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Some pharmacological studies with Cycleanine, a diphenylbisbenzylisoquinoline alkaloid from *Stephania hernandifolia*

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SUMMARY

Stephania hernandifolia belonging to the family Menispermaceae is the biggest storehouse of diphenylbisbenzylisoquinoline (DBBI) alkaloids. Exhaustive chemical processing of the bulb of *S. hernandifolia* by the application of modern separation techniques yielded a DBBI alkaloid which was identified as cycleanine using spectroscopic methods (UV, IR, ^{1}HNMR. ^{13}CNMR, Mass). Cycleanine showed significant anti-inflammatory activity against carrageenin induced paw oedema, comparable to that produced by diclofenac sodium, used as standard drug. It exhibited potent analgesic effects against chemical and thermal noxious stimuli. It was also found to possess anti-convulsive activity in the strychnine induced convulsion model.

Key words: *Stephania hernandifolia*; Cycleanine; Analgesic; Anticonvulsant; Anti-inflammatory

INTRODUCTION

Stephania hernandifolia belonging to the family menispermaceae is the biggest store house of diphenylbisbenzylisoquinoline (DBBI) alkaloids which are potent source of medicinal agents used to treat hypertension, malignancy and microbial infection (Seal *et al.*, 1997). The root of this plant which is used for fever, diarrhoea, dyspepsia and urinary diseases (Kirtikar *et al.*, 1984) was chemically processed for the isolation and identification of alkaloids viz. fangchinoline, dl-tetrandrine, d-isochondrodendrine, cycleanine, palmatine, tetrahydropalmatine. In the present study we report some pharmacological activities of cycleanine (**1**).

MATERIALS AND METHODS

Plant materials

Fresh plants with bulbs of *Stephania hernandifolia* (Menispermaceae), collected from Mungpoo, India was identified by Mr. Aloke Bhattacharya, Botanist, Botanical Survey of India, Calcutta. A voucher specimen (No. BM/UCM/008) has been preserved in our laboratory.

Instrumentation

The UV spectrum of cycleanine was recorded on an Hitachi U 2000 spectrophotometer in aldehyde free alcohol. The IR spectrum was taken on a Perkin Elmer 782 spectrophotometer in KBr pellets. The ^{1}HNMR spectrum was recorded in $CDCl_3$ solution on a Bruker AM 300L spectrometer with TMS as internal standard. The Mass spectrum of the compound was kindly supplied by Dr. B. C. Das, Institute de Chimie des Substances Naturelles, Gif-Sur-Yvette, France carried out at 70 ev using direct inlet system. Column chromatography was performed over Silica gel (60-120 mesh), using a mixture of chloroform and methanol of increasing polarity.

Animals

Swiss albino mice (weighing 25-30 g) and albino rats (120-150 g) of either sex were used. The animals were fed standard pellet diet and water

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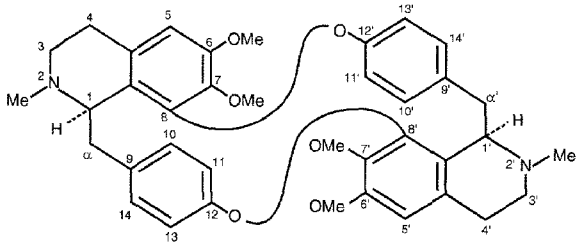
was provided *ad libitum*. The animals were housed in groups of 10 animals for mice and six animals for rat at 25±1°C. The animals were fasted for last 17 hours before the commencement of the experiment.

Drugs

The drugs used were acetic acid, acetylsalicylic acid (disprin), 1% carrageenin (SIGMA), cycleanine, diclofenec sodium, morphine sulphate, strychnine hydrochloride and sodium chloride.

Isolation and identification of cycleanine

Finely ground air-dried roots (1 kg) were powdered and soxhleted with petroleum ether (60-80°C) for 48 h. Defatted roots were then extracted with ethanol: acetic acid (95:5) for fifteen days by percolation. After removal of the solvent, the residual matter (30 g) was churned with 5% citric acid (5×100 ml) and filtered. The acid extract was washed with petroleum ether (40-60°C) and basified with ammonium hydroxide (pH 9.2). The buff colored precipitate was extracted with chloroform (5×100 ml). Chloroform was removed under reduced pressure to obtain the total base fraction (8 g) which was subjected to column chromatography using silica gel (60-120 mesh) as adsorbent and mixture of chloroform and methanol of increasing polarity as solvent. The compound (R_f 0.74, adsorbent: Silica gel G, developer; benzene: chloroform: methanol: :6 : 3 :1, indicator : Dragendorff reagent) that migrated was purified by repeated crystallization with acetone and identified as cycleanine (**1**) (300 mg; yield 0.03%) ($C_{38}H_{42}O_6N_2$, mp. 268-270°C) from spectroscopic analysis and by comparing with authentic sample.



