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Cocculus laurifolius leaf extract in
mice**

Anxiolytic and hypnotic effects of *Cocculus laurifolius* leaf extract in mice

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Abstract

The present study aims to assess the anxiolytic and hypnotic activity of ethanolic extract of *Cocculus laurifolius* leaf in mice. Anxiolytic activity was observed using behavioral paradigms while hypnotic activity was assessed by sodium pentobarbital induced hypnosis in mice. Animals were treated with the extract at dose 200 and 400 mg/kg orally for 30 days. Anxiolytic and hypnotic effect were observed on day 1, 7, 15 and 30 of treatment. Significant increase in the number of entries and time spent in open arm were observed in elevated plus maze. Similar results were observed in light/dark, open field and hole board test. The anxiolytic effect was substantially greater at dose 200 mg/kg in comparison to dose 400 mg/kg. Minimal hypnotic activity was observed with decrease in onset and prolongation in duration of sleep at dose 400 mg/kg, while dose 200 mg/kg did not produce any hypnotic effect. These results showed that the ethanolic extract of *C. laurifolius* leaf might have potential anxiolytic and hypnotic activity in mice.

Introduction

Approximately 450 million of the world population suffers from the mental and behavioral disorders, and according to WHO health report this ratio will increase up to 15% by 2020 (Reynolds, 2003). Globally most common mental disorders included anxiety and insomnia.

Anxiety is the most common psychiatric illness characterized by feeling of anxiousness, fear, apprehension and nervousness, accompanied with symptoms of sympathetic activity like elevated pulse rate, decrease in salivation and augmented muscle activity. Gamma aminobutyric acid (GABA) system is the widely accepted mediator that plays a central role in the pathophysiology of anxiety. Agents such as benzodiazepines, barbiturates, azapirones act as an allosteric modulator and direct agonist of GABA system, while certain anticonvulsant (valproate, gabapentin, pregabalin, and vigabatrin) increase brain GABA level by targeting

metabolic pathway (Bandelow et al., 2012).

Sleep plays an important role in the regulation of central nervous system and body physiological parameters. However, the disruption in sleep leads to dysregulation of neurotransmitters. Glutamate and GABA are primarily involved in the pathophysiology of insomnia. Different pharmacological agents like benzodiazepine hypnotics and zolpidem, monoamine oxidase inhibitors, antihistamines, barbiturates are commercially available and proved to be clinically effective for the treatment of insomnia (Meyerhoff et al., 2014).

In spite of clinical significance of anxiolytic and hypnotic drugs, the major complications occur with these agents are their susceptibility to adverse effects and dependency. These considerations provide evidence for the development of new agents with less adverse effects (Predictable SEAU, 2006). In the search for a novel drug product for the treatment of psychiatric illness, large number of medicinal plants such as *Areca catechu* (Dar



and Khatoon, 1997), *Capsicum annuum* (Jawad et al., 2017), *Crocus sativum* (Hosseinzadeh and Noraei, 2009), *Hypericum perforatum* (Kumar et al., 2000), *Melissa officinalis* (Akhondzadeh et al., 2003), *Morus alba* (Yadav et al., 2008), *Nigella sativa* (Raza et al., 2007), and *Ziziphus spina-christi* (Setorki, 2016) have been investigated for their therapeutic potential in variety of animal models.

Cocculus laurifolius (also known as laurel leaf snail tree) is an evergreen shrub or small tree of 1-2 m tall, belongs to the family Menispermaceae. The phytochemical analysis of leaves extract confirms the presence of phenolic compounds, flavanoids and tannins, responsible to produce antimicrobial and anti-oxidant activity. It has been established that water and ethanolic extracts of *C. laurifolius* possess selective biocide activity against several microorganisms (Ajaib et al., 2017). The ethanolic extract of leaves leads to the isolation of quaternary bases of the plant cocculidine and cocculine that exhibit hypotensive activity in dogs, cats and rabbits. While the quaternary aporphine bases O-methylisocorydine, isocorydine and boldine methochlorides possess considerable ganglion blocking activity on sympathetic and parasympathetic ganglia (Bhakuni, 1984). These aporphines bases act as a neuronal nicotinic acetylcholine receptor blocker (Iturriaga-Vásquez et al., 2007). The major alkaloids of *C. laurifolius*, isocorydine methochloride has been found to have neuromuscular blocking activity (Mukherjee et al., 1984). During the search of secondary metabolites from the ethanolic extract of *C. laurifolius* leaves, tetradrine (benzylisoquinoline alkaloid) was isolated, which is believed to possess the central nervous system effects and also exerts antidepressant effects in animal model (Bhakuni et al., 1980).

