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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Potentiating effects of *Lactuca serriola* on pentobarbital-induced sleep

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Abstract

Insomnia is the most common sleep disorder. The present study was carried out to evaluate the sedative-hypnotic effect of hydro-alcoholic extract (HAE) of lettuce and its water fraction (WF), ethyl acetate fraction (EAF) and n-butanol fraction (NBF). Extract and its fractions were injected to mice 30 min before the pentobarbital. Additionally, the effects of flumazenil on the hypnotic activity of the extract and its toxic effect were determined *in vivo* and *in vitro* situations. The HAE prolonged the pentobarbital-induced sleep duration at doses of 50-400 mg/kg (50 mg/kg; $P<0.05$, 100 and 200 mg/kg; $P<0.01$). In comparison to the control group, HAE at dose of 400 mg/kg increased sleeping time more than diazepam ($P<0.001$ *vs* control). Also flumazenil (2 mg/kg) reversed the effects of both diazepam ($P<0.001$ *vs* diazepam group) and 400 mg/kg of HAE ($P<0.001$ *vs* HAE group). The NBF was the only fraction which could increase sleep duration ($P<0.001$) and decrease sleep latency ($P<0.001$). Flumazenil reversed hypnotic effect of the extract and diazepam. The LD_{50}-value for HAE was found to be 4.8 g/Kg. HAE and its fractions did not show any neurotoxic effect in cultured PC12-cell line. HPLC profile of the extract showed five major peaks with different retention times. The results suggested that *Lactuca serriola* potentiated pentobarbital hypnosis without major toxic effect. Most probably, the main component (s) responsible for this effect was most likely to be non-polar agent (s) which is found in NBF of this plant.

Keywords: Insomnia; diazepam; *Lactuca serriola*; sleep; PC12

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Introduction

Sleep disorder is still a widespread complaint. Approximately 10-15% of adult population suffers from sleepless especially at night (McPherson et al., 2013). So to get rid of insomnia, they need to use hypnotic drugs. These drugs include benzodiazepines/non-benzodiazepines, antidepressants and antihistamines. Long-term administration of these agents leads to various adverse effects such as impaired cognitive function, memory and general daytime performance, tolerance and dependence (Cho et al., 2010). Recently, researchers have shown that some medicinal herbs have hypnotic properties. Sometimes these herbs can be a suitable replacement for chemical drugs (Attele et al., 2000; Phillipson, 2001; Carlini, 2003; Thomas and Christopher, 2004). Herbal extracts change some neurotransmitters such as GABAergic or serotonergic in the central nervous system and stimulate sleep via the mentioned mechanism (Currie and Wheat, 2007). *Lactuca serriola* L. (Compositae) is known by several names, that is, Prickly lettuce, Jagged lettuce, Kahu and Khas (Baquar, 1989). It is native to Himalaya, Siberia and Atlantic areas (Kirtikar and Basu, 1984) but is also cultivated in temperate lands of Europe, India, Pakistan and Iran. A brown viscid substance obtained following after the evaporation of the plant juice, called lactucarium, contains lactucone, lactucin, and

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lactucic acids (Dymock, 1972). The plant contains vitamins, beta carotene and iron (Usmanghani, 1997). Phytochemical investigations have revealed the presence of alkaloids, the bitter substance lettuce, oxalic acid, lactucopicrin (Baquar, 1989) and sesquiterpene esters (Marco et al., 1992).

Aerial parts of the herb have many effects such as sedative, hypnotic, expectorant, cough suppressant, diuretic, antiseptic, vasorelaxant and antispasmodic and are used to manage bronchitis, asthma, pertussis, gastrointestinal and other problems (Kirtikar and Basu, 1984; Baquar, 1989). Pharmacological studies have shown antipyretic (Agarwal, 1997), antibacterial (Yadava and Jharbade, 2008), analgesic, anti-inflammatory (Ahmad and Khan, 1992) and antioxidant activities for *Lactuca Serriola* (Kim, 2001; Tepe and Sokmen, 2007; Nabavi et al., 2012). Recent studies have shown that *Lactuca sativa* potentiated the pentobarbital-induced sleeping behaviours in mice (Ghorbani et al., 2013). Since, hypnotic effect of *Lactuca serriola* has been reported in ancient medicine, therefore, this study was aimed to investigate the effect of *Lactuca Serriola* on sleep.

