Research Article

Hypnotic Effect of A. absinthium Hydroalcoholic Extract in Pentobarbital-Treated Mice

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Background. Current drugs used in the management of insomnia are associated with side effects. The use of medicinal herbs for insomnia treatment has recently been suggested. Objective. The present study aimed to determine the hypnotic activity of the hydroalcoholic extract of Artemisia absinthium (A. absinthium) in mice. Method. The toxicity of A. absinthium extract is assessed by their lethal dose 50% (LD50), and cytotoxicity evaluation was also done with PC12 cell lines by MTT assay. A. absinthium extract (25, 50, 100, and 200 mg/kg) and 3 fractions (n-butanol fraction (NBF), ethyl acetate fraction (EAF), and aqueous fraction (AQF)) were administered intraperitoneally 30 minutes before 30 mg/kg pentobarbital intraperitoneal injection; after that, the sleeping time and sleep latency were recorded. Results. The LD50 value was 2.4 g/kg. The extracts tested showed no negative effect on the proliferation of PC12 cells. A. absinthium extract increased the duration of pentobarbital-induced sleep at doses of 100 and 200 mg/kg ($P < 0.01$-$P < 0.001$). Similarly, AQF, EAF, and NBF at 200 mg/kg could increase sleep duration ($P < 0.05$). The sleep latency was decreased by A. absinthium extract at doses of 100 and 200 mg/kg ($P < 0.05$-$P < 0.01$), AQF ($P < 0.05$), and EAF ($P < 0.05$). Besides, flumazenil reversed the hypnotic effect of A. absinthium extract ($P < 0.05$). Conclusion. A. absinthium extract probably demonstrated sleep-enhancing effects by regulating GABAergic system.

1. Introduction

Insomnia has a negative impact on the quality of life leading to impairment in memory, mood disorders, and longer reaction [1–3]. Controlled-release melatonin, doxepin, z-drugs (zolpidem, eszopiclone, and zaleplon), and the orexin receptor antagonist suvorexant are major drug classes used to treat insomnia. Although these hypnotic drugs may improve sleep quality, they are associated with unwanted side effects [4]. The use of herbal products, as one element of complementary and alternative medicine, is increasing globally [5]. Artemisia absinthium (A. absinthium) (Wormwood) is an important perennial shrubby medicinal plant that is globally distributed in Europe, Asia, Middle East, and North Africa [6]. A. absinthium is named by numerous common names. It is named as wormwood, green ginger, absinthe, absinthium, in English; Genepi in Latin; Absinth, Wermut in German; Damseeh and Afsanteen in Arabic [6]. A. absinthium is employed in folk medicine as a febrifuge, antiseptic, antispasmodic, anthelmintic, cardiac stimulant, for repairing deteriorating mental function and memory improvement [7, 8]. Recently, it has shown various pharmacological effects including neuroprotective [9], antidiabetic [10], and hepatoprotective [11] properties. Other Artemisia species such as A. persica showed anxiolytic [8] effects. A. absinthium contains compounds such as terpenoids (e.g., linalool), flavonoids (e.g., quercetin) that these constituents have been reported to have hypnotic effects.
2. Material and Methods

2.1. Reagents and Chemicals. Pentobarbital sodium, flumazenil, 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and penicillin-streptomycin were bought from Sigma (USA). Dulbecco’s Modified Eagles Medium (DMEM), fetal bovine serum (FBS), and other cell culture material were bought from Gibco Life Technologies (USA). Diazepam was obtained from Chemidarou Company (Iran). Tween 80 was purchased from Merck (Germany).

2.2. Plant and Extract. Aerial parts of *A. absinthium* were collected from the Allah Akbar mountain (Khorasan Razavi Province of Iran). The plant was identified in the Herbarium of Khorasan Razavi Agricultural and Natural Resources Research Center. A voucher specimen (No. 11856) has been deposited in this institute. The powder of the aerial parts of *A. absinthium* was percolated with ethanol 70% for 72 hr.

Extraction for three fractions, namely, *n*-butanol fraction (NBF), ethyl acetate fraction (EAF), and aqueous fraction (AQF). The yield of AQF, NBF, and EAF was 55%, 18%, and 27%, respectively. *A. absinthium* fractions were dried on a water bath. The *A. absinthium* extract and its fractions were dissolved in normal saline comprising 1% Tween 80 [16].

2.3. Animals. Male albino mice weighing 25–35 g were maintained at 22 ± 1°C with 12 hr of light and 12 hr of dark and had free access to water and food. Experiments were carried out in accordance with the ethical guidelines approved by the Animal Care Use Committee of Mashhad University of Medical Sciences (981134).