However, previously no such study has been reported on the anxiolytic and hypnotic activity of *C. laurifolius*. Hence, based on the evidence of possible central nervous system activity of plant leaves and its alkaloids, we have carried out the effect of ethanolic extract of *C. laurifolius* leaf for potential anxiolytic and hypnotic effects.

Materials and Methods

Collection of plant material

The fresh leaves of *C. laurifolius* were collected from the Botanic Garden GC University, Lahore in the month of October 2017, and was identified by Dr. Zaheer-uddin Khan, Herbarium Department of Botany, GCU Lahore with voucher specimen No. GCU-Herb-Bot 2997, for further reference. For the extraction of *C. laurifolius*, leaves were dried under shade for 15 days.

Preparation of ethanolic extract

After drying, the leaves were grounded and macerated

in ethanol (98% w/v) for 7 days. The solvent was filtered out through the filter paper to remove all debris. The extract was concentrated by the help of rotary evaporator. The concentrated extract of 14.8 g was obtained. This extract was stored at 4°C in an airtight container.

Chemicals

Ethanol was purchased from BDH Laboratory (England); sodium chloride was procured from Sigma Chemical Company (USA). All other reagents and drugs used in the study are of pharmaceutical standards.

Acute toxicity testing

The mice were divided into 5 groups: control, 5, 50, 300 mg and 200 mg/kg (each group contained three mice), where the limit test dose used for the study was 2,000 mg/kg. Food was restricted for mice with free excess to water, a night before performing the test. All mice were weighed prior to dose administration. Doses were prepared by dissolving the extract in distilled water according to 1 mL/100 g body weight of each mouse. Control group was kept on normal saline. All doses were administered via oral intubation. After drug administration, mice were observed for 24 hours for any mortality and toxic symptoms with special observation during initial 4 hours.

Animals

Swiss-albino NMRI, male mice weight (20-30 g) were procured and kept in the Animal house of Hafiz Muhammad Ilyas Research lab (HMI), Hamdard University. The animals were housed under a controlled environment at 50-60% humidity, 22-28°C temperature, and 12 hours light and dark cycle in plexiglas cages, with proper food and water supply. Mice were kept in a laboratory environment for adaptation, an hour before conducting tests. All tests were performed between 9 am to 4 pm, in a clean and noise free area.

Drug administration

Experimental mice were randomly divided into eight groups, each group containing (n=7) animals. Two groups were served as control (0.9% normal saline), four groups were treated with the extract of *C. laurifolius* (200 and 400 mg/kg). Diazepam (1 mg/kg) was used as a reference for both anxiolytic and hypnotic activity. The extract was dissolved in 1 mL distilled water to obtain the final concentration. All respective groups were administered orally via oral intubation, between 8 am to 10 am once daily for 30 days. Behavioral tests were conducted at day 1, 7, 15 and 30.

Elevated-plus maze test

The elevated-plus maze model consists of cross-shaped

platform consisting of two open and two closed arms, 50-70 cm above the ground. Each animal was placed in the middle of the apparatus facing towards the close arm and allowed to freely explore the environment for 5 min. The time spent in the open/close arm, percentage time spent in each arm (open/close), rearing and the total number of entries were recorded on day 1, 7, 15 and 30 (Lister, 1987).

Light-dark box test

The light-dark apparatus consisted of a perspex test box. The box is divided into two compartments, one 27 × 27 × 27 cm high transparent compartment, illuminated by 60-W incandescent bulb while the other black opaque compartment of 27 × 18 × 27 cm high. Both light and dark compartments were connected by a small opening (7 × 7 cm high) (Chaouloff et al., 1997). The procedure was performed in a noise-free dark room. Mice were kept in the room an hour before performing the procedure (Hascoë and Bourin, 1998). Each mouse was placed at the center of the light compartment facing towards the opening to observe the behavior activity for 5 min. The following behavioral parameters were observed: Time spent in each compartment (light/dark), the number of transition in each compartment (light/dark), rearing and the total number of transition in each compartment. All parameters were recorded on day 1, 7, 15 and 30.

