Original Article

Insignificant effect of cleistanthin A and cleistanthin B on motor function in animal models

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Abstract

Aim: To study the *in vitro* and *in vivo* effects of cleistanthin A and cleistanthin B on the cholinergic receptors and the motor function using mouse as an animal model. **Materials and Methods:** The cleistanthin A and cleistanthin B were isolated from the leaves of *Cleistanthus collinus* using column chromatography and purified. The effect of cleistanthins A and B on locomotor activity, motor co-ordination and grip strength in mice were evaluated using actophotometer, rota-rod apparatus and grip strength test respectively (*in vivo*). The effect of cleistanthins A and B on nicotinic acetylcholine receptor was studied using a mouse esophagus preparation (*in vitro*) and the concentration-response curves of carbachol and cleistanthins A and B were recorded using a data acquisition system (Biopac Inc. USA) through a variable transducer (500 g). **Results:** Cleistanthins A and B did not have any significant effect on locomotor activity, motor co-ordination and grip strength when they were used upto 400 mg/kg BW. Cleistanthins A and B did not show any significant inhibitory effect on nicotinic acetylcholine receptors in isolated mouse esophagus tissue preparation at 1, 3, and 10 μg doses. **Conclusion:** Cleistanthins A and B do not have significant activity on the motor system when used upto 400 mg/kg in mice and also they did not have any effect on nicotinic acetylcholine receptors when used upto 10 μg in the isolated mouse esophagus preparation.

Key words: Carbachol, cleistanthinA, cleistanthin B, nicotinic cholinergic receptor

INTRODUCTION

Cleistanthins A and B are two of the many phytoconstituents of *Cleistanthus collinus* Roxb. (Euphorbiaceae) plant.This plant is commonly used for suicidal and homicidal purposes in Southeast Asian countries including India and Srilanka. *Cleistanthus collinus* leaf extract in animals had shown neuromuscular junctional blocking action and inhibits the muscle contraction by blocking neuromuscular transmission in isolated rodent tissue preparations. The extract also produced myasthenia gravis like effect in animals.[1] It has also been reported in human beings that *Cleistanthus collinus* poisoning was associated with myasthenic crisis-like syndrome.[2] *Cleistanthus collinus* leaf extract contains more than twenty phytoconstituents and so far, it is not known which phytoconstituent is responsible for neuromuscular impairment. The present study was planned to study the effect of two phytoconstituents of *Cleistanthus collinusviz.* cleistanthins A and B on the motor function using mouse as an animal model.

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MATERIALS AND METHODS

Taxonomically identified *Cleistanthus collinus* Roxb. (Euphorbiaceae) plant parts were collected in the region of rural parts of Pondicherry and Villupuram district of Tamil Nadu, India. They were identified

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and certified by the Botanical Survey of India (BSI), Coimbatore (BSI/SC/5/23/08-09/Tech. 241). The leaves of *Cleistanthus collinus* plant were collected in the months of February - April every year. A voucher specimen of the plant is kept in the Department of Pharmacology, JIPMER, for further reference.

Isolation of cleistanthin A and B

Cleistanthins A and B were isolated from the leaves of *Cleistanthus collinus* plant. The acetone extract was obtained from defatted powdered leaves. This extract was dissolved in benzene and passed through a neutral alumina column and successively eluted with benzene, benzene: ethyl acetate (4:1), benzene: ethyl acetate (1:1), and methanol: chloroform (9.5:0.5) to collect the fractions of fatty alcohol, collinusin, cleistanthin A, and cleistanthin B, respectively. The fractions of cleistanthins A and B were purified, and the functional groups and facial arrangements of the molecules were confirmed by spectroscopic analysis.[3,4]

Animals

Healthy, adult, Swiss albino mice of either gender, weighing 20-30 g were obtained from central animal house, JIPMER, Pondicherry, India. The animals were housed in large, spacious, hygienic cages during the course of experimental period. The animal room was well maintained and the animals had 12 + 1 h day and night schedule with a temperature of 23-28°C maintained at standard experimental condition.[5] The animals were fed with standard rodent pellet feed supplied by Amrut laboratory animal feeds, Sangli (India) and water *ad libitum*. The animals were fasted 12 h prior to the experiment with free access to only water. The experimental protocol was approved by the Institute Animal Ethics Committee of JIPMER, Pondicherry, India. All the animal experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Drugs and chemicals

For *in vivo* experiment, cleistanthin A, cleistanthin B and diazepam were suspended in 0.5% carboxymethyl cellulose (CMC) and administered orally. For *in vitro* experiment carbachol (CCh) (Sigma Chemical Co., USA) was dissolved in distilled water and cleistanthins A and B (10 mg/ml) were dissolved in minimum volume of 95% ethanol and the required volume was made up with sufficient quantity of distilled water.