Materials and Methods

Experimental

Drugs and chemicals

Pentobarbital sodium, penicillin-streptomycin and flumazenil were bought from Sigma (USA). Diazepam was obtained from Chemidarou Company (Iran). Tween 80 was from Merck (Germany). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (USA). Flumazenil, pentobarbital and diazepam were dissolved in saline to make 30 mg/ml l solutions, respectively.

Preparation of extracts

The aerial parts of *Lactuca Serriola* were collected from the Garden of Ferdowsi University of Mashhad, Mashhad, Iran. The plant sample was identified at the herbarium of school of Pharmacy (Mashhad University of Medical Sciences, Mashhad, Iran). The aerial parts of lettuce were dried, powdered and subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h. The HAE extract was then dried on a water bath and the yield (19% w/w) was dissolved in saline containing 1% (v/v) of Tween 80.

For preparation of fractions, a part of hydro-alcoholic extract (HAE) was suspended in distilled water and transferred to a separator funnel. With solvent-solvent extraction, it was fractionated using ethyl acetate or *n*-butanol. The ethyl acetate fraction (EAF) and *n*-butanol fraction (NBF) were separated to obtain water fraction (WF) (Sadeghnia et al., 2012; Mortazavian et al., 2012; Mortazavian and Ghorbani, 2012; Ghorbani et al., 2012). The resulting fractions were dried on a water bath and working solutions were made up in saline and saline containing 1% Tween 80 for WF and EAF or NBF respectively (Rakhshandeh et al., 2012). The yields obtained from the extract fractionation were 73.5% water fraction (WF), 12.5% ethyl acetate fraction (EAF) and 14% n-butanol fraction (NBF).

Animals

A total 96 male albino mice weighing 22-30 g were employed in each experiment. The animals were kept at a controlled temperature with a 12 h light/dark cycle with free access to food and water. The study was approved by Animal Care Use Committee of Mashhad University of Medical Science in accordance with ethical guidelines the animals were randomly divided into 12 groups, each one consisting of 8 mice. At the beginning, hypnotic effect of HEA was evaluated in seven groups: saline as vehicle, diazepam (3 mg/kg) as positive control and HAE (25, 50, 100, 200 and 400 mg/kg). Also, in three groups of animals, effects of HAE-fractions including WF, EAF and NBF with dose of 400 mg/kg were investigated. The fractions were administered with dose of 400 mg/kg. Moreover, flumazenil was applied (2 mg/kg) as a diazepam antagonist for identifying the mechanism.

Sleep induction

The sleep evaluation method was based on the prolongation of pentobarbital-induced sleeping time (Rakhshandah and Hosseini, 2006; Ghorbani et al., 2012; Rakhshandeh et al., 2012). Briefly, the animals received (i.p.) a single dose of the vehicles, diazepam or the extracts. After 30 min, pentobarbital (30 mg/kg, i.p.) was given to induce sleep. The mice were considered asleep if stayed immobile and lost its righting reflex when positioned on its back. The time interval between pentobarbital injection and sleep onset was recorded as sleep latency. To evaluate the possible mechanisms involved in the hypnotic activity of *Lactuca serriola* extract, the animals were pretreated with flumazenil (2 mg/kg) as the antagonist of $GABA_A$-benzodiazepine receptor.

Neurotoxicity assessment

The PC12 cells were seeded (5000 cell/well) in 96-well plates and cultured for 48 h in DMEM supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37°C and 5% CO_2. Then, the medium was changed to a fresh one containing vehicle, HAE (200, 400, 800 and 1600 µg/ml) or the fractions *Lactuca serriola* (800 µg/ml) (Ghorbani et al., 2013). The cells were further incubated for 24 h. At the end of the treatment, the effect of extracts on cell proliferation was measured

using the previously described 3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assays (Rakhshandeh et al., 2012; Hadjzadeh et al., 2006; Tavakkol-Afshari et al., 2006; Alinejad et al., 2013). The assay was carried out in triplicate and repeated twice for confirmation. Level of cytotoxicity was expressed as the percent of surviving cells.

