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Hypnotic Effect of *Ocimum basilicum* on Pentobarbital-Induced Sleep in Mice

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Abstract

Background: Sleep disorders are accompanied by several complications, and currently used soporific drugs can induce unwanted effects such as psychomotor impairment, tolerance, amnesia, and rebound insomnia.
Objectives: The present study was carried out to investigate if *Ocimum basilicum* has a sleep-prolonging effect.
Materials and Methods: This work was an experimental study on 72 mice which were randomly divided into 9 groups: saline (control); diazepam (3 mg/kg, positive control); hydro-alcoholic extract (HAE) of Ocimum basilicum (25, 50, or 100 mg/kg); ethyl acetate fraction (EAF, 50 mg/kg); n-butanol fraction (NBF, 50 mg/kg); water fraction (WF, 50 mg/kg); and saline containing 10% DMSO (vehicle for EAF and NBF). All the test compounds were injected intraperitoneally (IP) 30 minutes before pentobarbital administration (30 mg/kg). Duration and latency of pentobarbital-induced sleep were recorded. Also, LD50 of HAE was determined and the cytotoxicity of HAE was tested on neural and fibroblast cells using the MTT assay.
Results: HAE increased the duration of pentobarbital-induced sleep at doses of 25, 50, and 100 mg/kg ($P < 0.001$). The hypnotic effect of HAE was comparable to that induced by diazepam. Similarly, WF, EAF, and NBF at 50 mg/kg could increase sleep duration. The sleep latency was decreased by HAE ($P < 0.01$ - $P < 0.001$) and NBF ($P < 0.001$), but not by WF and EAF. The LD50 value for HAE was found to be 2.4 g/kg. HAE had no effect on the viability of neuronal PC12 cells and L929 fibroblast cells.
Conclusions: The present data demonstrated that *Ocimum basilicum* potentiates sleeping behaviors without any cytotoxicity. The main component (s) responsible for the hypnotic effects of this plant is most likely a non-polar agent (s) which is found in NBF. Isolation of the active constituents may yield a novel sedative drug.

Keywords: L929, Pentobarbital, Ocimum Basilicum, PC12, Sleep

1. Background

Sleep can be disturbed by several factors such as illness, stress, and noise. Over time, sleep disorders may lead to serious physical complications. These include poor memory, slower reaction time, emotional disturbances, and changes in the immune response (1, 2).

Today, the most widely used drugs for sleep disorders are the benzodiazepines. However, administration of these drugs is accompanied by side effects including psychomotor impairment, drug dependence, tolerance, amnesia, rebound insomnia, and the potentiating effects of other central depressant drugs (3). Moreover, some patients with sleep disorders do not respond effectively to current therapeutics. Therefore, studies to find new hypnotic agents with fewer side effects and more efficacy have continued. Herbal agents have long been a valuable source for developing new therapeutics for disease treatment.

Ocimum basilicum (*O. basilicum*) is an annual herb belonging to the Lamiaceae family and grows mostly in tropical regions such as India, Africa, and South Asia (4). In traditional medicine, *O. basilicum* is used to treat many disorders such as anxiety, diabetes, cardiovascular diseases, headaches, neurological pain, and seizures (5, 6). It has been demonstrated that the ethyl acetate fraction (EAF) of *O. basilicum* decreases ischemia-induced oxidative stress in the brain and improves short-term memory and motor coordination (5). It was reported in some traditional medical texts that *O. basilicum* leads to sleep and a sedative state. Also, some biological activities of *O. basilicum* were screened by Ismail, and it was found that the essential oil of *O. basilicum* induces anticonvulsant and hypnotic activities (7). In folk medicine, maceration is the most widely used form of *O. basilicum* preparation. However, there is no pharmacological evidence for the sedative-hypnotic effect

of *O. basilicum* macerated extract.

2. Objectives

The present study was designed to evaluate the sleep-prolonging effects of hydro-alcoholic extract (HAE) of *O. basilicum*) and its fractions. Also, the safety of this plant was examined using neuronal and fibroblast cells.

