Acta Poloniae Pharmaceutica – Drug Research, Vol. 68 No. 1 pp. 75–81, 2011 ISSN 0001-6837
Polish Pharmaceutical Society

CNS ACTIVITIES OF ETHANOL EXTRACT OF AERIAL PARTS OF *HYGROPHILA DIFFORMIS* IN MICE

DILIPKUMAR PAL* and KRISHANU SAMANTA

Seemanta Institute of Pharmaceutical Sciences, Jharpokharia, Mayurbhanj -757 086, Orissa, India

Abstract: The ethanol extract of aerial parts of *Hygrophila difformis* (EEHD) was tested for possible pharmacological effects on experimental animals. EEHD significantly potentiated the sleeping time of mice induced by standard hypnotics, *viz*. pentobarbital sodium, diazepam, and meprobamate in a dose dependent manner. EEHD showed significant analgesic properties as evidenced by the significant reduction in the number of writhes and stretches induced in mice by 1.2 % acetic acid solution. It also potentiated analgesia induced by morphine and pethidine in mice. Pretreatment with EEHD caused significant protection against strychnine and leptazol-induced convulsions. The behavioral studies on mice indicate CNS depressant activity of the ethanol extract of *H. difformis*.

Keywords: *Hygrophila difformis*, sleeping time, general behavior, analgesic activity, anticonvulsant activity

Hygrophilla difformis Blume (commonly known as Water Wisteria, family: Acanthaceae) is a tropical aquarium plants used as environmental ornaments. It is very fast growing plant that requires high light and nutrients to get good green growth. Its rapid growth helps prevention of algae. It grows to a height of 20–50 cm with a width of 15 to 25 cm. It has slender lacey leaves and upright growth. It is found in marshy habitats on the Indian subcontinent in Bangladsh, Bhutan, India and Nepal. The plant is used as anticoagulant by tribal people (1). It has also antioxidant properties (2). The aerial parts of *H. difformis* on preliminary chemical analysis are found to contain hygrophiloside (3). The ethanol extract of *H. difformis* (EEHD) showed marked CNS depressant action compared to other extracts of it in preliminary pharmacological screening. However, no work has been reported on the CNS activities of this plant. Keeping this in view, the present study has been undertaken to investigate various CNS activities such as behavioral, sedative-hypnotic, analgesic and anticonvulsant effects of EEHD in mice to substantiate the folklore claim.

EXPERIMENTAL

Preparation of extracts

The aerial parts of *H. difformis* were collected from fields of Midnapur District, West Bengal in the month of December and were authenticated by Dr. M.S. Mondal, Additional Director, Central National Herbarium, Botanical Survey of India, Howrah and West Bengal. A voucher specimen has been preserved in our laboratory for future reference (DPKS1). Shade-dried, powdered, sieved (40 mesh size) plant materials were exhaustively extracted successively with petroleum ether (40–60^{o}C), benzene, chloroform, ethanol and distilled water using a Soxhlet extractor. The extracts were concentrated to dryness in vacuum. The yield of ethanol extract (EEHD) was 9.17% w/w.

Chemical investigation of EEHD

The ethanol extract was subjected to silica gel preparative TLC, where two compounds were isolated using diethyl ether : ethanol : ammonia (1: 0.9: 1 drop, v/v) as solvent system. Compound A (R_f value: 0.71, λ_{max}: 239 nm) having characteristic IR (Perkin Elmer, IR-297) peaks at 3456 cm^{-1} (alcohol, amine, amide group), 1413 cm^{-1}, 2073 cm^{-1} (aldehyde group), 704 cm^{-1}, 1016 cm^{-1}, 1641 cm^{-1}, (aromatic) suggesting the structural similarities with aromatic compounds containing aldehyde group (4–7).

Compound B (R_f value: 0.22, λ_{max}: 229 nm) showed characteristic IR (Perkin Elmer, IR-297) peaks at 1387 cm^{-1}, 3440 cm^{-1} (alcohol group), 1458 cm^{-1}, 2926 cm^{-1} (alkane group), 1740 cm^{-1}, 2856 cm^{-1} (aldehyde group), 1630 cm^{-1} (aromatic), 1119 cm^{-1},

* Corresponding author: phone: +91-3244-243265, +91-9937135904 (M), fax: +91-6791-222034; e-mail: drdilip2003@yahoo.co.in

1213 cm^{-1} (aromatic aldehyde group), 1049 cm^{-1} (aromatic aldehyde group) suggesting the structural similarities with the polyphenolic type of compounds (4–7).