Determination of LD_{50}

Acute toxicity of HAE was tested using the method Akhila et al. (2007) as described previously (Ghorbani et al., 2012). Briefly, different doses (0.8-6.4 g/kg) of the extract were administrated to mice (two mice were used for each dose). Then, the treated animals were monitored for mortality for 24 h. The highest and the lowest doses which did not kill any mouse were recorded. Mean of these two doses was taken as the median lethal dose (LD_{50}) of *Lactuca serriola* extract.

Characterization of the extract by HPLC

The quality of hydoalcoholic extract of *Lactuca serriola* was characterized by HPLC-UV fingerprint. The chromatographic separation was carried out with a reverse-phase Waters C18 analytical column (250 × 4.6 mm, 5 µm particle size). An isocratic elution was carried out by the mobile phase of methanol: acetonitrile: water (60:20:20% v/v, pH 5.9 adjusted with phosphoric acid) at the flow rate of 1 ml/min. The UV detector wavelength was set at 330 nm. A sample of the extract was dissolved in distilled water and passed through 0.45 µm membrane filter. Then, 20 µl of the sample (500 µg/l) was injected to the HPLC column.

Statistics

All 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. Differences were considered significant at $P<0.05$.

Results

Effect of HAE on sleep

Effects of different doses of HAE on sleeping time and sleep latency induced by pentobarbital are shown in Tables 1 and 2, respectively. HAE at the doses of 50-200 mg/kg significantly increased sleeping time compared to the control group (saline) (50 mg/kg; 32±1, $P<0.05$) (100 and 200 mg/kg; 34±1.7, 43±1.3 ($P<0.01$). In comparison to control group, HAE at dose of 400 mg/kg increased sleeping time more than diazepam (59±3.4, $P<0.001$*vs* control). In Table 1, it can also be seen that flumazenil (2 mg/kg) reversed the effects of both diazepam (25.7±1.2, $P<0.001$*vs* diazepam group) and 400 mg/kg of HAE (32.5±2.1, $P<0.001$*vs* HAE group). Therefore, there was decreased sleeping time in pentobarbital-induced hypnotic test.

Diazepam (3.4±0.36 min, $P<0.001$) and HAE at doses of 200 (4.6±0.36, $P<0.001$) and 400 (4.1±0.46, $P<0.001$ mg/kg) significantly reduced the latency to sleep in comparison to the vehicle (control). As it can be observed in Table 2, flumazenil (2 mg/kg, i.p.) reversed the effects of diazepam (7.1±0.63 min, *vs* diazepam $P<0.001$) and 400 mg/kg of HAE (6.28±0.42 min, *vs* HAE $P<0.05$). Therefore, in flumazenil-treated mice, there was an increased latency to sleep in pentobarbital-induced hypnotic test.

Effect of HAE-fractions on sleep

As shown in Table 3, only n-butanol fraction (NBF) was able to significantly increase sleep time (54±1.6 *vs* control, $P<0.001$). However, effects of water fraction (WF) and ethyl acetate fraction (EAF) were statistically non-significant. Also in comparison with vehicle, NBF significantly reduced latency to sleep (3.8±0.45, $P<0.001$) (Table 4).

Toxicity assessments

The highest dose which did not kill any mice and the lowest dose caused the death of one mouse were 6.4 and 3.2 g/kg respectively. Mean of these two doses was calculated as the lethal dose (LD_{50}) which was 4.8 g/kg. Result of MTT showed that none of the HAE concentrations decreased the proliferation of PC12 cells. In the presence of 200, 400, 800 and 1600 µg/ml of the extract, survival of the cells was 110±1.5, 108±2.1, 112±1.3 and 105±2.6%, respectively, as compared to control. Similarly, the HAE fractions exhibited no cytotoxicity. The level of viability was 115±2.5, 112±1.6 and 106±3.2 for WF, EAF and NBF respectively (Table 5).