(1)

Cycleanine: Mol. Formula: $C_{38}H_{42}O_6N_2$; mp.: 268-270°C; [α]22: -15.94° ($CHCl_3$) ; UV (nm, EtOH): 213 (4.75), 263 (3.87), 290(3.91); IR (cm^{-1}) : 3500; ^{1}HNMR (300 MHz, $CDCl_3$): 2.56, 3.44, 3.84, 5.75, 6.27, 6.57, 7.02, 7.26; 13 CNMR: 25.0, 37.8, 42.4, 44.9, 56.1, 59.7, 76.9, 109.7, 114.0, 117.3, 126.0, 128.1, 128.7, 129.8, 130.5, 139.3, 143.8, 151.9, 154.3; MS (m/z): 622 (M^+), 621, 313, 312, 311, 204, 190, 174, 159, 146, 145.

Preparation of drug

Cycleanine (100 mg) was dissolved in 20 ml of acetone and 10 ml of methyliodide was added to it and kept in a refrigerator for seven days. The mixture was then filtered and the residue (250 mg) was dried. Methiodide salt of cycleanine thus prepared was freely soluble in water and was taken as drug for pharmacological investigations.

Pharmacological studies

Safety evaluation

The LD_{50} was determined by the standard method of Litchfield and Wilcoxon (Litchfield and Wilcoxon 1949). The drug cycleanine methiodide was administered i.p. to group of 10 swiss albino mice (weight 25-30 g) at doses upto 10 mg/kg. The animals were observed for 24h mortality.

Analgesic activity

The analgesic activity was tested by the following methods:

i) Acetic acid induced writhing (chemical stimulus): Albino male mice were divided into five groups, each group containing ten mice. Writhing response was induced by 1% acetic acid at a dose of 10 ml/kg, i.p (Turner, 1965). The test and the standard drugs were administered 15 minutes prior to i.p. injection of acetic acid. The number of abdominal constrictions (writhings) and stretchings with a jerk of the hind limbs were counted between 5 and 15 minutes after administering acetic acid (Koster *et al.*, 1959). The analgesic effect of the drugs were calculated by the percentage inhibition of writhing episode over that of control group. The results were compared with acetylsalicylic acid (Disprin; 200 mg/kg i.p.)

ii) Tail immersion : The experiment was carried out following the method of Palanichamy (Palanichamy and Nagarajan, 1990). All the mice were screened by exposure to the thermal stimulus.

Those animals showing positive responses within 5 seconds were selected for the experiment.

The mice were divided into four groups each containing 10 animals. The first group received normal saline as control (10 ml/kg, i.p.). The 2nd and 3rd groups received two doses of test drug, i.e., 1 mg/kg and 2 mg/kg, i.p. and the 4th group received one dose of standard drug i.e., 5 mg/kg, i.p. of morphine. The tail (up to 5 cm) was then dipped in a beaker of water at 55±1°C. The cut off time was 30 seconds. The time in seconds taken to withdraw the tail clearly out of the water was taken as the reaction time. The first reading (0 min.) was taken immediately after administration of the agents and then 30, 60 and 90 minutes later.

Anticonvulsant activity

Strychnine antagonism by cycleanine and diazepam was examined by the method of Chen (Chen and Portman, 1952). Albino mice were divided into eleven groups containing 10 mice in each group. The control group of animals received normal saline at a dose of 10 ml/kg i.p. Convulsive responses were produced by strychnine at doses of 1.5 mg/kg (50% convulsion) and 2 mg/kg (100% convulsion). Effect of cycleanine (0.5 to 2.0 mg/kg , i.p.) was studied on strychnine induced convulsions in separate groups of mice. Strychnine induced convulsion was recorded at 5 min and 15 min respectively. Cycleanine was administered 15 min prior to strychnine and the recovery from strychnine induced convulsion for each group of mice (n=10) was recorded at 5 min and 15 min respectively. Accordingly percentage of convulsion was recorded. Similar experiments were performed with diazepam at a dose of 5 mg/kg, i.p.