Animals and treatment

Adult Swiss albino mice of either sex (22 ± 2 g) obtained from B.N. Ghosh & Co., Kolkata were acclimatized to normal laboratory conditions for one week and given pellet diet (Hindustan Lever Ltd., India) and water *ad libitum*. All experiments were performed between 8 am to 12 pm to minimize circadian influences. The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethical Committee and was cleared before starting. The animals were handled as per guidelines of committee for the purpose of control and supervision on experimental animals (CPCSEA), New Delhi. For the pharmacological testing, the ethanol extract of C. rotundus (EEHD) was dissolved in propylene glycol.

Toxicity study

An acute toxicity study related to the determination of LD_{50} value was performed with different doses of EEHD into different group of mice, each containing 10 animals, acc. to the method described by Litchfield and Wilcoxon (8).

Effect on sleeping time

Mice were divided into 4 groups, each group containing 6 mice. The animals of group I served as the control (normal saline, 0.9 % w/v NaCl, 5 mL/kg); groups II, III, and IV received EEHD at a low, medium and high dose (400 mg/kg, 500 mg/kg and 600 mg/kg, respectively). Normal saline and the extracts were injected intraperitoneally 30 min prior to the administration of pentobarbital sodium (40 mg/kg, *i.p.*), diazepam (3 mg/kg, *i.p.*) and meprobamate (100 mg/kg, *i.p.*). The sleeping time was noted by recording the interval between the losses and regaining of righting reflex (9, 10).

Analgesic properties

The analgesic activity was tested by the following methods:

I) Acetic acid-induced writhing (chemical stimulus) method

This method, involved intraperitoneal injection of freshly prepared 1.2% (v/v) acetic acid. The number of abdominal constrictions (writhing) and stretching with a jerk at the hind limbs and bending of trunk were counted between 5 and 15 min after administration of acetic acid (11–14). The analgesic effect of the drugs was calculated by the percentage inhibition of writhing episode over that of the control group. The results were compared with those of acetylsalicylic acid, (68 mg/kg), paracetamol (68 mg/kg), and morphine sulfate (1.15 mg/kg).

II) Thermal stimulus by Eddys hot plate method

The analgesic actions were studied using Eddys hot plate method (15). The reaction time was taken as the interval extending from the instant the mouse reached the hot plate till the animal licked its feet or jumped out of the cylinder. The reaction time was recorded at 30, 45, 60, 90, 120, 150, and 180 min after intraperitoneal injection of EEHD at doses of 200, 300, and 400 mg/kg. The temperature of the hot plate was maintained at 55± 0.5°C. A cut off reaction time of 30 s was chosen in order to avoid the physical injury. Morphine and pethidine were used as reference drugs (at doses of 5 and 10 mg/kg, *i.p.*, respectively). EEHD was given individually and also 15 min prior to the administration of reference drugs to investigate the potentiation of morphine and pethidine activity (16, 17).

Anticonvulsant activity

The anticonvulsant property of EEHD (200–500 mg/kg, *i.p.*) was tested against two standard drugs, strychnine (2 mg/kg, *i.p.*) and leptazol (80 mg/kg, *i.p.*). The average survival time (min) and percentage of mortality after 24 h were recorded (17–20). Phenytoin sodium (25 mg/kg, *i.p.*) was used as a standard.

Behavioral effects

The effects of EEHD (400, 500, and 600 mg/kg, *i.p.*) on righting reflex, pinna reflex, corneal reflex, awareness, grip strength, touch and pain responses on mice were observed by conventional methods. Chlorpromazine (5 mg/kg, *i.p.*) was used as a reference drug (21–23).

Statistical analysis

Results are expressed as the mean ± SEM. ANOVA followed by Dunnett's '*t*' test was performed as a *post hoc* test of significance taking vehicle treated animals as control. A p value of < 0.05 was considered as statistically significant.