Experimental design

The effect of cleistanthins A and B on locomotor activity, motor co-ordination and grip strength in mice were evaluated using actophotometer, rota-rod apparatus and grip strength test respectively (*in vivo*). The effect of cleistanthins A and B on nicotinic acetylcholine receptor was studied using an isolated mouse esophagus tissue preparation (*in vitro*).

Effect of cleistanthins A and B on motor function

Either gender of Swiss albino mice were used for the experiment. The animals were divided into the eight groups and each group consists of six animals. Group-1 served as control; group-II served as standard drug treatment (diazepam 5 mg/kg; *p.o.*); group-III to V were administered cleistanthin A (100, 200 and 400 mg/kg respectively) and group-VI to VIII were administeredcleistanthin B (100, 200 and 400 mg/kg respectively). Both investigational and standard drugs were suspended in CMC and administered orally in the morning hours. The locomotor activity was tested using an actophotometer at pre-dose and at 0.5, 1, 2, 3, 4, 5 and 6 h after the test drug administration. The total counts in 10 minutes were recorded. Motor co-ordination was tested using a rota-rod apparatus and the time in seconds taken for the mouse to fall from rotating rod was recorded. Grip strength test was performed for each animal individually to test the grip strength. During the experiment the animals were observed for various visual parameters like straub reaction, piloerection, catatonia, loss of righting reflex, ataxia and arching and rolling.[6,7]

Effect of cleistanthins A and B on nicotinic cholinergic receptor

A mouse was sacrificed by a blow on the head and exsanguinated, and the neck region was cut open and esophagus was identified. The esophagus was carefully removed, and transferred to a Petri dish containing Kreb's solution. The tissue was mounted on an organ bath (Biopac Inc., USA) containing Kreb's solution maintained at 32 ± 1°C and areated. The concentration-response curves of ACh was recorded in the absence and the presence of cleistanthin A, cleistanthin B using a data acquisition system (Biopac Inc., USA) through a variable transducer (500 g).[8]

Statistics

The results were presented as mean ± SEM. One-way ANOVA followed by Bonferroni's post-hoc test was used to find out the statistically significant difference between groups. Instat 3.0 version (GraphPad Software, Inc., USA) was used to analyze the data. Microsoft Office Excel 2007 was used to calculate AUC. $P < 0.05$ was considered statistically significant.

RESULTS

Both cleistanthin A and cleistanthin B administered group showed arching and rolling, which was not observed in control and standard drug (diazepam) treatment group. Diazepam treated animals developed grade-1 catatonia 0.5 h after administration and the effect was prolonged upto 5 h, which was not observed in control, cleistanthin A and cleistanthin B treated animals. No animal in the experimental groups had Straub reaction, pilo-erection, and loss of righting reflex or ataxia.

Cleistanthins A and B did not significantly affect the locomotor activity [Figures 1 and 2], motor co-ordination [Figures 3 and 4] and grip strength [Figures 5 and 6] when they were used upto 400 mg/kg BW. Diazepam significantly reduced the locomotor activity, motor co-ordination and grip strength of mice when compared to those in the vehicle treated group.

In isolated mouse esophagus tissue preparation both cleistanthins A and B did not any significant ($P > 0.05$) inhibitory effect on nicotinic acetylcholine receptors at 1, 3, and 10 μg doses.

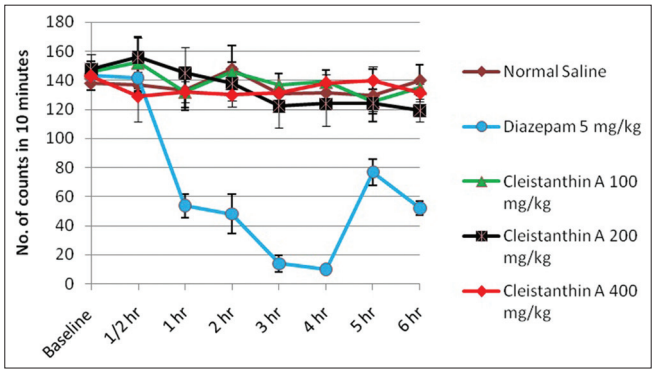

Figure 1: Effect of cleistanthin A on locomotor activity using actophotometer in mice. Values are mean ± SEM, $n = 6$ in each group