3. Materials and Methods

3.1. Drugs and Chemicals

Dimethyl sulfoxide (DMSO, code D4540), penicillin-streptomycin (code P4333), sodium pentobarbital (code P3761), and 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-Diphenyl-2H-tetrazolium bromide (MTT, code M-5655) were bought from Sigma (St. Louis, MO, USA). Diazepam was purchased from the Chemidarou Company (Tehran, Iran). Dulbeccos Modified Eagles Medium (DMEM, code 12800-082) and fetal bovine serum (FBS, code 10270-106) were purchased from Gibco Life Technologies (Grand Island, NY, USA).

3.2. Plant Collection and Extraction

The leaves of *O. basilicum* were collected from Mashhad, Iran in the month of July and dried in the shade. A voucher sample was preserved for reference in the herbarium of the Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences (herbarium number: 12937 obl).

HAE was prepared using the maceration method (8). The powder of the leaves of *O. basilicum* (100 g) was macerated in 800 mL of 70% ethanol for 48 hours. The extract was then filtered through a 0.106 mm filter and dried in a water bath. The yield of the dried extract as related to the weight of the dried leaves was 21%.

For the preparation of different fractions from HAE, 10 g of dried extract were suspended in 200 mL of distilled water and transferred to a separator funnel. Using solvent-solvent extraction, it was fractionated with ethyl acetate or n-butanol. The EAF and n-butanol fraction (NBF) were separated to obtain water fraction (WF) (9, 10). All the fractions were dried in a water bath (Memmert, Germany) and stored at -20°C until used. Then, the WF was dissolved in saline, and the EAF and NBF were dissolved in distilled water containing 10% DMSO.

3.3. Animals

Male albino mice weighting 20 - 30 g were maintained at a controlled temperature (22 $\pm$ 1°C) with a 12 hours light/dark cycle and free access to water and food. The study was carried out in accordance with the ethical guidelines of Mashhad University of Medical Sciences (code 910240, 2012.07.05). The animals were randomly divided into 9 groups consisting of 8 mice each. First, to determine if HAE has a sleep-prolonging effect, the animals received saline (control group) and diazepam (as positive control) or different doses of HAE. In the second experiment, to determine the most effective fraction of HAE, animals were treated with WF, EAF, NBF, or 10% DMSO (vehicle for EAF and NBF).

3.4. Evaluation of Pentobarbital-Induced Sleep

The hypnotic assessment method was based on the prolongation of sleep induced by pentobarbital. A single dose of HAE (25, 50, 100 mg/kg), fractions of HAE, diazepam (3 mg/kg), or other vehicles were injected intraperitoneally (IP) into the mice. After 30 minutes, pentobarbital (30 mg/kg IP) was administrated to induce sleep (11-13). Flumazenil (1 mg/kg) was administrated 30 minutes before diazepam or HAE (12). All materials were injected with a volume of 10 mg/kg (11). The onset of sleep is the time that the mice stayed immobile and lost their righting reflex. The time interval between administration of pentobarbital and onset of sleep was considered sleep latency.

3.5. LD50 Determination

Nine groups, each containing two mice were used for determination of LD50 of HAE. Groups 1 - 8 were injected IP with 25, 50, 100, 200, 400, 800, 1,600, and 3,200 mg/kg of HAE and group 9 received normal saline as a vehicle. Mortality rate was observed and recorded for a 24 hours period. The highest dose which did not kill any mice and the lowest dose which led to the death of one animal were recorded. The mean of these two doses was considered the median lethal dose (14, 15).

3.6. Neurotoxicity Assessment

The possible cytotoxicity of *O. basilicum* was tested on rat pheochromocytoma-derived cells (PC12) and murine fibroblast cells (L929). PC12 cells have several neuronal characteristics and, therefore, are useful in the in vitro model for evaluating the neuroprotective or neurotoxic activity of drugs and plant extracts. Also, the L929 cell is considered to be a standard cell line for cytotoxicity assays according to the US pharmacopeia (USP) and is frequently used for testing the possible toxic effects of materials.