RESULTS

Acute toxicity tests in mice established that EEHD is a safe drug and no mortality was observed up to 2.5 g/kg, *i.p.* Three doses of EEHD (400, 500, and 600 mg/kg) potentiated the sleeping time induced by standard hypnotics *viz*. pentobarbitone

Table 1. Effect of EEHD on sleeping time (min) induced by pentobarbitone, diazepam and meprobamate in mice.

Treatment	Sleeping time (min) induced by		
	Pentobarbitone (40 mg/kg, *i.p.*)	Meprobamate (100 mg/kg, *i.p.*)	Diazepam (3 mg/kg, *i.p.*)
Control (NS, 5 mL/kg, *i.p.*)	40.6 ± 0.74	61.8 ± 0.80	75.2 ± 0.77
EEHD (400 mg/kg, *i.p.*)	71.2 ± 1.05[a]	96.5 ± 1.15[a]	118.6 ± 1.75[a]
EEHD (500 mg/kg, *i.p.*)	90.5 ± 2.00[a]	117.2 ± 2.03[a]	148.9 ± 2.00[a]
EEHD (600 mg/kg, *i.p.*)	118.8 ± 2.04[a]	138.7 ± 2.10[a]	192.8 ± 2.30[a]

Values are the mean ± SEM from 6 animals in each group; Statistical analysis done by ANOVA followed by *post hoc* test of significance, Dunnett's '*t*'test. [a] $p < 0.05$ *vs*. vehicle control. NS: normal saline, *i.p.*: intraperitoneal.

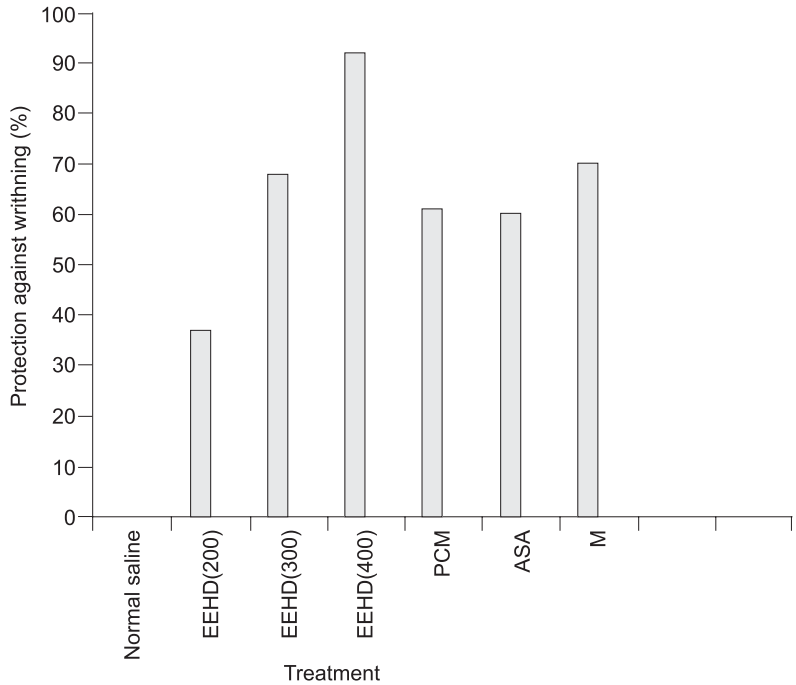

Figure 1. Influence of EEHD (200, 300, 400 mg/kg) *i.p.* on the writing and stretching induced in mice by acetic acid (writhing test). The activity was compared with PCM, ASA, M. Values are mean ± SEM from 6 animals in each group.

(75.4%, 122.9%, and 192.6%, respectively), diazepam (57.7%, 98%, and 156.4%, respectively) and meprobamate (56.1%, 89.6%, and 124.4%, respectively) (Table 1).