Carrageenin induced paw oedema in rat

The rats were divided into five groups comprising six rats in each group. In all the groups, acute inflammation was produced by subplanter injection of 0.1 ml of freshly prepared 1% suspension of carrageenin in normal saline in the right hind paw of the rats and paw volume was measured plethismometrically at '0' h and 3h after carrageenin injection (Chattopadhyay *et al.*, 1986; Winter *et al.*, 1962). Animals were treated with saline (10 ml/kg i.p.), cycleanine methiodide (1.0 mg/kg i.p. and 2.0 mg/kg i.p), acetylsalicylic acid (5 mg/kg i.p with 2% gum acacia) and diclofenec sodium (5 mg/kg i.p) 30 min before carrageenin injection. Mean increase in paw volume was measured and the percentage inhibition was calculated according to the following formula (Awe *et al.*, 1998):

$$\text{Percentage inhibition of oedema} = \frac{(Vc - Vt)}{Vc} \times 100$$

Where Vc is control paw volume and Vt is drugs treated paw volume (both standard and test drugs).

Statistical analysis

Values are expressed as Mean±SEM and the significance of difference of data obtained was evaluated statistically using the Student's t test.

RESULTS

Safety evaluation

Acute toxicity tests in mice established the LD_{50} of cycleanine to be 4 mg/kg, i.p.

Analgesic activity

i) Acetic acid induced writhing (chemical stimulus): Cycleanine exhibited a dose dependent and significant analgesic activity in the acetic acid induced writhing test. It was found that, the administration of the two doses (1 mg/kg and 2 mg/kg) of cycleanine showed less significant analgesic activity in the acetic acid induced writhing episode in comparison to acetylsalicylic acid (200 mg/kg). The results are shown in Table 1.

ii) Tail immersion (thermal stimulation): Using thermal stimulation (55±1°C) the two doses of cycleanine (1 mg/kg and 2 mg/kg i.p.) produced an elevation of pain threshold after 30 min followed by progressive diminution of the activity within 90 min. It was also found that morphine (5 mg/kg) alone produced very significant analgesic activity from 30 minutes onward and this effect lasted for more than 80 mins. Morphine was used as standard drug. The results are shown in Table 2.

Anticonvulsant activity

Strychnine at a dose of 1.5 and 2.0 mg/kg i.p. induced toxic type of convulsion with clonus of mice. The degree of convulsion was measured visually. The animals were observed for a period of 5 and 15 minutes during the experiment. The doses

Table 1. Effect of cycleanine and disprin on acetic acid induced writhing episodes in mice

Drug	Writhing episodes for 5 min. (Mean±SEM)	Percentage of inhibitory activity
Normal saline (0.9%, w/v; 10 ml/kg)	-	-
1% v/v acetic acid (10 ml/kg)	25.7±1.03	-
Cycleanine (1 mg/kg) + Acetic acid (10 ml/kg)	15.1±1.37*	41.24
Cycleanine (2 mg/kg) + Acetic acid (10 ml/kg)	06.1±1.07*	76.13
Disprin (200mg/kg) + Acetic acid (10 ml/kg)	1.20±0.54*	95.30

No. of animals used for each group (n=10)
*Denotes significant reduction as compared to control ($p<0.05$).
Route of administration for each treatment was i.p.

Table 2. Effect of cycleanine on pain threshold in thermal-induced pain (55±1°C)

Drug	Mean reaction time (in sec.)±SEM			
	0	30	60	90
Normal saline (0.9%,w/v; 10 ml/kg)	4.7±0.37	4.9±0.26	4.8±0.20	4.75±0.29
Cycleanine (1 mg/kg)	6.5±0.67*	15.5 ±1.97*	12.6±1.86*	6.4 ±0.85
Cycleanine (2 mg/kg)	10.5±2.6*	24.2±2.80*	24.0±3.15*	22.3±3.30*
Morphine (5 mg/kg)	6.5±0.56	>30±0	>30±0	>30±0

No. of animals used for each group (n = 10)
*Denotes significant reduction as compared to control ($p< 0.05$).
Route of administration for each treatment was i.p.

of strychnine hydrochloride (1.5 and 2.0 mg/kg, i.p.) which produced 50% and 100% convulsion respectively were used in this study. Cycleanine at doses 0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg, i.p. protected 70%, 80%, and 80% of mice against strychnine (2.0 mg/kg) induced convulsion only at 5 min. duration. On the other hand diazepam at dose of 5 mg/kg, i.p protected 100% of mice against convulsions induced by 1.5 mg/kg,i.p. of strychnine hydrochloride over a period of both 5 and 15 min. duration. It was observed that different combinations of strychnine hydrochloride and cycleanine in both the duration showed significant protective action against convulsion (Table 3).