Hole-board paradigm

The apparatus was a plexiglass box of 40 × 40 × 30 cm, with four holes equally spaced at 3 cm diameter on the

floor. The experiment was performed in a clean and noise-free area. Mice were divided into four groups stated previously. Each mouse was placed in the center of the apparatus and allowed to explore for 5 min. The total number of head dips, rearing and latency to first head dip were observed.

Sodium-pentobarbital-induced hypnosis

In sodium pentobarbital-induced hypnosis, sleeping time and the onset of sleep were observed by change in the time of drug administration and time of loss of righting reflex, while the duration of sleep was represented by time to recover from righting reflex (Nugroho et al., 2012). On the day of test session, mice of respective groups were administered with normal saline (10 mL/kg), diazepam, 1 mg/kg and ethanolic extract of *C. laurifolius* leaf (200 and 400 mg/kg) via oral intubation. After 30 min of administration, mice were injected with sodium pentobarbital 40 mg/kg intraperitoneally (Mujumdar et al., 2000) and placed in separate cages. The onset of sleep (loss of righting reflex) and the duration of sleep were the observed parameters (Yemitan et al., 2001). Test sessions were conducted on day 1, 7, 15 and 30 of treatment.

Statistical analysis

Data entry and analysis were performed using the Statistical Package for the Social Sciences (SPSS) version 20. All results were presented as mean ± SEM. One-way ANOVA and post-hoc Tukey were used for analysis. A probability value of <0.05 was considered as significant between the control and treated groups.

Box 1: Open Field Test

Principle

Open-field is used to measure locomotion, exploration and anxiety activities simultaneous. The number of line crosses and the frequency of rearing (the mice stood on their hind legs in the maze) are usually used as measures of locomotor activity. The number of central square entries and the duration of time spent in the central square are measures of exploratory behavior and anxiety.

Requirements

Digital camera (Canon A 4000 IS); Red lamp (60-Watt); Mouse; Rectangular arena; Transparent floor

Procedure

Step 1: The open-field is a rectangular arena of opaque acrylic walls of 37 cm in height and transparent floor (80 × 80 × 15 cm)

Step 2: The bottom of the arena is divided into 25 equal squares with dimensions of 16 × 16 cm

Step 3: The apparatus was lightened with indirect light and trial was conducted in a noise free room

Step 4: Each mouse was placed in the center of arena and the number of square crossed with four paws was recorded for 5

min

Step 5: The observed parameters were the number of square crossed (peripheral and central squares) and total number of rearing

Notes

1. Walls may be opaque or clear. The clear walls are preferred for measurement of anxiety, while opaque walls for novel object recognition
2. Walls are easily detachable for easy cleaning
3. Grid floor insert available for video tracking
4. A high frequency of line crosses and rearing indicates increased locomotion and exploration and/or a lower level of anxiety
5. A high frequency/duration of central square entries/duration of time spent indicates high exploratory behavior and low anxiety levels
6. Repeated exposure to the open field apparatus results in time-dependent changes in behavior

References

Archer, 1973; Walsh and Cummins, 1976; Tatem et al., 2014 (<https://bit.ly/2U283fY>)