EEHD exhibited a dose dependant and significant analgesic activity in the acetic acid induced writhing test. As can be seen in Figure 1, EEHD with a dose of 200 mg/kg, *i.p.* exhibited percentage of protection 37%. This dose dependent effect reached 92% with a dose of 400 mg/kg, *i.p.* Analgesic compounds: acetyl salicylic acid (68 mg/kg, *i.p.*), morphine sulfate (1.15 mg/kg, *i.p.*), and paracetamol (68 mg/kg, *i.p.*) gave 60%, 70%, and 61% protection, respectively. From Table 2, it is also found that EEHD not only produced analgesia in mice but also potentiated the analgesic action of morphine and pethidine.

Strychnine and leptazol at the doses of 2 mg/kg, *i.p.* and 80 mg/kg, *i.p.*, respectively, induced tonic type of convulsions with clonus in mice. The degree of convulsions was measured visually. Table 3 and Figure 2 show that EEHD increased the average survival time and decreased the percentage mortality in a dose dependent manner against strychnine and leptazol-induced convulsions. The compounds showed percentage protection in comparison to phenytoin sodium (25 mg/kg), which completely inhibited the convulsions produced by strychnine

Table 2. Effect of EEHD on analgesia induced by morphine and pethidine in mice (by hot plate method).

Treatment	Resting value	Average maximum reaction time (s) at min							
		15	30	45	60	90	120	150	180
Control (NS, 5 mL/kg, *i.p.*)	4.8 ± 0.06	10.1 ± 1.23	7.6 ± 0.05	5.3 ± 0.03	5.1 ± 1.09	4.2 ± 1.02	4.1 ± 0.72	3.7 ± 1.01	3.1 ± 0.32
EEHD (200 mg/kg, *i.p.*)	4.9 ± 0.79	-	19.8 ± 1.01[a]	14.3 ± 1.16[a]	11.2 ± 0.9[a]	9.5 ± 0.82[a]	6.0 ± 0.04	5.0 ± 0.70	4.5 ± 0.65
EEHD (300 mg/kg, *i.p.*)	5.1 ± 0.94	27.3 ± 1.04[a]	24.6 ± 1.31[a]	18.4 ± 1.09[a]	14.2 ± 1.24[a]	12.8 ± 1.36[a]	9.0 ± 0.97[a]	6.5 ± 0.83	5.2 ± 0.71
EEHD (400 mg/kg, *i.p.*)	4.5 ± 0.08	> 30[a]	> 30[a]	> 30[a]	> 30[a]	28.4 ± 1.91[a]	25.2 ± 1.40[a]	20.2 ± 0.80[a]	14.1 ± 1.12[a]
Morphine (5 mg/kg, *i.p.*)	5.6 ± 0.94	> 30[a]	19.3 ± 0.42[a]	18.6 ± 1.04[a]	14.4 ± 0.94[a]	9.9 ± 1.18[a]	8.1 ± 0.82[a]	6.5 ± 0.72	5.0 ± 0.34
EEHD (200 mg/kg, *i.p.*) + Morphine	5.3 ± 0.87	> 30[a]	> 30[a]	27.6 ± 1.49[a]	24.6 ± 0.80[a]	21.2 ± 0.95[a]	12.2 ± 1.30[a]	9.2 ± 0.86[a]	6.7 ± 0.80
EEHD (300 mg/kg, *i.p.*) + Morphine	5.9 ± 0.62	> 30[a]	> 30[a]	> 30[a]	28.6 ± 1.10[a]	25.3 ± 1.70[a]	15.9 ± 2.60[a]	11.6 ± 1.23[a]	10.1 ± 0.30[a]
EEHD (400 mg/kg, *i.p.*) + Morphine	5.9 ± 0.94	> 30[a]	> 30[a]	> 30[a]	> 30[a]	> 30[a]	> 30[a]	28.4 ± 1.50[a]	21.3 ± 1.85[a]
Pethidine (10 mg/kg, *i.p.*)	5.0 ± 0.84	25.2 ± 1.27[a]	23.1 ± 1.39[a]	15.6 ± 0.52[a]	11.4 ± 1.05[a]	9.5 ± 0.91[a]	6.1 ± 0.91[a]	4.4 ± 0.94	3.9 ± 0.80
EEHD (200 mg/kg, *i.p.*) + Pethidine	5.7 ± 0.79	28.5 ± 1.00[a]	27.5 ± 1.22[a]	17.8 ± 1.97[a]	16.1 ± 1.28[a]	16.0 ± 1.39[a]	11.6 ± 1.70[a]	9.3 ± 0.94[a]	6.2 ± 0.72
EEHD (300 mg/kg, *i.p.*) + Pethdine	5.6 ± 1.12	> 30[a]	> 30[a]	28.3 ± 1.48[a]	21.4 ± 1.07[a]	17.5 ± 1.58[a]	14.4 ± 1.18[a]	11.8 ± 1.12[a]	9.2 ± 0.70[a]
EEHD (400 mg/kg, *i.p.*) + Pethidine	5.8 ± 0.95	> 30[a]	> 30[a]	> 30[a]	> 30[a]	> 30[a]	28.9 ± 1.56[a]	23.5 ± 1.85[a]	18.6 ± 1.25[a]