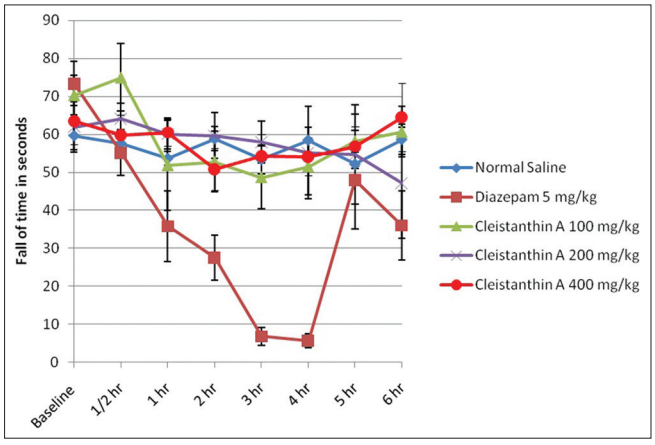

Figure 3: Effect of cleistanthin A on motor co-ordination using rota-rod apparatus in mice. Values are mean ± SEM, $n = 6$ in each group

DISCUSSION

Both cleistanthin A and cleistanthin B were able to produce arching and rolling in micebut both had no significant effect on motor co-ordination, locomotor activity and grip-strength when compared to control group. Arching and rolling is a non-specific sign which only indicates toxicity.[9]

Locomotor activity indicates alertness of central nervous system activity and it can be easily measured using actophotometer instrument. The negative results signify that both the cleistanthins do not have any significant effect on the central nerve system. Motor co-ordination is an important parameter to study muscle relaxation.[7] Cleistanthins A and B do not alter the motor co-ordination indicating they do not exert muscle relaxant activity in mice. Grip strength test is also one of the important tests for muscle co-ordination. This test is mainly used to evaluate the rodents neuromuscular function or muscular strength which may be affected by various drugs like muscle relaxants, sedative drugs

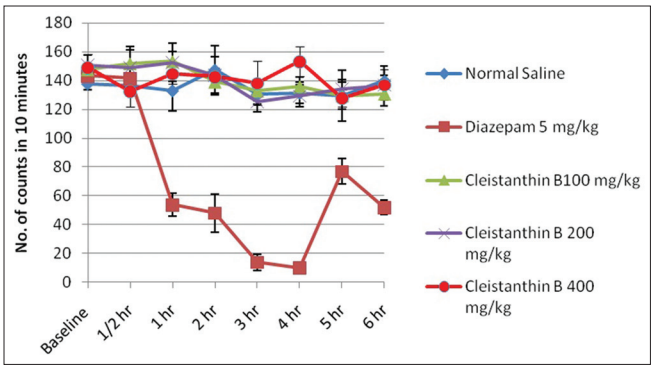

Figure 2: Effect of cleistanthin B on locomotor activity using actophotometer in mice. Values are mean ± SEM, $n = 6$ in each group

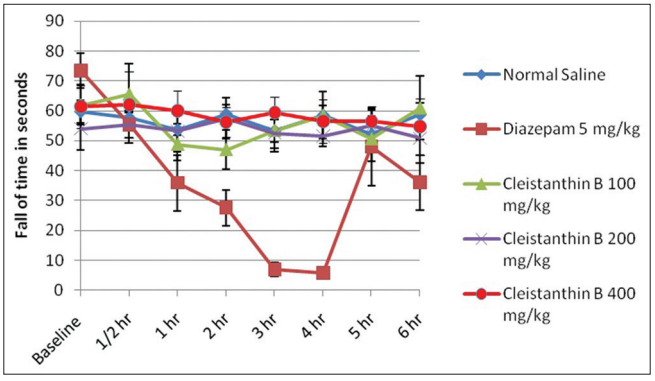

Figure 4: Effect of cleistanthin B on motor co-ordination using rota-rod apparatus in mice. Values are mean ± SEM, $n = 6$ in each group

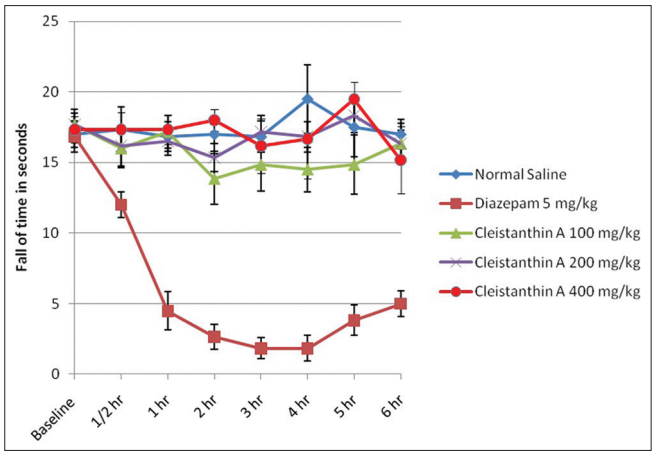

Figure 5: Effect of cleistanthin A on grip strength in mice. Values are mean ± SEM, $n = 6$ in each group