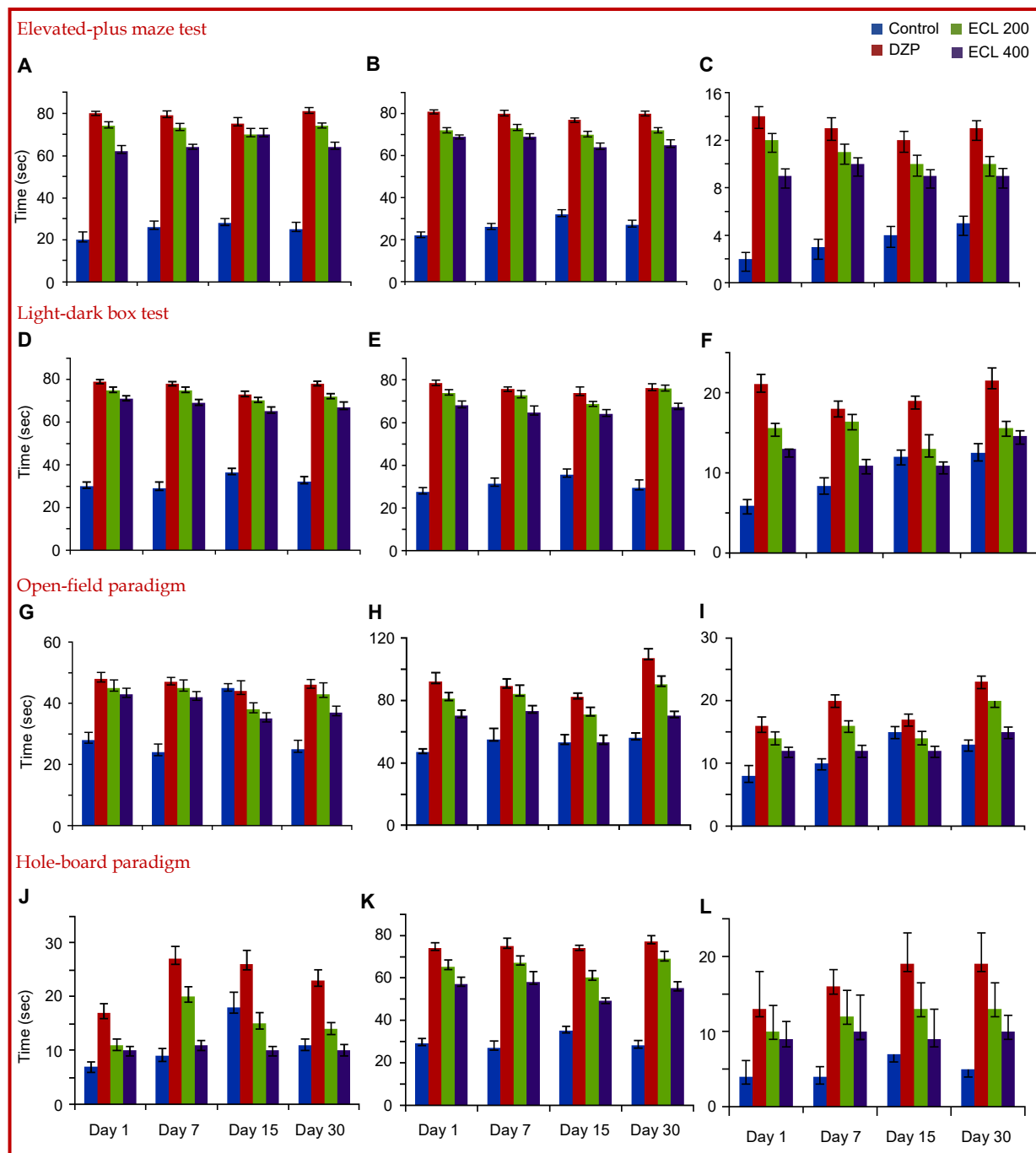


Figure 1: Effect of *Cocculus laurifolius* extract on A) percentage entries in open arm, B) percentage time spent in open arm and C) total number of rearing in elevated-plus maze test; Effect of *Cocculus laurifolius* extract on D) percentage number of entries, E) percentage time spent and F) total number of rearing in light area in light-dark box test; Effect of *Cocculus laurifolius* extract on G) number of center square crossed, (H) number of peripheral square crossed and I) number of total number of rearing in open-field paradigm; Effect of *Cocculus laurifolius* extract on J) latency to first head dip, K) number of head dips and L) total number of rearing in hole-board paradigm; Data expressed as mean \pm SEM; n=7

Results

Acute toxicity test

There are no toxic symptoms and signs of mortality were observed after a single oral dose of 5, 50, 300 and 2,000 mg/kg of extract. The behavioral patterns of mice

were observed for initial 4 hours, followed by 24 hours after administration in the control and treated groups. The treated groups did not show any significant change from the control in behavior patterns, skin and eye coloration, body temperature, food intake and no sign of mortality were observed. All mice showed minimal