Values are the mean ± SEM from 6 animals in each group; Statistical analysis done by ANOVA followed by *post hoc* test of significance, Dunnett's '*t*' test, [a]$p < 0.05$ *vs*. resting value (average reaction time before treatment). Results of (EEHD + morphine) and (EEHD + pethidine) were significant ($p < 0.05$) *vs*. EEHD. NS: normal saline; > 30: animals fail to react within 30 s (30 s response latency). *i.p.*: intraperitoneal.

Table 3. Effect of EEHD on average survival time on strychnine- and leptazol-induced convulsion in mice.

Treatment	Survival time (min) after treatment of	
	strychnine (2 mg/kg, *i.p.*)	leptazol (80 mg/kg, *i.p.*)
Control (NS, 5 mL/kg, *i.p.*)	6.2 ± 0.90	12.6 ± 1.15
EEHD (200 mg/kg, *i.p.*)	130.5 ±1.00[a]	141.6 ± 1.07[a]
EEHD (300 mg/kg, *i.p.*)	160.2 ±1.13[a]	175.7 ± 1.20[a]
EEHD (400 mg/kg, *i.p.*)	209.8 ±1.66[a]	218.6 ± 1.95[a]
EEHD (450 mg/kg, *i.p.*)	355.8 ±2.21[a]	380.0 ± 2.75[a]

Values are the mean ± SEM from 10 animals in each group; Statistical analysis done by ANOVA followed by *post hoc* test of significance, Dunnett's '*t*'test. [a] $p < 0.001$ *vs*. control. NS: normal saline, *i.p.*: intraperitoneal.

Table 4. Effect of EEHD on behavioral profiles in mice.

Treatment	Awareness Response	Touch response	Pain response	Righting reflex	Pinna reflex	Corneal reflex	Grip strength
Control (NS, 5 mL/kg, *i.p.*)	0	0	0	0	0	0	+
Chlorpromazine(5 mg/kg,*i.p.*)	4+	4+	4+	4+	4+	4+	4+
EEHD (400 mg/kg, *i.p.*)	2+	3+	4+	2+	2+	3+	2+
EEHD (500 mg/kg, *i.p.*)	3+	4+	4+	3+	3+	4+	3+
EEHD (600 mg/kg, *i.p.*)	4+	4+	4+	4+	4+	4+	4+

Key for scoring: 0, no effect (normal); +, slight depression; 2+, moderate depression; 3+, strong depression; 4+, very strong depression. *i.p.*: intraperitoneal. NS: normal saline. Number of animals used for each group (n = 6).