2.4. Grouping and Treatment. A total of 66 adult male albino mice were divided into 6 groups as below:

- **Group 1**: Control (normal saline)
- **Group 2**: Positive control or Diazepam (3 mg/kg)
- **Groups 3–6**: Treatment group or different doses of *A. absinthium* extract (25, 50, 100, and 200 mg/kg)
- **Groups 7–9**: Treatment group with AQF, EAF, and NBF (200 mg/kg)
- **Group 10**: Diazepam (3 mg/kg) plus flumazenil (2 mg/kg)
- **Group 11**: *A. absinthium* extract at a dose of 200 mg/kg plus flumazenil (2 mg/kg)

2.5. All Agents Were Administered Intraperitoneally. Thirty min after the intraperitoneal administration of the agents, pentobarbital (30 mg/kg IP) was administered to induce sleep. Time elapsed between the administrations of pentobarbital until loss of righting reflex and immobility was considered as the sleep latency, while the time from the loss to its recovery was considered the sleeping time. Mice were given flumazenil (2 mg/kg) 30 min before diazepam or *A. absinthium* extract administration [17].

2.6. Toxicity Assessment. The possible neurotoxicity of *A. absinthium* extract and its fractions was tested on PC12 cells. Cells were seeded in 96-well plates in DMEM culture medium supplemented with 10% FBS and 1% penicillin streptomycin. After 24 hours, the culture medium was changed to fresh one having vehicle (1% Tween 80) or *A. absinthium* extract (200, 400, and 800 µg/mL) and its fractions (800 µg/mL). After 24 hours, the MTT (0.5 mg/mL in final volume) was added to culture medium of each well and incubated for 2 hours. Afterwards, the medium was discarded and DMSO was added to each well to dissolve any formazan crystalsformed. The absorption of dye was measured at 570 nm [15]. For the determination of LD50, a series of doses of *A. absinthium* extract were administered intraperitoneally to mice. The number of deaths in each group within 24 hours was recorded. The maximum dose which did not cause death and the minimum dose causing death of one animal were noted. The average of these two doses was defined as the median lethal dose [18].

2.7. Data Analysis. All results are presented as mean ± SEM. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tamhane’s T2 post-hoc test. Values with *p* < 0.05 were considered as statistically significant.

3. Results

3.1. Assessment of Toxicity. The highest dose of *A. absinthium* extract that did not result in death in any mouse and the lowest dose that resulted in death in one mouse were 1.6 and 3.2 g/kg, respectively. The LD50 value of *A. absinthium* extract was 2.4 g/kg. Results showed no significant decrease in the proliferation of PC12 cells at the concentrations of 100, 200, 400, and 800 µg/ml of the extract and 800 µg/ml of the fractions (Table 1).

3.2. Effects of *A. absinthium* Extract on Pentobarbital-Induced Sleeping Behaviors in Mice. The results show that diazepam (3 mg/kg) and *A. absinthium* extract (100 and 200 mg/kg) significantly increased the sleep time compared with the control group (*P* < 0.001, *P* < 0.01, and *P* < 0.001, resp.). Flumazenil (2 mg/kg) and *A. absinthium* extract (200 mg/kg) significantly reversed the effects of the diazepam (3 mg/kg) (*P* < 0.001) (Table 2).

As illustrated in Table 3, diazepam (3 mg/kg) and *A. absinthium* extract (100 and 200 mg/kg) significantly
reduced the sleep latency compared with the control group (P < 0.001, P < 0.05, and P < 0.01, resp.). Flumazenil (2 mg/kg) and A. absinthium extract (200 mg/kg) significantly reversed the augmented effects of diazepam (3 mg/kg) in pentobarbital-treated mice (P < 0.001 and P < 0.05, respectively) (Table 3). Effects of A. absinthium extract fractions on pentobarbital-induced sleeping behaviors in mice are as follows:

The results show that all fractions (200 mg/kg) significantly increased the sleep time compared with the control group (P < 0.05) (Table 4). EAF and AQF (200 mg/kg) significantly reduced the sleep latency compared with the control group (P < 0.05) (Table 5).

4. Discussion

Based on experimental result, A. absinthium extract reduced the sleep latency and augmented the total sleep in pentobarbital-induced sleeping mice. Previous studies have shown that a wide range of herbal medicine ingredients including alkaloids, flavonoids (e.g., quercetin and luteolin), terpenoids (e.g., linalool), and steroids have hypnotic properties [12]. A. absinthium contains numerous phytochemical ingredients, namely, lactones, terpenoids (e.g., γ-terpinene, 1,4-terpinol, trans-thujone, cadinene camphene, bornyl acetate, trans-sabinyl acetate, myrcene, guaiazulene, chamazulene, camphor, and linalool), essential oils, tannins, organic acids, resins, and phenols. The plant also has flavonoids (e.g., quercetin), flavonoid glycosides such as quercetin-3-O-D-glucoside, isorhamnetin-3-O-rhamnose glucoside, quercetin-3-O-rhamnoglucose, isorquercitin, and isorhamnetin-3-O-glucoside, and phenolic acids (coumaric, chlorogenic, syringic, salicylic, and vanillic acids) [6, 19, 20]. Benzodiazepines such as diazepam enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the GABAA receptor and thereby increase pentobarbital-induced sleeping time [21]. Indeed, GABA opens the Cl– channels accompanied by a membrane hyperpolarization that cause CNS depression and sedative and hypnotic action [22, 23]. Therefore, drugs that affect these systems can be considered in the treatment of insomnia. Accordingly, we observed that pretreatment of mice with flumazenil (as the antagonist of GABAA benzodiazepine receptor) reduces the prolonged sleep effect of diazepam. Furthermore, we found that flumazenil reverses the hypnotic effect of A. absinthium extract. We prepared three fractions from A. absinthium extract: the AQF, which contains solubilizing the polar agents and water-soluble plant ingredients (e.g., glycosides, tannins, and quaternary alkaloids); EAF, which contains ingredients with intermediate polarity; and the NBF, which contains nonpolar agents like sterols, alkanes, and some terpenoids [14]. In our study, all fractions augmented sleep duration, suggesting that the extract and its various fractions may have bioactive constituents with hypnotic property. To evaluate the fact that the hypnotic effect is associated with no negative impact on neurons, we utilized PC12 cells. No cytotoxic effect for A. absinthium