Table I					
Effect of <i>Cocculus laurifolius</i> extract on latency time and duration of sleep in sodium-pentobarbital induced hypnosis					
Groups	Doses	Day 1 ^a	Day 7 ^a	Day 15 ^a	Day 30 ^a
Latency time of sleep					
Normal saline	10 mL/kg	7.3 ± 0.3	8.1 ± 0.6	9.9 ± 0.5	9.7 ± 0.5
DZP	1 mg/kg	4.6 ± 0.2 ^c	5.3 ± 0.4 ^c	4.9 ± 0.4 ^c	5 ± 0.3 ^c
ECL I	200 mg/kg	7.9 ± 0.4	8.4 ± 0.4	10.2 ± 0.6	9.9 ± 0.8
ECL II	400 mg/kg	6.3 ± 0.5	6.1 ± 0.4 ^b	6 ± 0.7 ^c	6.1 ± 0.6 ^c
Duration of sleep					
Normal saline	10 mL/kg	28.2 ± 0.4	27.5 ± 0.9	24.2 ± 0.8	26 ± 0.5
DZP	1 mg/kg	42.7 ± 0.7 ^c	42.9 ± 1.1 ^c	41.4 ± 1.2 ^c	44.4 ± 1.4 ^c
ECL I	200 mg/kg	28.1 ± 0.5	28.1 ± 0.5	26 ± 0.4	26 ± 0.5
ECL II	400 mg/kg	34 ± 1 ^c	33.7 ± 1 ^c	34.1 ± 0.5 ^c	32.2 ± 1.1 ^c
Data expressed as ± SEM; n=7; ^a p<0.05; ^b p<0.01; ^c p<0.001 when compared with control groups					

increment in body weight without any significant difference between the control and treated groups. The maximum dose of 2,000 mg/kg did not show any considerable behavioral changes, except, drowsiness and sedation but these effects were diminished later. To further ameliorate the dose level for pharmacological study 1/10th i.e. 200 mg/kg and 1/5th i.e. 400 mg/kg of the (maximum dose 2,000 mg/kg) was selected according to OECD guideline 423.

Elevated-plus maze test

Effect of oral administration of ethanolic extract of *C. laurifolius* leaves and diazepam at day 1, 7, 15 and 30 in the elevated-plus maze were shown (Figure 1A-C). After day 1 treatment with the extract at doses (200 and 400 mg) had the tendency to significantly increase the percentage number of entries in open arm, percentage number of time spent in open arm and the total number of rearing as compare to the control. However, after 30 days of treatment, the extract had significantly increased all parameters of elevated-plus maze except the total number of rearing, which had been slightly reduced at dose 200 mg. The reference anxiolytic drug diazepam at 1 mg/kg, significantly increased the percentage number of entries in open arm, percentage number of time spent in open arm and the total number of rearing in elevated-plus maze model as compared to the control at day 1, 7, 15 and 30.

Light/dark test

The percentage number of entries and time spent in the light area along with the total number of rearing were significantly greater as compared to the control, from day 1 to 30 days of treatment with extract (200 and 400 mg/kg). In diazepam 1 mg/kg, all parameters were relatively increased in the same fashion as that of the extract after 1 to 30 days treatment as compared to the control (Figure 1D-F).

Open field test

The number of center squares crossed had been significantly increased with diazepam 1 mg/kg. Similar effects were observed by ethanolic extract of *C. laurifolius* leaves 200 and 400 mg/kg comparable to the control (Figure 1G-I). Meanwhile, the number of peripheral squares crossed and the total number of rearing were considerably augmented by extract at 200 and 400 mg/kg on day 1, 7, 15 and 30 of test sessions.

Hole board test

Latency to first head dip significantly reduced by ethanolic extract of *C. laurifolius* leaves 200 and 400 mg/kg at 1, 7, 15 and 30th day test session. Similarly, the total number of head dips and rearing showed considerable escalation in comparison to the control. Similar results were observed with diazepam 1 mg/kg (Figure 1J-L).

Hypnotic activity

Ethanolic extract of *C. laurifolius* leaves at dose 200 mg/kg failed to produce a significant increase in the latency time to sleep, while the duration of sleep was also decreased in comparison to the control. While at dose 400 mg/kg, a significant decrease in the latency to sleep and increase in the duration of sleep were observed in comparison to the control. Results obtained from the extract at dose 400 mg/kg were comparable to diazepam 1 mg/kg, to produce hypnotic activity (Table I).