The cells were cultured in 96-well plates for 24 hours in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). Then, the culture medium was changed to a fresh one containing the vehicle (DMSO 1%) or HAE (50, 100, 200, 400, and 800 μg/mL). The cells were incubated for 24 hours at 37°C in an atmosphere of 5% CO_2. Then, cell proliferation was evaluated using the MTT assay as previously described (16). Briefly, the MTT solution was added to a culture medium to make a final concentration of 0.5 mg/mL and incubated for 2 hours. The medium was then discarded and the resulting formazan dye was dissolved with DMSO. The optical density of the dye was measured at 545 nm.

3.7. Statistics

All values are expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tamhane's T2 post hoc test using the Instat software package (Graphpad, San Diego, CA). Differences were considered significant at $P < 0.05$.

4. Results

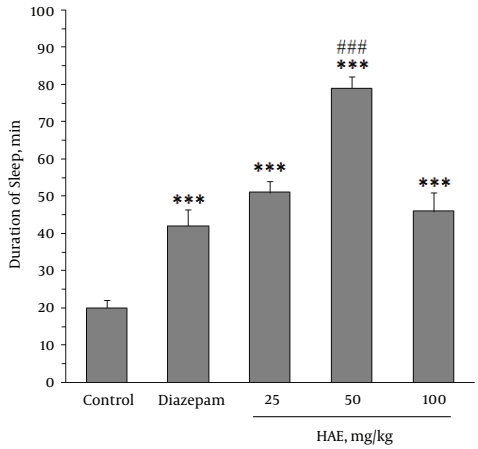
4.1. Effects of O. Basilicum on Duration of Sleep

Sleep duration in the negative control group that received normal saline before pentobarbital was 20 ± 2 minutes (Figure 1). As expected, the reference drug diazepam was able to increase the duration of sleep (42 ± 4.6 minutes, $P < 0.001$ vs. control). The HAE at doses of 25, 50, and 100 mg/kg could significantly increase sleep duration to 51 ± 3 minutes ($P < 0.001$), 79 ± 3 minutes ($P < 0.001$), and 46 ± 5 minutes ($P < 0.001$), respectively. The effect produced by 50 mg/kg was significantly greater than that produced by 25 or 100 mg/kg ($P < 0.001$).

As expected, pretreatment of mice with flumazenil decreased the sleep-prolonging effect of diazepam (42 ± 4.5 minutes and 20 ± 3 minutes for diazepam and flumazenil plus diazepam, respectively, $P < 0.001$). Similarly, the effect of HAE on sleep duration was significantly inhibited by flumazenil (79 ± 3 minutes and 30 ± 2 minutes for HAE and HAE plus diazepam, respectively, $P < 0.001$). Flumazenil alone had no effect on sleep duration (Figure 2).

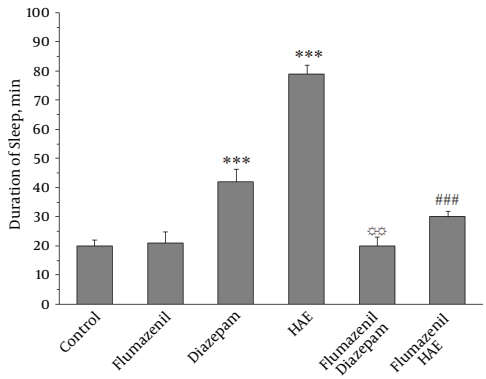
As shown in Figure 3, all three fractions of HAE exhibited sleep-prolonging activity. Duration of sleep in groups receiving 50 mg/kg of WF, EAF, and NBF was 40 ± 4.5 minutes ($P < 0.001$ vs. control), 30 ± 2 minutes ($P < 0.001$ vs. vehicle) and 42 ± 2 minutes ($P < 0.001$ vs. vehicle), respectively. Among the fractions, NBF induced the maximum prolongation of sleep.

Figure 1. Effect of Hydro-Alcoholic Extract of *Ocimum basilicum* on Sleep Duration in Mice



The animals were treated with saline (control), diazepam (3 mg/kg), or hydro-alcoholic extract (HAE) 30 minutes before administration of pentobarbital (30 mg/kg IP). ***P < 0.001 vs. control; ###P < 0.001 vs. groups of 25 and 100 mg/kg.