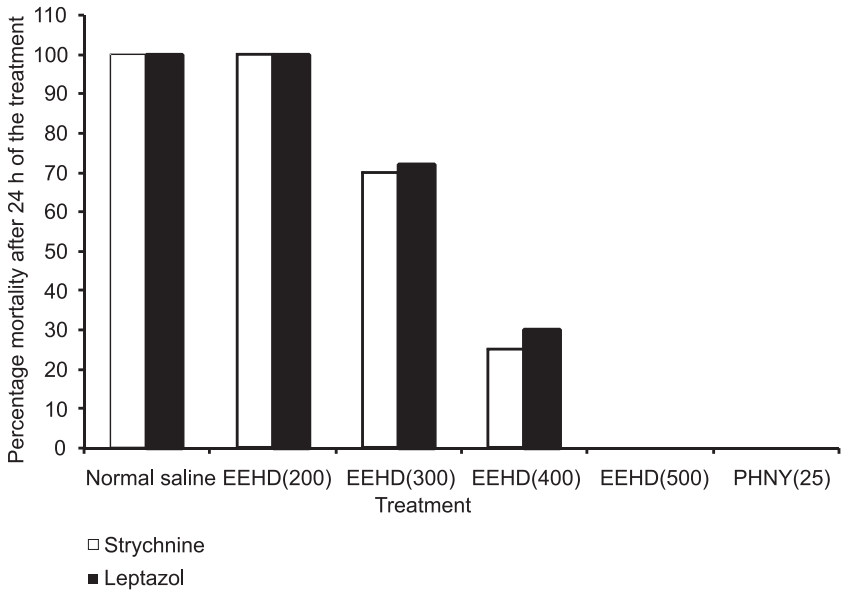

Figure 2. Anticonvulsant effect of EEHD and PHNY (phenytoin) on strychnine (2 mg/kg, b.w.)/leptazol(80 mg/kg, b.w.) induced convulsions in mice. Results are expressed as % mortality. Respective doses of the extracts and PHNY (mg/kg, b.w.) are in parenthesis.

and leptazol (Fig. 2). It was observed that different combinations of strychnine or leptazol with EEHD did not show any significant protective action against convulsions.

The results obtained from general behavioral profiles are shown in Table 4. It was noted that EEHD depressed awareness and alertness, touch and pain responses, grip strength, altered righting, pinna and corneal reflexes when compared to the control (normal saline 0.9%, w/v, 5 mL/kg). However, chlorpromazine hydrochloride (standard) produced a significant depression of these responses in comparison with EEHD.

DISCUSSION AND CONCLUSION

Pentobarbital, diazepam and meprobamate were used to induce sleep in this study. Benzodiazepines are believed to act at specific binding sites that are closely linked to γ-aminobutyric acid (GABA) receptors, the binding of benzodiazepines enhancing GABA-ergic transmission. Although the cause of prolongation of diazepam-induced sleeping time is not known, the enhancement of GABA-ergic transmission might be related to its sedative activity. Prolongation of pentobarbital-induced sleeping time might be due to tranquilizing action as well as CNS depressant action. Although the exact mechanism responsible for the sedation action of meprobamate is not clear, it might be due to CNS depressant action or due to enhancement of GABA-ergic transmission (12, 16, 17, 24). EEHD potentiated significantly the duration of pentobarbital, diazepam- and meprobamate-induced sleep in mice, suggesting probable tranquilizing action as well as CNS depressant action (13, 22).

Pal et al., found that analgesic activity of *Celsia coromandeliane* is probably mediated by inhibition of a post synaptic specific sensitive mechanism either by depleting endogenous levels of norepinephrine *via* dopamine-β-hydroxylase inhibition or by blocking norepinephrine effects at the receptor level (25). Analgesic and anticonvulsant activities can also be mediated by other mechanisms. The increase of brain serotonin and GABA level is responsible for analgesic and anticonvulsant activities (16, 17, 20, 25–27). It was found that EEHD increased the brain serotonin and GABA level in mice (unpublished data). Therefore, analgesic and anticonvulsant activities produced by EEHD may be releted to the increased brain serotonin and GABA level in mice (25).

Gupta et al. established that inhibition of the touch response, righting reflex, and grip strength is probably produced due to a pronounced CNS depressant action (19). Reduction of pinna reflex and awareness may be due to synapses block of the afferent pathway or due to overall CNS depressant action (28, 29). In this study, the mechanism whereby EEHD depressed awareness, touch and pain responses, righting reflex, pinna reflex, corneal reflex and grip strength may also be due to synapses block of the efferent pathway or by overall CNS depressant action.

EEHD enhanced sleeping time, analgesic, and anticonvulsant activities and reduced different behavioral reflexes. It can be concluded from the present discussion that the ethanol extract of *H. difformis* exhibited strong CNS depressant action.

Acknowledgments

The authors are thankful to Principal and President, S.I.P.S., Jharpokharia, Orissa, India for providing necessary facilities.

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Received: 19. 10. 2009