Screening of isolated phytosterol from leaves of *Holoptelea integrifolia* (Roxb.) Planch for its anticonvulsant activity in experimental animals

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Abstract

Objective: The majority of scientific documentation suggested prominent role of Phytosterols towards anticonvulsant activity. The main objective of the work was to evaluate anticonvulsant activity of *Holoptelea integrifolia* isolated Phytosterol (HIIP) from petroleum ether extract (PEHI) of leaves of *H. integrifolia* (Roxb) Planch

Methods: The anticonvulsant activity of different doses of HIIP (10 and 30 mg/kg-p.o.) was evaluated using Pentylenetetrazole (PTZ) induced convulsions in mice and lithium-pilocarpine induced status epilepticus in rats.

Results: HIIP-30 mg/kg was more potent than HIIP-10 mg/kg for showing anticonvulsant activity.

Conclusions: The results indicate that HIIP shows anticonvulsant activity which was dose dependent.

Keywords: Anticonvulsant, Pentylenetetrazole, Lithium-pilocarpine, *Holoptelea integrifolia* Isolated Phytosterol

1. Introduction

In traditional system of medicine, bark and leaves of *Holoptelea integrifolia* (HI) used as bitter, astringent, acid, thermogenic, anti-inflammatory, digestive, carminative, laxative, anthelmintic, depurative, repulsive, urinary astringent and in rheumatism [1, 2]. In our previous studies the anticonvulsant activity of petroleum ether and methanol extract of leaves of *Holoptelea integrifolia* in experimental animals was evaluated and it was found that petroleum ether extract (PEHI) has shown comparable effects with the standard drug and more significant anticonvulsant activity than methanolic extract (MHI) [3]. On the similar lines in this present study a phytosterol (HIIP) was isolated from petroleum ether extract and it was studied for anticonvulsant activity.

2. Materials and methods

2.1 Chemicals and Drugs

Pentylenetetrazole (Sigma, USA), Diazepam and Clonazepam (Campose injection, Ranbaxy, India), Lithium carbonate (Glenmark Pharmaceuticals, India), and pilocarpine (FDC Limited, India) were used in the study. All other chemicals were of analytical grade. PTZ, Diazepam inj., Lithium carbonate, Pilocarpine nitrate were dissolved in distilled water just before administration. A gastric catheter was used for oral drug administration. All the solvents used for the extraction were of AR grade.

2.2 High Performance Thin Layer Chromatography (HPTLC) Instrumentation

HPTLC system of CAMAG, Muttenz, Switzerland, Anchorn Enterprises (I) Pvt. Ltd, Mumbai, consisting of sample applicator (Linomat 5), Twin trough chamber with lid (20×10 cm, CAMAG, Muttenz, Switzerland), UV cabinet (Actron, Mumbai) with dual wavelength (254/366 nm) and the HPTLC photo documentation (Actron, Mumbai) were used for study. UV spectra was recorded using CAMAG TLC Scanner – IV.

LC/MS was recorded using SHIMADZU- LC/MS 2020, IR spectra was recorded using SHIMADZU-IR PRESTIGE-21, NMR spectra was recorded using Mercury plus 300 MHZ NMR Spectrometer.

2.3 Plant Material, Extraction and Isolation of *Holoptelea integrifolia* phytosterol (HIIP) from petroleum ether extract by preparative TLC

The dried and powdered leaves (1kg) of *Holoptelea integrifolia* was extracted with petroleum
ether (b.p. 60-80°C) for three times. After evaporation of the solvent under reduced pressure, the yield obtained was 4.8% w/w.

The petroleum ether extract was prepared in petroleum ether as a sample solution applied on Precoated silica gel aluminium plates 60F254, 20 cm x 10 cm with 250 µm thickness with CAMAG Linomat V (Switzerland) was used. The plates were washed by methanol and activated at 120 °C for 20 min before the start of chromatography. The sample solution was applied by using CAMAG microlitre syringe on the plates. The distance between the 2 bands was 5 mm with constant application rate of 1.0 µl/s was applied.

The composition of mobile phase used for isolation of phytosterol was Chloroform: Ethyl acetate, in the volume ratio of 4:6 (v/v) and 20 ml of mobile phase was used per chromatography. The plates were developed in 20 cm x 10 cm twin trough glass chamber saturated with filter paper Whatmann No.1 in mobile phase for 20 min at room temperature, and length of chromatogram run was 8.0 cm.