Figure 2. Effect of Flumazenil on the Sleep-Prolonging Effect of *Ocimum basilicum*

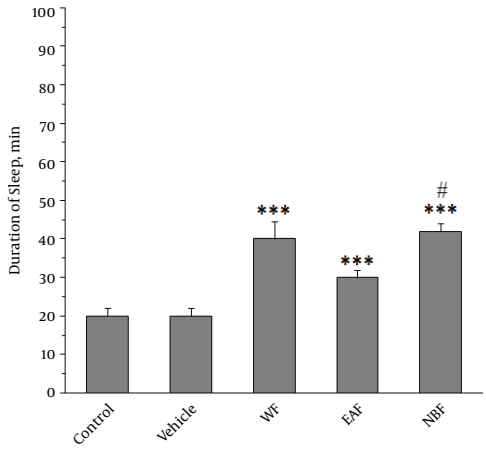


The animals were treated with saline (control) and 3 mg/kg of diazepam or 50 mg/kg of hydro-alcoholic extract (HAE) before injection of pentobarbital (30 mg/kg IP). Flumazenil (1 mg/kg) was administrated 30 minutes before diazepam or HAE. ***P < 0.001 vs. control; ###P < 0.001 vs. HAE; ¤¤ P < 0.01 vs. diazepam. Values are presented as mean ± SEM (n = 8).

4.2. Effects of O. Basilicum on Sleep Latency

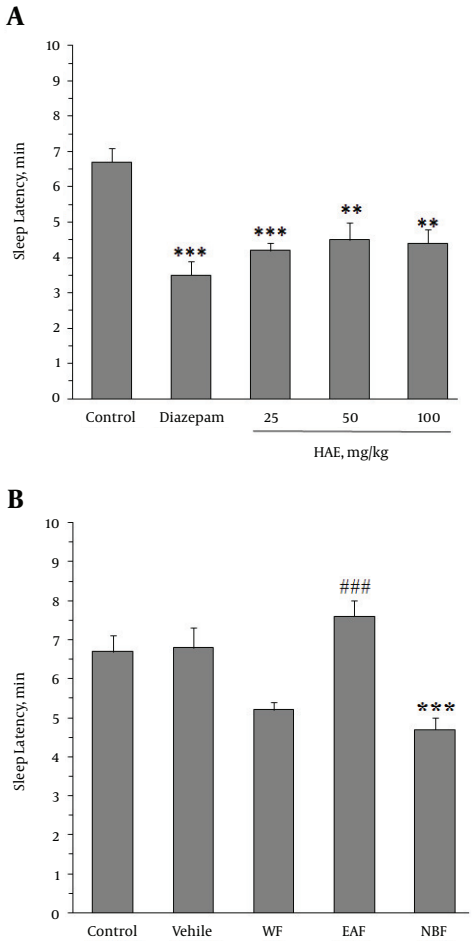
Figure 4 shows the time elapsed between the administration of pentobarbital and the onset of sleep. When compared to the control (6.7 ± 0.4 minutes), diazepam significantly decreased sleep latency to 3.5 ± 0.4 minutes ($P < 0.001$). The latency time in animals receiving 25, 50, and 100 mg/kg of HAE was 4.2 ± 0.2 minutes ($P < 0.001$), 4.5 ± 0.5 minutes ($P < 0.01$), and 4.4 ± 0.4 minutes ($P < 0.01$), respectively. The effect of diazepam was not statistically dif-

Figure 3. Effects of Fractions of *Ocimum basilicum* Extract on Sleep Duration in Mice



The animals were treated with 10% DMSO (vehicle) or 50 mg/kg of water fraction (WF), ethyl acetate fraction (EAF) or n-butanol fraction (NBF) before administration of pentobarbital (30 mg/kg, IP). ***P < 0.001 vs. control and vehicle; #P < 0.05 vs. EAF group; Values are presented as mean ± SEM (n = 8).