Discussion

In the present study, the ethanolic extract of *C. laurifolius* leaves showed no sign of toxicity and mortality. The behavioral changes were observed after a single oral dose of the extract. However, sedation and

drowsiness were observed at a higher dose but no considerable change in body weight was observed. Generally, the significant change in the body weight is the indication of adverse effect from drug and chemical (Auletta, 1995). Furthermore, no significant change in the body weight and temperature were reported after administration, which postulated that the acute oral extract up to dose 2,000 mg/kg is safe (Adeneye et al., 2006). The present study also provides evidence for the beneficial effect of extract in anxiety and insomnia. The anxiolytic screening test of ethanolic extract of *C. laurifolius* leaves was conducted by exposing mice to a dilemma, whether to explore the new environment or to avoid it due to fear and potential danger (Bourin and Hascoët, 2015). For this purpose, well-reported animal models like the elevated plus maze, light/dark, open field and hole board tests are employed, which have prescient validity of consistent results in standard anxiolytic drugs (Belzung and Griebel, 2001).

In the present study, the ethanolic extract of *C. laurifolius* leaves (200 and 400 mg/kg) produce a significant increase in the number of entries, time spent in open arm and rearing after 1 to 30 days of treatment. While similar results were seen after 30 days of treatment with a slight deviation of results at day 7 and 15. These results were comparable with diazepam (1 mg/kg). According to the previous published studies, the number of entries and time spent in open arm were sensitive to agents that act via GABA-A (gamma amino butyric acid receptor A) receptor complex, while diazepam is among the benzodiazepines that exert anxiolytic properties by directly targeting the GABA-A receptor (Nutt and Malizia, 2001).

Anxiolytic activity of ethanolic extract of *C. laurifolius* leaves (200 and 400 mg/kg) was further evaluated by another animal model light-dark test. In the light-dark test, treated mice showed a significant increase in the number of entries and the time spent in light area at day 1 to 30 treatment as compared to control, which indicates the exploratory behavior of the animal, similar to *Artocarpus lacucha* leaves (Hossain et al., 2010). Rearing was also significantly increased. On the other hand, increase in the number of center squares crossed was observed in the open field test that indicates the decrease in anxiety and contributed as a measure of central exploration. While escalation in peripheral squares crossed and the number of rearing explains the exploratory activity of animal with the reduction in anxiety (Prut and Belzung, 2003). Similar outcomes were observed by extract at dose 200 and 400 mg/kg at day 1, 7, 15 and 30 test session.

In addition to this, a significant reduction in latency to first head dip, with augmentation in head dipping behavior and rearing by ethanolic extract leaves of *C. laurifolius* leaves at dose 200 and 400 mg/kg. The increase in the number of head dips and rearing

narrate the release of anxiety and anxiolytic effect of drug substance (Takeda et al., 1998).

The result from the above tests reveals that ethanolic extract of *C. laurifolius* leaves might have possessed potential anxiolytic activity at both doses (200 and 400 mg/kg), in which 200 mg/kg, is the more potent anxiolytic dose. The mechanism of anxiolytic activity of the extract might be due to the presence of flavonoids, since flavonoids exerts their activity through GABA receptors. Previously many traditional plants like *Morus alba*. leaves (Yadav et al., 2008), *Cissus sicyoides* (Almeida et al., 2009), *Cocculus hirsutus* (Marya et al., 2012) and *Hibiscus rosa sinensis* (Begum and Younus, 2018) have been reported for their anxiolytic effect due to affinity of flavonoids for benzodiazepine receptors (Jäger and Saaby, 2011). Meanwhile, there might be a possible involvement of serotonergic pathway in producing the anxiolytic effect, as serotonin has been primarily implicated in anxiety-like behavior in rodents (Sfinchez et al., 1993). Plants like *Citrus aurantium* (Costa et al., 2013), *Nigella sativa* (Perveen et al, 2009), *Cinnamomum cassia* (Yu et al., 2007) are proved to produce anxiolytic effect via acting on the serotonin pathway. Moreover, the contributing alkaloids of *C. laurifolius*, aporphine and benzyloquinoline have also been reported to produce anxiolytic activity via acting on dopaminergic (Yan et al., 2015), as dopamine receptors play an important role in mediating anxiolytic activity (Zarrindast and Khakpai, 2015). In addition to this, plants like *Ficus carica* (Bhanushali et al., 2014), *Nymphaea lotus* (Fajemiroye et al., 2018) and *Asparagus racemosus* (Singh et al., 2009) have been reported to produce anxiolytic effect by acting on noradrenergic pathway. So, it can be assumed that the anxiolytic effect produce by *C. laurifolius* leaves might be exhibited due to its possible involvement with noradrenergic pathway, as noradrenergic receptors play a significant role in anxiety (Massé et al, 2006). However, further studies are needed to justify this assumption as well as to identify the specific phytochemical constituent and along with endogenous mediator involved responsible to produce anxiolytic activity.

