Research Paper

Lactuca sativa seed and Albizia julibrissin flower combination synergistically promote sleep and suppress locomotor activity in mice

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ABSTRACT

Among medicinal plants, Albizia julibrissin and Lactuca sativa have been claimed in traditional Chinese and Iranian folk medicine to have profound hypnotic and anti-anxiety effects. In the present study, hydro-alcoholic extracts of Albizia julibrissin flower (AJE) and Lactuca sativa seeds (LSE) were tested in combination for hypnotic and anxiolytic effects in mice. Elevated-plus maze, locomotor activity meter and ketamine-induced sleep time were used in mice to evaluate the hypnotic effects of these plant extracts. Administration of plant extracts alone did not produce significant anxiolytic effects combination of the extracts however, became profoundly sedative. When used alone, AJE and LSE at doses ranging from 50-400 mg/kg significantly reduced the mobility of animals compared to control group. Ketamine-induced sleeping test showed that AJE at only 400 mg/kg dose has hypnotic effect. When used in combination, the two extracts at doses lower than 400 mg/kg were somehow more effective in causing sedation in animals. The lack of anxiolytic effects by AJE and SLE (alone or in combination) is probably due to their potential sedative properties.

Key words: Anxiety, insomnia, sedative, Albizia julibrissin, Lactuca sativa.

INTRODUCTION

Insomnia and anxiety are the most common psychiatric disorders that cause great suffering for patients and inflicting a huge burden on social resources. The prevalence of anxiety disorders across the world varies from 2.5 to 7% by country and globally an estimated 284 million people experienced an anxiety disorder in 2017, making it the most prevalent mental disorder (Dattani et al., 2018). The statistics for insomnia are even worse. It is estimated that 10 to 30% of the population, some even as high as 50 to 60% suffer from some kind of sleep disorder (Bhaskar et al., 2016). Daily fatigue and drowsiness, memory problems, decreased attention and concentration, absence from work are only some of the problems involved in insomnia (Zailinawati et al., 2012; Zhang et al., 2012). Anxiety can contribute to the development of depression, physical illness, and emotional and psychological symptoms (Richards et al., 2020). Pharmacological therapy is the most commonly used treatment for insomnia and anxiety. Several groups of drugs (synthetic and natural) have been used throughout the history of anxiety and insomnia treatment. Among the synthetic drugs, benzodiazepines and antidepressants have been mostly prescribed with a wide range of side effects and drug interactions. Chronic use of these sedative-hypnotic drugs beyond 4 weeks is generally not recommended because of their various side effects such as ataxia, cognitive impairment, dizziness, and falls, fractures and dementia (Guo et al., 2020). Furthermore, long-term administration of these drugs results in tolerance and dependence (Sys et al., 2020). It is because of these problems that the use of herbal extracts is gaining ground among clinicians as well as patients as an alternative therapy for insomnia and anxiety. Single plant extracts such as Fumaria indica, Passiflora incarnata, Piper methysticum, Hypericum perforatum, Stachys lavandulifolia, Valeriana officinalis, Melissa officinalis, Ocimum basilicum L. are used traditionally in various part of the world to treat nervous
tension, anxiety and to aid sleep (Alambayan and Garg, 2020; Gross et al., 2019; Guadagna et al., 2020; Singh et al., 2013).

However, since stress causes different symptoms such as agitation, anxiety, sleep disorder, etc. a single plant extract might not be the solution for curing this multifactorial illness. Therefore, combinations of two or more plant extracts with different mechanisms of action are an alternative approach to increase the success therapeutic rate. In the European Union, preparations of the above mentioned extracts are available either as single extracts or in combination with other herbal extracts for the treatment of stress symptoms (Keck et al., 2020). Likewise, in the Middle Eastern pharmaceutical markets, plant extracts such as Kava-lava, California poppy, lavender, valerian, passiiflora, Motherwort are abundantly used as a sedative and hypnotic (Devesa et al., 2015). In view of the effectiveness of some herbal products that contain more than one plant extract, the present study was intended to compare the effects of hydro-alcoholic extracts of Albizia julibrissin flower (AJE) and Lactuca sativa seeds (LSE) alone or in combination in a mice model of anxiety and sleep condition. Albizia julibrissin Durazz (Leguminosae) (AJ) is a plant that is widely distributed in Asia. Traditionally, the stem bark of AJ is used as a folk medicine to treat swelling, traumatic injuries, and insomnia (Tang and Eisenbrand, 1992; Wang et al., 2019). Lactuca sativa (LS) belonging to Asteraceae family is an important leafy vegetable known for its medicinal properties. As per traditional knowledge, it is used in the treatment of insomnia, anxiety, neurosis, dry coughs, rheumatic pain, etc (Harsha and Anilakumar, 2012). The whole plant has also been used for the treatment of stomach problems, to stimulate digestion, and to enhance appetite and relieve inflammation (Harsha and Anilakumar, 2012). Considering the wide-ranging activities of LS and AJ, and the high consumption of these plants in Iranian and Chinese folk medicine as hypnotic and sedative, we decided to compare the action of these plants separately and in combination in mice for anxiolytic, sedative, and hypnotic properties.