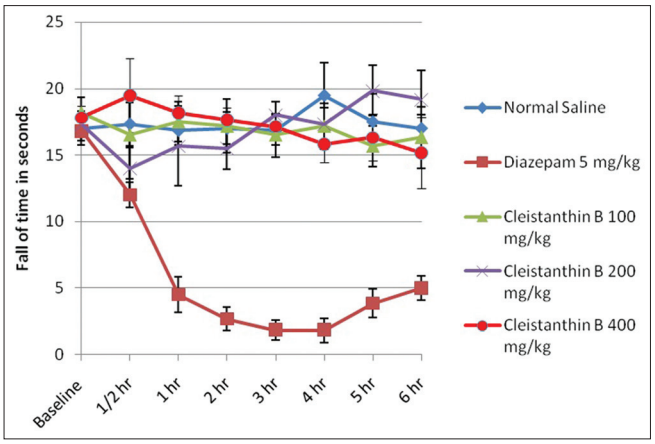

Figure 6: Effect of cleistanthin B on grip strength in mice. Values are mean ± SEM, $n = 6$ in each group

Table 1: Effect of cleistanthins A and B on mouse esophagus in the presence of carbachol

EC_{50} of carbachol (μg)	EC_{50} of carbachol (μg) in the presence of cleistanthin A			EC_{50} of carbachol (μg)	EC_{50} of carbachol (μg) in the presence of cleistanthin B		
	1 μg	3 μg	10 μg		1 μg	3 μg	10 μg
36.14	39.44	46.88	87.39	13.81	54.32	57.63	43.58
51.17	45.23	73.34	47.09	50.19	39.44	48.54	50.54
60.08	81.27	54.78	75.98	42.75	54.32	62.59	65.90
54.32	39.44	113.84	108.88	32.83	46.06	54.32	46.88
50.43±5.11	51.35±10.07	72.21±14.94	79.84±12.87	34.90±7.88	48.54±3.60	55.77±2.95	51.72±4.93

EC: Effective concentration. The values are representing EC_{50} of carbachol for each tissue. The mean±SEM EC_{50} values of carbachol are given in the last row

and toxic substances.[9] Cleistanthins A and B do not alter the grip strength showing that they do not affect the muscular strength or neuromuscular function in mice. From the above findings it can be concluded that cleistanthins A and B do not affect the locomotor activity (alertness of central nervous system activity), motor co-ordination (skeletal muscle relaxation) and grip strength (neuromuscular function or muscular strength) in mice.

Cleistanthins A and B had no significant ($P > 0.05$) inhibitory action on CCh induced contractions in mouse esophagus preparation when they were used upto 10 μg doses [Table 1]. Previous studies have demonstrated the presence of nicotinic acetylcholine receptors in the mouse esophagus and the blockade by different drugs like d-tubocurarine, and pancuronium.[8,10-13] So the model used in our study to evaluate the effect of cleistanthins A and B on nicotinic acetylcholine receptors. However it might be possible that our study did not pick up the actual difference as the number of mouse esophagus preparations used was small i.e., only four for cleistanthins A and B each. Further cleistanthins A and B may have their inhibitory effect on CCh induced contractions in mouse esophagus preparation at doses higher than those used in the present study (10 μg).This needs to be explored further.

Previous studies done on sciatic nerve-anterior tibialis muscle preparations in rat showed that administration of *Cleistanthus collinus* leaf extract led to neuromuscular junctional block[14] and also neuromuscular disorder like myasthenia gravis when administered by intraperitoneal route in rats.*Cleistanthus collinus* leaf extract on isolated mouse phrenic nerve-diaphragm inhibited the muscle contraction by decreasing the excitability of the nerve and muscle membranes and also by neuromuscular transmission blocking action.[15] There was also a report of myasthenic crisis-like syndrome in human beings due to *Cleistanthus collinus* poisoning.[2]

All these studies done on animals and a report on a patient indicate *Cleistanthus collinus* leaf extract impairs motor function. However our study did not find any effect of cleistanthins A and B on motor function or nicotinic acetylcholine receptors at neuromuscular junction. In one of our previousstudies we demonstrated the blockade of cholinergic nicotinic receptor by cleistanthins A and B using the isolated rabbit vas deferens though higher doses of ACh (in milligrams) was required to stimulate the nicotinic receptors.[3] The present study contradicts our

previous finding but it suffers from certain drawbacks as discussed above.

CONCLUSION

From the results it can be concluded that Cleistanthin A and cleistanthin B did not have any significant effect on rodents motor function when used up to 400 mg/kg and have no action on the nicotinic acetylcholine receptors when used up to 10 μg.

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