Figure 4. Effect of *Ocimum basilicum* on Sleep Latency in Mice



The animals were treated with saline (control) and diazepam (3 mg/kg) or hydro-alcoholic extract (HAE). **P < 0.01 vs. control; ***P < 0.001 vs. control; B, Mice were treated with 10% DMSO (vehicle) or 50 mg/kg of water fraction (WF), ethyl acetate fraction (EAF) or n-butanol fraction (NBF). ***P < 0.01 vs. control; ###P < 0.01 vs. WF and NBF. Values are presented as mean ± SEM (n = 8).

ferent from that observed with 25, 50, or 100 mg/kg of HAE.

Among the different fractions of HAE, EAF was not able to significantly change sleep latency. However, NBF significantly decreased the latency time to 4.7 ± 0.3 minutes (P < 0.001). Although WF could decrease sleep latency from the control level (6.7 ± 0.4 minutes) to 5.2 ± 0.2 minutes, this effect was not statistically significant.

4.3. Toxicity Assessments

The highest dose, which did not kill any mice, and the lowest dose, which led to the death of one mouse, were 1.6 and 3.2 g/kg, respectively. The mean of these two doses (2.4 g/kg) was considered as LD50.

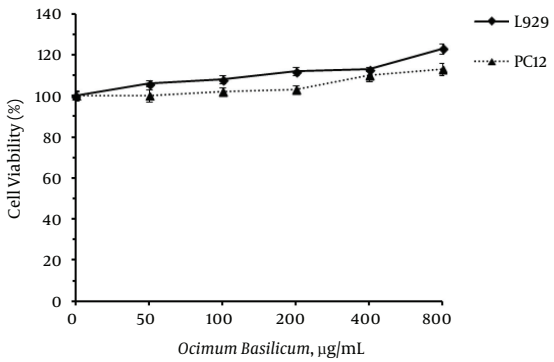
The possible cytotoxicity of HAE of *O. basilicum* was evaluated on PC12 and L929 cells (Figure 5). It was found that up to 24 hours none of HAE concentrations decreased proliferation of PC12 cells. In the presence of 50, 100, 200, 400, and 800 μg/mL of the extract, cell viability was 100 ± 3, 102 ± 2, 103 ± 2, 110 ± 3, and 113 ± 3%, respectively, as compared to the vehicle (100 ± 2.5%). Regarding L929 cells, the viability was 106 ± 1.5, 108 ± 2, 112 ± 2, 113 ± 1, and 123 ± 2.5% in the presence of 50, 100, 200, 400, and 800 mg/mL, respectively. Again, there was no significant difference in the cell viability when compared to the vehicle (100 ± 2%).

5. Discussion

The present study showed that *O. basilicum* further enhances sleep behavior, confirming that this plant has a hypnotic action as claimed in traditional medicine. To our knowledge, this is the first pharmacological study showing the effects of the macerated extract of this plant on sleep duration and sleep latency. Also, this is the first work to determine the LD50 value for HAE of *O. basilicum* and to assess possible cytotoxicity of this extract on neuronal cells. Yet, results of this study are preliminary and need to be confirmed by further clinical trials.

The hypnotic assessment method was based on prolongation of sleep induced by pentobarbital, which is the most commonly used method for screening sedative-hypnotic agents (11-13). In agreement with the previously published works and as expected, diazepam significantly

Figure 5. Effect of *Ocimum basilicum* on the Viability of PC12 and L929 Cells



The cells were cultivated for 24 hours in culture media containing hydro-alcoholic extract of *O. basilicum*. Data are mean ± SEM (n = 8).

increased pentobarbital-induced sleeping time, indicating that our study method was optimized (17, 18). The effect of HAE of *O. basilicum* on sleep latency was lower than that of diazepam. However, the sleep-prolonging effect of HAE was comparable to that of diazepam, and, even at a dose of 50 mg/kg, HAE had a greater effect. Also, the hypnotic effect of HAE of *O. basilicum* is greater when compared with the effect of essential oil of *O. basilicum* reported by Ismail (4-fold and 2-fold increase in sleep duration, respectively) (7).