| Table 1: Effects of A. absinthium on viability of PC-12 cell. Values are expressed as the mean ± SEM (n = 4). |
|---------------------------------------------------------------|
|                   | Saline | Extract 200 | Extract 400 | Extract 800 | EAF 800 | NBF 800 | AQF 800 |
|-------------------|--------|-------------|-------------|-------------|---------|---------|---------|
| 100.0 ± 2.198     | 93.75 ± 1.315 | 93.25 ± 1.797 | 95.00 ± 1.080 | 94.00 ± 1.472 | 96.00 ± 0.7071 | 93.25 ± 1.377 |

| Table 2: Effects of A. absinthium extract (25, 50, 100, and 200 mg/kg) or diazepam (3 mg/kg) on duration of sleep. Values are expressed as the mean ± SEM (n = 6). **p < 0.01 and ***p < 0.001 vs. saline; ###p < 0.001 vs. the same group without flumazenil (2 mg/kg). |
|---------------------------------------------------------------|
|                   | Saline | Diazepam | Diazepam + flumazenil | Extract 25 | Extract 50 | Extract 100 | Extract 200 | Extract 200+ flumazenil |
|-------------------|--------|----------|------------------------|-----------|-----------|-----------|-----------|------------------------|
| 21.33 ± 1.909     | 40.33 ± 1.022 | 22.00 ± 0.6831### | 22.83 ± 0.9458 | 25.67 ± 1.687 | 30.67 ± 2.275 ** | 32.50 ± 1.176 *** | 22.67 ± 1.453 ### |

| Table 3: Effects of A. absinthium extract (25, 50, 100, and 200 mg/kg) or diazepam (3 mg/kg) on the sleep latency. Values are expressed as the mean ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001 vs. saline; #p < 0.05, ###p < 0.001 vs. the same group without flumazenil (2 mg/kg). |
|---------------------------------------------------------------|
|                   | Saline | Diazepam | Diazepam + flumazenil | Extract 25 | Extract 50 | Extract 100 | Extract 200 | Extract 200+ flumazenil |
|-------------------|--------|----------|------------------------|-----------|-----------|-----------|-----------|------------------------|
| 8.833 ± 0.4773    | 3.667 ± 0.2108 | 8.333 ± 0.5578 ### | 8.667 ± 0.5578 | 7.833 ± 0.4773 | 5.833 ± 0.8333 * | 5.333 ± 0.7149 ** | 8.5 ± 0.6# |

| Table 4: Effects of aqueous fraction (AQF), ethyl acetate fraction (EAF), or n-butanol fraction (NBF) of A. absinthium extract on the duration of sleep. Values are expressed as the mean ± SEM (n = 6). *p < 0.05 vs. saline. |
|---------------------------------------------------------------|
|                   | Saline | EAF | NBF | AQF |
|-------------------|--------|-----|-----|-----|
| 21.33 ± 1.909     | 28 ± 1* | 28.33 ± 1.16* | 28 ± 1.4* |

| Table 5: Effects of aqueous fraction (AQF), ethyl acetate fraction (EAF), or n-butanol fraction (NBF) of A. absinthium extract on sleep latency. Values are expressed as the mean ± SEM (n = 6). *p < 0.05 vs. saline. |
|---------------------------------------------------------------|
|                   | Saline | EAF | NBF | AQF |
|-------------------|--------|-----|-----|-----|
| 8.833 ± 0.4773    | 5.8 ± 0.9* | 6.5 ± 0.67 | 5.8 ± 0.7* |
extract was found at 24 h period in PC12 cells. Acute toxic study demonstrated the lethal dose (LD (50)) of A. absinthium extract was 2.4 g/kg. Based on these results, A. absinthium possesses hypnotic activities in a relatively safe dose range.

5. Conclusion

Taken together, all the data presented here demonstrate that A. absinthium extract increased hypnotic effects in pentobarbital-treated mice. The GABAergic system might be involved in the mechanisms of these effects. Further studies are needed to support this evidence.

Data Availability

Some data will be available from the corresponding author upon reasonable request.

Disclosure

The results described in this paper were extracted from a medical student thesis proposal.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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