HPLC profile of HAE

A simple and reliable HPLC fingerprint has been developed for the standardization of the hydoalcoholic extract. HPLC profile of HAE was recorded under UV 330 nm. The corresponding HPLC chromatogram is

Table 1: Effects of *Lactuca serriola* hydro alcoholic extract on sleeping time in pentobarbital-induced hypnotic test

Saline	DZP	DZP+FLU	EXT25	EXT 50	EXT 100	EXT 200	EXT 400	EXT 400 + FLU
23±1.3	48±1***	25.7±1.2$^{###}$	28±1.17	32±1*	34±1.7**	43±1.3**	59±3.4***	32.5±2.1$^{###}$

Diazepam: 3 mg/kg; Pentobarbital: 30 mg/kg; Flumazenil: 2 mg/kg; *P<0.05; **P<0.01; ***P<0.001 significantly different from control. $^{###}$P<0.001 significantly different from the same group plus flumazenil (2mg/kg). DZP: diazepam; EXT: extract; FLU: flumazenil (2 mg/kg)

Table 2: Effects of *Lactuca serriola* hydro alcoholic extract on sleeping latency in pentobarbital-induced hypnotic test

Saline	DZP	DZP+FLU	EXT25	EXT 50	EXT 100	EXT 200	EXT 400	EXT 400 + FLU
7.7±0.56	3.4±0.36***	7.1±0.63###	6.6±0.36	5.7±0.42	5.7±0.48	4.6±0.36***	4.1±0.46***	6.28±0.42#

***P<0.001 significantly different from control group. ###P<0.001, # P<0.05 significantly different from the same group plus flumazenil, DZP: diazepam; EXT: extract; FLU: flumazenil

Table 3: Effects of hydro alcoholic extract fractions of *Lactuca serriola* on sleeping time in pentobarbital-induced hypnotic test significantly different from control.

Saline	WF	EAF	NBF
23±1.3	27.7±0.92	28.1±1.6	54±1.6***

***P<0.001

Table 4: Effects of hydro alcoholic extract fractions of *Lactuca serriola* on sleeping latency in pentobarbital-induced hypnotic test.

Saline	WF	EAF	NBF
7.7±0.56	7.3±0.56	8.8±0.6	3.8±0.45***

***P<0.001 significantly different from control.

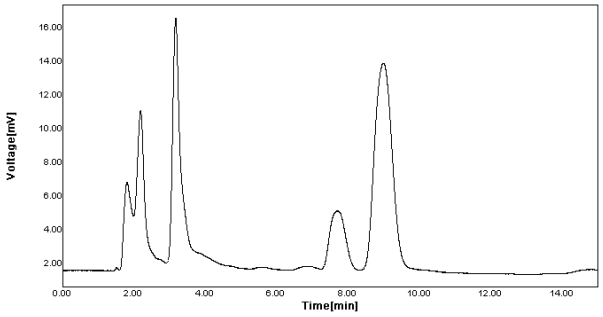

Fig. 1: HPLC fingerprints of hydro-alcoholic extract of *Lactuca serriola*. Chromatogram detected by UV at 330 nm.

presented in Figure 1. The extract revealed 5 major peaks with retention time (RT) values in the range of 1.9 to 9.7 min for 20 µl application volume (Fig. 1).

Discussion

In our previous study, we reported hypnotic effect of *Lactuca sativa* and the result showed that it potentiated pentobarbital effect of sleep (Ghorbani et al., 2013). Diazepam belongs to the benzodiazepine group which has a binding site on GABA receptor type-ionophore complex ($GABA_A$) (Huang et al., 2007) and this mechanism can be useful in the onset of sleep and increase of sleep duration (Herrera-Ruiz et al., 2007). HAE administration at doses of 50-400 mg/kg produced sedative effect similar to that observed with 3 mg/kg of diazepam. The HAE may have the effect via GABA ergic system. The inhibitory action of GABA is via opening of chloride channels and hyperpolarization of the membrane, all of them lead to CNS depression, sedative and hypnotic activity (Alnamer et al., 2012). Therefore, the drugs that influence these systems can be important in insomnia disorder.

