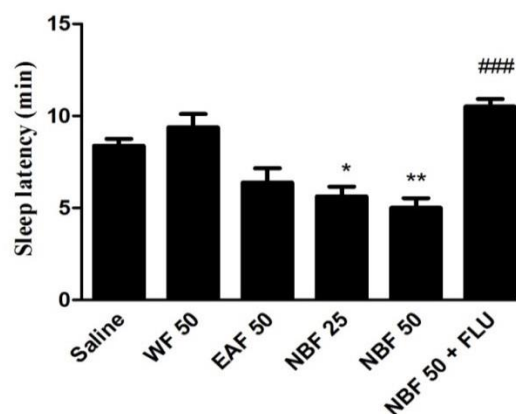


Figure 4. Effects of different fractions of *P. eldarica* extract on sleep latency. The saline containing 1% Tween (vehicle), water fraction (WF), ethyl acetate fraction (EAF), or *n*-butanol fraction (NBF), was administered (ip) to mice 30 min before injection of pentobarbital (30 mg/kg, ip). Data are expressed as mean ± SEM of 7 animals in each group. * $p < 0.05$, ** $p < 0.01$ vs saline; ### $p < 0.001$ vs the same group plus flumazenil (FLU, 2mg/kg).

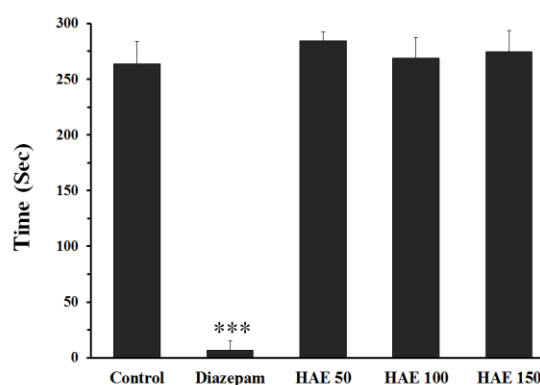


Figure 5. The effects of hydroalcoholic extract of *P. eldarica* on motor performance in rats. The animals were placed on a rotating rod and the length of time they could maintain their balance on the turntable against the movement's strength was recorded. Then, the extract was injected (i.p.) and after 30 min the animals were subjected to the test again. Control group received saline containing 1% of Tween 80 as vehicle. Data are expressed as mean ± SEM of 7 animals in each group. *** $p < 0.001$ vs the control and all three doses of the extract.

Toxicity assessments

The maximum dose which did not kill any mice and the minimum dose that led to death of one mouse were 3.2 and 6.4 g/kg, respectively. Therefore, LD₅₀ of HAE of *P. eldarica* is about 4.8 g/kg.

MTT assay showed that none of HAE concentrations and HAE fractions reduced the viability of PC12 cells (Figure 6). In the presence of 100, 200, 400 and 800 µg/ml of HAE, the percent of viable cells was 99 ± 3.7 , 93.5 ± 4 , 90.5 ± 3.3 , and $99.2 \pm 7\%$, respectively. Also, in comparison with untreated cells ($100 \pm 0.8\%$), incubation with 800 µg/ml of WF, EAF and NBF showed cell viability of $102 \pm 4.7\%$, $99 \pm 4.2\%$ and $100.8 \pm 3.2\%$, respectively.

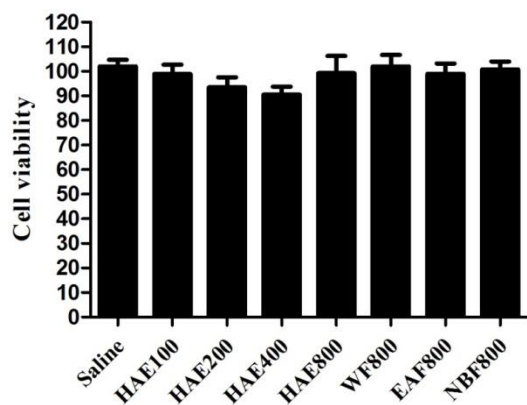


Figure 6. Effects of hydroalcoholic extract of *P. eldarica* on viability of neuronal PC12 cell. The cells were cultured for 24 hr in the medium containing saline (vehicle), 100-800 µg/ml hydroalcoholic extract (HAE), 800 µg/ml water fraction (WF), 800 µg/ml ethyl acetate fraction (EAF) and 800 µg/ml *n*-butanol fraction (NBF). Values are expressed as mean \pm SEM ($n = 4$).