MATERIALS AND METHODS

Preparation of the plant material

The flowers of AJ were collected from Isfahan province and LS seeds were purchased from PakanBazr (Isfahan, Iran). For the preparation of hydro alcoholic extracts, air-dried and powdered samples of the plants (1500 g) were macerated with ethanol:water (8:2) for 72 h. The extracts were then shaken, filtered, and evaporated in a rotating evaporator at 70°C under reduced pressure to give a residue of 250 and 300 g of AJ and LS, respectively. The residues were dissolved in normal saline for final suitable concentrations.

Folin Ciocalteu test (Determination of total phenolic content)

Total phenolic content was estimated spectrophotometrically using Folin-Ciocalteu reagent as prescribed by Everette et al. (2010). Briefly, the diluted reagent was mixed with plant samples. After 5 min, sodium carbonate solution (20%) was added to the mixture followed by incubation at room temperature for 120 min, subsequently, UV absorbance was measured at 765 nm using a UV-visible spectrophotometer. Total phenolic was quantified by a standard curve obtained from various concentrations of gallic acid (50-500 µg/ml in methanol). The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per gram of dried extract.

Animals

Male Syrian mice (Pasteur institute, Iran) weighing 25-30 g were housed in a cage (six in each cage) and kept in a room with controlled temperature and light. Animals had free access to food and water except during behavioral tests. Cages were cleaned twice weekly, but never on the day of testing. Two hours before experiments, mice were acclimated to the main environment.

Treatment

Mice received a single intraperitoneal (i.p.) injection of drugs (diazepam, 1.5 mg/kg; ketamine 100 mg/kg), plant extracts (different doses), or vehicle (normal saline) and were used for just one test. The treatments were randomized throughout the day, between 08:00 and 13:00 h, to control for diurnal variation in activity. A minimum of six mice was used for each treatment group. All procedures were approved by the ethical committee of the Isfahan University of Medical Sciences, and conducted in accordance with the internationally accepted principles for laboratory animal use and care. Diazepam and ketamine were purchased from Chemidarou Co. Iran, and Rotexmedica Co. Germany, respectively.

Elevated plus-maze

The EPM test was first designed by Montgomery in 1955 and validated and described by Lister later in 1987 (Lister, 1987). This is one of the most widely used tests for measuring anxiety-like behavior and is based on the natural hatred of mice for open and elevated areas. The apparatus comprised two open arms (35×5 cm) and two closed arms (30×5×15 cm) that extended from a common central platform (5×5 cm). The floor and the walls of each arm were wooden and painted back. Testing was conducted in a
quiet room that was illuminated only by a dim light. Mice were injected with different doses of the plant extracts 30 min before their placement on the EPM. To begin a test session, mice were placed on the open arm facing the center of the maze. An entry into an open arm was defined as the animal placing all four paws over the line marking that area. The number of entries and the time spent in the open and closed arms were recorded during a 5-min test period. The percentage of open arm entries (100×open/total entries) was calculated for each animal. Between each trial, the maze was wiped clean with a damp sponge and dried with paper towels.

**Locomotor activity**

The actions of plant extracts on spontaneous locomotor activity were measured automatically by breaking of infrared beams as described in details elsewhere (Rabbani et al., 1995). The units of the activity counts were arbitrary and based on the beam breaks by movement of mice. Each mouse was injected with the plant extract, diazepam or vehicle (normal saline) and then, after 30 min interval, placed in a novel cage in the infrared apparatus. The locomotor activity was measured at 5 min interval for the next 15 min.

**Ketamine-induced sleeping time**

The effect of plant extract on ketamine-induced sleeping time was carried out as previously described (Mimura et al., 1990). Ketamine was injected intraperitoneally at 100 mg/kg to induce sleep in mice. Mice were pre-treated with vehicle, different doses of plant extracts and diazepam 30 min prior to administration of ketamine. The interval between the administrations of ketamine until the loss of the righting reflex was recorded as onset of sleep. While the time from the loss to regaining reflex as the duration of sleep. Diazepam was used as standard drug. Minimum of six mice were used for each treatment group.

**Statistics**

Statistical analysis was performed using One-Way Analysis of Variance (ANOVA) with post-hoc Tukey test. P<0.05 was considered significant. All data are expressed as mean ± SEM. Also, if necessary, Student t-test was used to compare between two groups.