In the present study, the potentiated effect of HAE on sleep was observed. It not only prolonged the sleeping time, but also decreased the latency of falling asleep. In order to determine if benzodiazepine receptor participates in the hypnotic effects of HAE, flumazenil as a specific antagonist of the benzodiazepine receptor was administered. Pre-treatment with flumazenil significantly reduced the effects of HAE. Therefore, it is possible for HAE to increase sleep via benzodiazepine receptor. It has been found that some plant compounds such as flavonoids, terpenes and saponins show hypnotic effect (Rakhshandah et al., 2004; Rakotonirina et al., 2001). Recent studies have shown that *Lactuca Serriola* contains lactucin and lactucopicrin that have sedative properties (Mohammad, 2013).

To obtain a better insight into the nature of the compounds responsible for the effect of HAE, three fractions were prepared: (1) The WF solubilizing the polar agents and water-soluble plant constituents (e.g. glycosides, quaternary alkaloids, tannins); (2) the EAF extracting compounds of intermediate polarity; and (3) the NBF bearing non-polar agents like sterols, alkanes and some terpenoids (Seidel, 2006; Ghorbani et al., 2012). The present data showed that NBF was the only fraction which could significantly prolong the sleep duration or decrease the sleep latency. Therefore, it can be concluded that active compounds responsible for the effects of lettuce are non-polar agents concentrated in NBF. Unexpectedly, however, the strength of the effects induced by NBF was not more than that of HAE. It raises this possibility that some polar agents or compounds with intermediate polarity in WF and EAF also contribute to the sleep prolonging effect of HAE and potentiate the effects induced by NBF, although their effect per se was not statistically significant. The other possibility could be that the sleep prolonging effect of HAE and NBF reached the plateau level at dose of 400 mg/kg.

The phytochemical studies have represented the presence of alkaloids, the bitter substance lettuce, oxalic acid, lactucopicrin (Baquar, 1989), and sesquiterpene esters in *Lactuca Serriola* (Marco et al., 1992). Lactucin, a sesquiterpene lactone of Lactuca species, has been reported to have a sedative property in the spontaneous locomotor activity test (Wesołowska et al., 2001). Also, Janbaz et al. (2013) showed that *Lactuca serriola* extract has a spasmolytic activity and

Table 5: Effect of hydoalcoholic extract of *Lactuca serriola* and its fractions on PC12 cell viability

Control	EXT200	EXT400	EXT800	EXT1600	WF	EAF	NBF
100±2	110±1.5	108±2.1	112±1.3	105±2.6	115±2.5	112±1.6	106±3.2

probably bocks calcium channel to induce the sleeping effect (Janbaz et al., 2013). Recent studies have shown that classical calcium channel blockers, like nifedipine, verapamil and diltiazem prolonged sleeping time in mice treated with the pentobarbital (Zhao et al., 2006) which support our findings.

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The toxicity assay showed that LD50 value for HAE of Lactuca serriola is 4.8 g/kg. This dose is so far from its hypnotic doses (50-400 mg/kg). Also, HAE even at high concentrations did not decrease viability of neuronal and fibroblast cells. Therefore, it seems that hypnotic effect of Lactuca serriola accompanied with no neurotoxicity.

The construction of chromatographic fingerprints plays an important role in the quality control of complex herbal medicines. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might appropriately represent the chemical integrities of the herbal medicines and therefore be used for authentication and identification of the herbal products (Wagner et al., 2011). In the present chromatographic technique, in order to obtain a good resolution within a short analysis time, the composition of mobile phase was optimized. Acidic mobile phase was used in order to suppress the ionization of phenolic hydroxyl groups. This acidification was beneficial, leading to good peaks separation and better peak shape. Various mobile phase compositions were evaluated. Methanol, acetonitrile and water containing little amount phosphoric acid were chosen as the mobile phase because all peak components could be resolved under this condition. The HPLC fingerprint showed high stability and reproducibility, and thus could be used for quality control of the hydro-alcoholic extract and *Lactuca* products.

In conclusion, this work showed that *Lactuca serriola* had significant sedative-hypnotic effect. Further chemical and pharmacological analyses of the extract are needed to isolate and characterize the active components responsible for the sedative effect of *Lactuca serriola*.

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