Discussion

The present study showed that extract of *P. eldarica* enhances sleep behavior in mice. To our knowledge, this is the first study which shows that this plant increases sleep duration and decreases sleep latency. In the present work, the hypnotic evaluation method was based on

prolongation of sleep induced by pentobarbital. This technique is of the most used method for screening sedative-hypnotic agents (Ghorbani *et al.*, 2013; Hosseini *et al.*, 2014; Ghorbani *et al.*, 2012; Rakhshandeh *et al.*, 2012). In consistency with the previous reports and as expected, diazepam significantly enhanced the sleeping time induced by pentobarbital, indicating that our experimental method was well optimized (Emamghoreishi and Heidari-Hamedani, 2006; Rakhshandeh *et al.*, 2007). The effect of HAE of *P. eldarica* on sleep latency was dose-dependent and at doses of 150 and 200 mg/kg was comparable to that of diazepam. However, the effect of HAE (at doses of 100-200 mg/kg) on sleep duration was even greater than that of diazepam. Because HAE did not affect the animals' performance on the rotarod test, it seems that its effects on sleeping time and sleep latency are not due to affecting motor movement.

To obtain a better insight into the nature of compounds responsible for sleep prolonging effect of this plant, three fractions were prepared from HAE of *P. eldarica*. The WF comprises polar compounds including tannins, glycosides, and some alkaloids. The EAF contains constituents of intermediate polarity such as flavonoids. The NBF contains low polar agents including alkanes, sterols, and terpenoids (Seidel, 2006; Tian *et al.*, 2011; Edewor-Kuponiyi, 2013). Our data showed that among these three fractions, NBF was the only one which could significantly prolong the duration of sleep and could decrease the sleep latency. Therefore, it can be concluded that the active constituents responsible for sleep prolonging effects of *P. eldarica* are low polar agents in NBF. Consistent with this possibility, the effect of 50 mg/kg NBF on sleep duration was even greater than that induced by 50 mg/kg of HAE.

Phytochemical studies have shown that a wide variety of compounds may be involved in hypnotic effects of medicinal

plants. These compounds include alkaloids, terpenoids (e.g. linalool), steroids, and flavonoids (e.g. quercetin) (Edewor-Kuponiya, 2013; Sharma et al., 2012). Previous studies have revealed that *P. eldarica* needles contain terpenoids as well as some flavonoids such as quercetin (Kaundun et al., 1997; Lee, 2008). It has been shown that quercetin is able to cross the blood-brain barrier and to induce some effects in the central nervous system including neuroprotective and antioxidant actions (Paulke et al., 2006; Youdim et al., 2004; Ishisaka et al., 2011). Kambe et al. (2010) demonstrated that quercetin increases non-rapid eye movement sleep in dark period in rats. The depressant activity of some terpenoids in the central nervous system has also been reported by Meckes et al. (1996). Although we did not investigate the exact compound responsible for sleep prolonging effect of *P. eldarica*, quercetin and some terpenoids might be involved in this effect. Further studies should be performed to test this hypothesis and to isolate these compounds from *P. eldarica*.

Several neurotransmitters have been shown to be involved in the regulation of sleep behavior. GABA is released from neurons located in the anterior hypothalamus and inhibits wake-promoting areas of the hypothalamus and brainstem (Datta, 2007; Murillo-Rodriguez et al., 2011). Barbiturates such as pentobarbital favor binding of GABA to its receptor by acting at GABA receptors ionophore complex. Diazepam, a benzodiazepine agonist, increases the affinity of GABA for its receptor and thereby enhances hypnotic action of pentobarbital (Gottesmann, 2009). Accordingly, we observed that pretreatment with flumazenil inhibits sleep-prolonging effect of diazepam. Also, we found that in the presence of flumazenil, *P. eldarica* is unable to increase the duration of sleep or decrease sleep latency time. In agreement with our finding, Kambe et al. reported that

quercetin, which is found in *P. eldarica*, alters the sleep-wake cycle partly via activation of GABA receptors (Kambe et al., 2010). Also, it has been reported that some natural flavonoids possess selective affinity for benzodiazepine site of the GABA receptor (Wasowski and Marder, 2012). Therefore, it is rational to assume that sleep-prolonging action of *P. eldarica* is mediated, at least in part, by potentiating GABAergic system.

Evaluation of the toxicity of *P. eldarica* showed that LD₅₀ value for HAE is 4.8 g/kg. This value was markedly higher than hypnotic doses of *P. eldarica* (50-200 mg/kg). Similarly, HAE and its fractions did not diminish the viability of neuronal cells even at high concentrations. Therefore, it seems that sleep prolonging effect of *P. eldarica* is not accompanied by a neurotoxic action.

In conclusion, the present data indicate that HAE of *P. eldarica* potentiates sleeping behaviors without major toxic effect. Most probably, the active components responsible for this effect are non-polar agents which are found in NBF of this plant. Isolation of these compounds from *P. eldarica* may offer novel sedative-hypnotic agent.

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