**RESULTS**

**Gallic acid standard curve**

Using the standard curve of gallic acid, the total phenolic content of the AJE and LSE were found to be 75.72 and 77.32 mg GAE/gr of dried plant extract, respectively.

**Elevated plus maze (EPM)**

As shown in Figure 1, diazepam at 1.5 mg/kg significantly increased the time spent (Figures 1A and 1B), and the percentage of entries (Figures 1C and 1D) to the open arms (P<0.05). Various doses of the plant extracts (6.25–400 mg kg) were tested to determine the minimum effective dose. The AJE and LSE only at a dose of 200 mg/kg significantly increased the time spent on the open arms by 99.5%, 69.9%, respectively (Figures 1A and 1B, P<0.05). Other doses of the plant extracts had no effects on the measured EPM parameters. Administration of the plant extracts at doses ranging from 6.25-400 mg/kg, led to a decrease in the number of entries. This attenuation was significant at 100 and 400 mg/kg (P<0.05, Figures 1C and 1D). At the rest of the tested doses, the general trend of effects was a decrease in movement into either arm of the apparatus.

**Locomotor activity**

As shown in Figure 2, diazepam at 1.5 mg/kg also decreased (by 84.24%) the total locomotor activity (P<0.05, Figures 2B and 2C). Four doses (50, 100, 200, and 400 mg/kg) of the AJE and LSE were tested for their effects on locomotor activity. Figure 2A shows the locomotor activity counts measured at three-time intervals of 5, 10, and 15 min. All doses of the plant extracts significantly decreased the locomotor activity at three-time intervals (Figure 2A). The cumulative locomotor activity counts that measure the total activity during 15 min recording, showed that AJE at 50, 100, 200, and 400 mg/kg doses significantly decreased the activity by 96.6%, 97.7%, 99.3%, 99.1%, respectively (P<0.05, Figure 2B). In a similar fashion, the LSE at 50, 100, 200 and 400 mg/kg significantly decreased the cumulative locomotor activity by 85%, 87.2%, 93.5%, 91.1%, respectively (Figure 2C, P<0.05).

**Ketamine-induced sleeping time**

The effects of AJE and LSE alone and in combinations were evaluated for their action on ketamine. As shown in Figure 3, diazepam at 1.5 mg/kg caused a significant increase in total sleep time (100.1%) (P<0.05, Figure 3). When injected 30 min before ketamine, AJE only at doses of 200 and 400 mg/kg significantly increased the total sleep time by 40.2% and 235%, respectively, compared to the control group (P<0.05, Figure 3). In a similar fashion but less prominent, LSE at doses of 200 and 400 mg/kg caused a significant increase (47.5% and 63.2%, respectively) in the total sleep time (P<0.05, Figure 3). In combinational studies, AJE was
used at 25, 50, and 400 mg/kg and LSE at 50, 100, 200, and 400 mg/kg. When AJE/LSE at doses of 25/50, 25/100, 25/200, and 25/400 mg/kg were tested only a 25/400 dose proved to be significantly different from control values (Figure 3, P<0.05). We then increased the dose of AJE to 50 and repeated the same experiment. AJE/LSE at 50/50, 50/100, 50/200 and 50/400 mg/kg significantly increase the total sleep time by 32%, 69.5%, 63.6%, 43%, (P<0.05, Figure 3). Finally, the dose of AJE was raised to 400 mg/kg and LSE tested at 50, 100, 200, and 400 mg/kg. AJE/LSE at doses of 400/50, 400/100, 400/200, and 400/400 mg/kg caused a significant increase in sleeping time by 180%, 160%, 210%, and 157%, respectively (P<0.05, Figure 3).

**DISCUSSION**

In the present study, we used the EPM model of anxiety to evaluate the anxiolytic effects of the hydro-alcoholic extract of AJ flower and LS. This is a model that uses the natural fear of rodents to avoid open and elevated places. As expected, diazepam produced significant increases in open arm time and the number of entries into the open arms. These data are in agreement with the results of other studies, where diazepam and other benzodiazepines have shown to produce robust anxiolytic effects in a variety of anxiolytic screening procedures, including conflict model, EPM procedures, and other no punishment procedures (Golda and Petr, 1989; Panhelainen and Korpi, 2012; Rabbani et al., 2008). The AJE and LSE only at a dose of 200 mg/kg exhibited anxiolytic-like activity by increasing the time stayed in the open arms. Since the lower dose or higher doses of the plant extracts did not change the EPM parameters, the effect observed at 200 mg/kg could be due to other actions of the extracts such as sedation and muscle relaxation. Data from the number of entries proves such actions. The number of entries into the open arms not only was not increased, but there was attenuation in all tested doses. The marked sedation in animals could be the reason for not being able to travel between the arms of the apparatus. Lack of sensitivity of the method in detecting the anti-anxiety action could be another reason for not seeing clear anxiolytic action by these plants. The sensitivity of the EPM test is thought to be correlated to the compounds that act through GABA receptors, a conclusion that fully justifies the anti-anxiety effect of diazepam (Borsini et al., 2002).