Carrageenin induced paw oedema

The anti-inflammatory effect of different doses of cycleanine against carrageenin induced inflammation

Table 3. Effect of cycleanine and diazepam on strychnine induced convulsion in mice

Group	Drug	% Convulsion	% Convulsion (Pretreatment)	
			5 minutes	15 minutes
1	Normal saline (0.9% w/v, 10 ml/kg)	-	-	-
2	Strychnine (1.5 mg/kg)	50	-	-
3	3 Strychnine (2.0 mg/kg) 100	100	-	-
4	Cycleanine (0.5 mg/kg) + Strychnine (1.5 mg/kg)	-	0	20
5	Cycleanine (0.5 mg/kg) + Strychnine (2.0 mg/kg)	-	30	100
6	Cycleanine (1.0 mg/kg) + Strychnine (1.5 mg/kg)	-	10	50
7	Cycleanine (1.0 mg/kg) + Strychnine (2.0 mg/kg)	-	20	100
8	Cycleanine (2.0 mg/kg) + Strychnine (1.5 mg/kg)	-	0	50
9	Cycleanine (2.0 mg/kg) + Strychnine (2.0 mg/kg)	-	20	100
10	Diazepam (5.0 mg/kg) + Strychnine (1.5 mg/kg)	-	0	0
11	Diazepam (5.0 mg/kg) + Strychnine (2.0 mg/kg)	-	0	30

Route of administration for each treatment was i.p.

Table 4. Effect of cycleanine and diclofenec on carrageenin induced rat hind paw oedema

Drug	Paw volume increase after 3 h (ml) Mean±SEM	Percentage of inhibitory activity
Normal saline (0.9% w/v; 10 ml/kg)	0.37±0.06	-
Cycleanine (0.7 mg/kg)	0.19±0.02*	48.60
Cycleanine (1.4 mg/kg)	0.08±0.01*	78.30
Diclofenec (1.0 mg/kg)	0.15±0.03*	59.50
Diclofenec (2.0 mg/kg)	0.06±0.01*	81.08

No. of animals used for each group (n=6)
Denotes significant reduction as compared to control ($p<0.05$).
Route of administration for each treatment was i.p.

is shown in Table 4. The agent significantly reduced the paw volume ($p<0.05$) as compared to the control rats. Diclofenec showed a similar type of reduction ($p<0.05$).

DISCUSSION

Pain and inflammation are an essential prelude to repair process (Bairy *et al.*, 1991). Acetic acid is believed to trigger the production of irritant substances within the peritoneum which cause the writhing response (Bartolinin *et al.*, 1987). Cycleanine depressed the production of irritants and thereby reduced the number of writhings in the animals. However, acetylsalicylic acid (200 mg/kg) reduced the number of writhings in the animals more than the different doses of cycleanine employed. Cycleanine exhibited potent analgesic effects against both chemical and thermal noxious stimuli. This is evidenced by the significant reduction of the analgesic effect produced by it in the tail immersion test.

The convulsant action of strychnine is due to interference with post synaptic inhibition that is mediated by glycine (Ramanjaneyulu *et al.*, 1984). The main site of action of strychnine is on the spinal cord and convulsion occurs after removal of the rest of the nervous system (Sutinski *et al.*, 1964). The appearance of the induced convulsive state is also related to a decrease in gamma amino butyric acid (GABA) content of brain (Killan *et al.*, 1960). A definite correlation of decreased GABA level with a state of convulsion and vice-versa has not been ascertained. Different dose combinations of strychnine with cycleanine and diazepam indicated that cycleanine has potent anti-convulsive activity. Though the exact mechanism is not clear, excessive liberation of GABA from the neural junction by cycleanine in this action is a possibility.

Inhibition of carrageenin-induced inflammation in rats is one of the most suitable test procedures to screen anti-inflammatory agents. The devlopment of carrageenin-induced oedema is biphasic, the first phase is attributed to the release of histamine, 5-HT and kinins, while the second phase is related to the release of prostaglandins (Banerjee *et al.*, 2000). The results of the present investigation suggest that cycleanine has better anti-inflammatory effect than that of diclofenec sodium against carrageenin induced paw-oedema. The study reveals that cycleanine possesses analgesic, anticonvulsive and anti-inflammatory activities. The bioactivity profile of cycleanine needs to be studied further using additional parameters.

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