According to our results, the hypnotic effect of HAE was not dose-dependent in the range of given doses, and the maximum effect occurred with a dose of 50 mg/kg. Therefore, we used this dose to investigate the effects of different fractions of HAE. The fractions of HAE were prepared to obtain better insight into the nature of compounds responsible for the hypnotic effect of *O. basilicum*: 1, WF which extracts water-soluble constituents (e.g., glycosides, quaternary alkaloids, tannins); 2, EAF which extracts compounds of intermediate polarity (e.g., some flavonoids); and 3, NBF which extracts low polar agents (e.g., sterols, alkanes, and some terpenoids) (19, 20). Although WF, EAF, and NBF at a dose of 50 mg/kg were all able to increase sleep duration, not one of them could enhance sleep duration to the level induced by 50 mg/kg of HAE. Because HAE contains the active constituents of all the above-mentioned fractions, it can be concluded that an additive effect was caused by the interaction between these constituents when HAE was administrated. Therefore, both polar and non-polar constituents in *O. basilicum* extract are involved in the sleep-prolonging effect of this plant. Among WF, EAF, and NBF fractions, NBF not only induced the maximum prolongation of sleep duration, but also was the only fraction to induce a significant decrease in sleep latency. On the other hand, EAF failed to show any effect on sleep latency. Therefore, NBF contains a higher concentration of constituents responsible for the hypnotic effect of *O. basilicum*.

A wide variety of phytochemicals has been reported to have sedative-hypnotic effects. These include terpenoids (e.g., linalool, eugenol), flavonoids (e.g., quercetrin, luteolin), alkaloids (e.g., rosmarinic acid), steroids (e.g., beta-sitisterol), and saponins (21, 22). It has been shown that *O. basilicum* contains high amounts of linalool, eugenol, and rosmarinic acid (7). Linck and coworkers demonstrated that linalool increases barbiturate-induced sleeping time in mice (23). Similarly, the sleep-prolonging effect of eugenol was reported by Sharma et al. (22). Rosmarinic acid is found in methanolic extract of *O. basilicum* and can be isolated from the NBF of plant extracts (24, 25). It plays a major role in the sedative-hypnotic actions of some medicinal plants (26).

Several neurotransmitters are involved in the regulation of sleep behavior. Neurons located in the anterior hypothalamus release gamma-aminobutyric acid (GABA) to inhibit wake-promoting areas in the hypothalamus and brainstem (27, 28). Barbiturates such as pentobarbital act on the GABA receptor's ionophore complex and favor the binding of GABA. Benzodiazepines such as diazepam increase the affinity of GABA for its receptor and thereby enhance pentobarbital-induced sleeping time (29). Consistent with this, we observed that pretreatment of mice with flumazenil decreases the sleep-prolonging effect of diazepam. Also, we found that flumazenil inhibits the hypnotic effect of *O. basilicum* extract. In agreement with our finding, Awad et al. reported that rosmarinic acid, which is found in the extract of *O. basilicum*, can act as a GABA transaminase inhibitor and therefore increases the brain level of GABA (30). Therefore, it is rational to suggest that the sleep-prolonging effect of *O. basilicum* is mediated, at least in part, through the potentiating of the GABAergic system. It is now accepted that some natural compounds interact with the GABAergic system to increase sleep behavior (31, 32).

The toxicity assay showed that the LD50 value for HAE of *O. basilicum* is 2.4 g/kg. This dose is far from its hypnotic doses (25 - 100 mg/kg). Also, HAE, even at high concentrations, did not decrease the viability of neuronal and fibroblast cells. Therefore, it seems that the hypnotic effect of *O. basilicum* is not accompanied by neurotoxicity.

In conclusion, present data demonstrated that the macerated extract of *O. basilicum* potentiates sleeping behaviors without any cytotoxicity. The compounds responsible for this effect are mainly found in NBF. Isolation of the active compounds may yield a novel sedative-hypnotic agent.

Acknowledgments

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Footnote

Authors' Contributions: Study concept and design: Ahmad Ghorbani and Hassan Rakhshandeh; collection, analysis and interpretation of data: Vafa Baradaran Rahimi, Ahmad Ghorbani, and Hassan Rakhshandeh; drafting of the manuscript: Vafa Baradaran Rahimi and Ahmad Ghorbani; and critical revision of the manuscript for important intellectual content: Vahid Reza Askari, Vafa Baradaran Rahimi, Ahmad Ghorbani, and Hassan Rakhshandeh.

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