In the locomotor activity test, four doses of plant extracts were evaluated. The locomotor activity measured at three-
Figure 2: Effects of hydro-alcoholic extracts of *Albizia julibrissin* flower and *Lactuca sativa* seeds on (A), spontaneous locomotor activity during the total 15 min of testing, (B and C), spontaneous locomotor activity during three 5-min intervals. The locomotor activity counts (mean ± SEM) were measured over a 15-min period, beginning 30 min after the administration of normal saline, diazepam and different doses of plant extracts. Data are presented as mean ± SEM for each group of six mice. ***P< 0.001 compared to control group.
time intervals was enormously suppressed by all four doses of the plant extracts. Cumulative locomotor activity (total counts for 15 min), was also greatly decreased by AJE and LSE, even to a greater extent than diazepam. These effects further prove the great relaxing action of the plant extracts. In ketamine-induced sleeping time, administration of either plant extracts did slightly lengthen the sleeping time. Interestingly, when the extracts were administered together, the effect was huge and incomparable to the individual action of the extracts. Similar work has been done on another genus of the plants. For example, in phenobarbital sleeping tests, separate administration of aqueous extract of FlosAlbiziae flower and lactuca sativa seed oil has been reported to potential sleeping time (Ghorbani et al., 2013; Wesołowska et al., 2006; Ye et al., 2015). The genus of the plants and the drug used to induce sleep were different in these studies, and more importantly, the combination of these two plant extracts was not tested before. This synergistic action of plant extracts that we observed, suggests that the combination of AJE and LSE may be more desirable for the treatment of insomnia. These data clearly show a marked sedative effect, with virtually no anxiolytic action at the tested doses, a behavior somehow similar to drugs such as zolpidem (Maroo et al., 2013). It is hard to tell from the current data what are the possible mechanisms underlying the hypnotic effects of AJE and LSE. Unlike synthetic drugs with known mechanisms of action, medicinal herbs normally contain a myriad of chemicals. Without knowing every active constituent of these herbs it is impossible to determine the exact mechanism of action.

Among many other constituents, lactucopicrin and lactucin are thought to be the major compounds in the lettuce responsible for many of its CNS-related actions (Wesołowska et al., 2006). Unlike lactucopicrin, lactucin is presumed to be the predominant substance exhibiting the sedative property including the sleep-promoting effect. In lettuce, phytol, a diterpenoid isolated from the ethanol fraction of lettuce was found to raise the levels of gamma-aminobutyric acid, a sleep-promoting neurotransmitter, in the brain (Ghorbani et al., 2013). Previous data have shown that quercetin is a major flavonoid constituent of Albizia julibrissin Durazz flowers. The active ingredients of AJE namely, quercetin, and isoquercetin have been shown to dose-dependently increase the hypnotic activity of pentobarbital sleep time (Ye et al., 2015). In the present study, the separate administration of plant extracts did not affect the ketamine-induced sleep time as much as the co-administration of the plants did. The plant extract may work through receptors other than the NMDA that ketamine is antagonizing. Although most constituents of these plant extracts are identified, it is impossible to single out the individual ingredient responsible for the pharmacological action. Reports state that the ethanol
extract of LS is rich in sesquiterpene lactones, carotenoids, polyphenols (like vanillin, epicatechin, chlorogenic acid, etc.), and jubiloside C1 (Harsha and Anilakumar, 2012). The latter is a saponin-containing compound from the stem bark of A. julibrissin (Jung et al., 2013). Reviewed articles suggest that these compounds may be responsible for the anti-anxiety effect of both plants and are probably present in small amounts in the flowers of AJE and seeds of LSE (Harsha and Anilakumar, 2013; Jung et al., 2013). Sedative action of A. julibrissin is thought to be attributed to flavonol glycoside (Kang et al., 2000).

CONCLUSION

In conclusion, this study showed that although the separate administrations of AJE and LSE have hypnotic effects and prominently suppressed locomotor activity, when they were combined, the effect was quite different. The combination of AJE/LSE at a minimum dose of 25/400 mg/kg showed a greater hypnotic effect than diazepam at 1.5 mg/kg. Regardless of the mechanisms involved, we can conclude that the combination of these two herbs is an acceptable alternative to benzodiazepines or zolpidem in the short-term treatment of insomnia. Further studies are underway to evaluate decisively the effectiveness and safety of this polyherbal combination in insomnia.

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REFERENCES