The hypnotic activity of ethanolic extract leaves of *C. laurifolius* leaves was observed by sodium-pentobarbital-induced hypnosis in comparison with diazepam. The extract did not exhibit any hypnotic activity at dose 200 mg/kg, however, dose 400 mg/kg produce minimal hypnotic activity with inhibition in the onset of sleep and prolonged duration of sleep in comparison with control. The hypnotic effect of the extract at dose 400 mg/kg increases with the duration of treatment at day 30, while dose 200 mg/kg does not show any alterations. This indicates that extract might have possessed hypnotic activity at higher doses. It has been reported that prolongation of sleep may be due to hepatic enzyme metabolism and the effect of

pentobarbital on the central nervous system involves activation of inhibitory GABAergic system (Aguirre-Hernández et al., 2007). The prolongation in the duration of sleep by the extract might be due to the involvement of GABAergic system.

According to Bhakhuni et al., (1980) leaves of *C. laurifolius* contains tetrandrine, this compound has been previously reported to increase the hypnotic effect induced by pentobarbital via acting on serotonergic pathway, like ferulic acid (Zhao et al., 2004; Tu et al., 2012). Although, some studies also supported the involvement of saponins in producing hypnotic activity of plant extract (Jiang et al., 2007; Rakhshandah and Hosseini, 2006; Yao et al., 2010). While tannins have also been suggested to produce hypnotic activity by non-specific central nervous system depression via acting on GABAergic pathway (Takahashi et al., 1986). However, further investigations are needed to support these assumptions.

The ethanolic extract of *C. laurifolius* leaves which were previously reported for its antimicrobial, anti-oxidant, neuromuscular and ganglionic blocking activity, has been evaluated for anxiolytic and hypnotic activity. In the light of pharmacological activity of behavioral animal models in mice on acute and repeated treatment, and statistical significance of the results, it has been evaluated that the ethanolic extract of *C. laurifolius* leaves might have potential anxiolytic activity and minimal hypnotic activity at higher dose of 400 mg/kg, while 200 mg/kg fails to produce hypnosis. Previously, different medicinally active plants; *Capsicum annuum* (Jawad et al., 2017), *Citrus aurantium* (Carvalho-Freita, 2002), *citrus limon* (Lopes Campêlo et al., 2011) and *Magnolia dealbata* (Martinez et al., 2006) have also been reported to possess anxiolytic and hypnotic activities on different doses. Furthermore, no signs of toxicity after 30 days of treatment with extract was observed, which reflects the safety profile of extract. These effects may be attributed due to the presence of rich content of flavonoids (Ajaib et al., 2017), alkaloids (erythrina and isocorydine methochloride), aporphines and quaternary bases in ethanolic extract of *C. laurifolius*. However, there is a need for further investigation on the pharmacological activity of these active principals of the plant.

Furthermore, behavioral testing on different doses of ethanolic extract of *C. laurifolius* leaves is needed to enlighten the therapeutic significance of this plant in the treatment of anxiety and insomnia. Moreover, it is also important to study the involvement of neuronal pathway (GABAergic, serotonergic or dopaminergic pathways) by which ethanolic extract of *C. laurifolius* leaves produces anxiolytic and hypnotic effect.

Conclusion

The acute and repeated dosing of ethanolic extract of *C. laurifolius* leaves are effective in the treatment of anxiety and insomnia.

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Ethical Issue

All the research protocol, procedures, euthanasia as well as ethical clearance were approved by the Board of Advanced Studies and Research, Hamdard University, Karachi (No. PS-MP-16-039). The animals used in the study were handled as per specifications described in Helsinki Resolution 1964. The acute oral toxicity test was conducted according to Organization for Economic Co-operation and Development (OECD) guideline 423 on Swiss-albino NMRI male mice of (20-30 g) (Class, 2001).

Conflict of Interest

Authors declare no conflict of interest

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