TLC plates were dried with the help of air dryer. Later on, densitometric scanning was performed with CAMAG TLC Scanner IV at 540 nm. The TLC Plate was dipped in Anisaldehyde Sulphuric acid reagent and then dried in oven at 110°C. Concentration of the compound was then determined. The yield of HIIP obtained was 6 mg for a total of 40 preparative TLC Plates. In order to get sufficient quantity of HIIP, TLC plate of 1mm thickness was used. 20 gm of PEHI has given 228 mg, HIIP yield by using this method [4-11].

2.4 Preparation of test samples
The test drug HIIP was prepared individually as suspension in distilled water with tragacanth (1%w/v) as a suspending agent. For the all pharmacological studies freshly prepared suspensions were used.

2.5 Animals
Albino rats of either sex weighing between 180-220 gm were procured from Central Animal House, Rajah Muthiah Medical College & Hospital, Faculty of Medicine, Annamalai University, Annamalai Nagar 608002, Tamil Nadu, India for experimental purpose. The animals were acclimatized to laboratory conditions for 7 days. The animals were supplied with commercially available standard diet. They were maintained at 25 ± 2 °C and relative humidity of 45 to 55% and under standard environmental conditions (12 hour. light 12 hour. dark cycle). Water was allowed ad libitum under hygienic conditions. All animal studies were performed in accordance guideline of CPCSEA and Institutional Animal Ethical Committee (IAEC) of Central Animal House, Rajah Muthiah Medical College & Hospital, Annamalai University, Tamil Nadu, India (CPCSEA registration number 160/1999 /IAEC/CPCSEA, Proposal no:1029). All experiments were carried out between 12:00- 16:00 h

2.6 Acute toxicity study
Healthy adult female Wistar albino mice were subjected for acute toxicity studies as per OECD-425 guidelines for isolated compound, HIIP. The test substances were administered orally in a single dose by gavages using a stomach tube. Mice were fasted prior to dosing (food was withdrawn overnight and water was withdrawn 3-4 h before drug administration). Following the period of fasting, the mice were weighed and the test substance, HIIP was administered. After the administration of the substances, food was withheld for 2 h in mice. Mice were observed for its onset and duration of behavioural changes, toxicity and mortality upto 24h and observations were done for a period of 14 days after acute toxicity. For determining LD50 value, HIIP was administered in mice as per OECD 425 guidelines, the isolated compound HIIP was given as 100,200,300,400 and 500mg/kg/p.o/b.wt. If the first animal survived; the second animal received a higher dose. If the first animal died or appeared moribund, the second animal received a lower dose.

2.7 Evaluation of anticonvulsant activity using Pentylenetetrazole (PTZ) induced seizures model
60 min after as per below mentioned drug treatment Clonic seizures were induced in mice by subcutaneous injection of 80mg/kg Pentylenetetrazole. The latency to the onset of clonic convulsions in non-protected mice and lethality during the following 24 hour was recorded and compared with those of vehicle treated control mice to assess the anticonvulsant activity [12-14]. One group received clonazepam 0.1 mg/kg - i.p. as a reference standard 30 min before PTZ. The animals were observed for onset of convulsion up to 30 min after PTZ. Each animal was then placed into individual plastic cages and were observed initially for 30 min and later up to 24 hrs. The following parameters were recorded during test session of initial 30 min and up to 24 hrs respectively: Latency (onset of clonus), Onset of tonic-clonic convulsions, Status of animal after 1hr, Status of animal after 24 hrs, percent protection are observed and measured.

Group 1: Control group of mice treated with vehicle 10 ml/kg/p.o/b.wt
Group 2: Test group of mice treated with low dose of HIIP-10 mg/kg/p.o/b.wt
Group 3: Test group of mice treated with high dose of HIIP-30 mg/kg/p.o/b.wt
Group 4: Test group of mice treated with (std.) Clonazepam - 0.1 mg/kg/p.o/b.wt

2.8 Lithium pilocarpine induced status-epileptics
HIIP was screened for anticonvulsant activity using Lithium pilocarpine induced status epilepticus model in rat. Status epilepticus was induced by administration of pilocarpine...
(30 mg/kg i.p) 24 h after lithium carbonate (3 mEq/kg i.p). The effect of HIIP was studied on the rearing with forelimb clonus by administering both doses 30 min. before injection of pilocarpine [15]. Diazepam was used as a reference standard in a dose of 1 mg/kg i.p.

**Group 1:** Control group of rats treated with vehicle

**Group 2:** Test group of rats treated with low dose of HIIP-10 mg/kg i.p/b.wt

**Group 3:** Test group of rats treated with high dose of HIIP-30 mg/kg i.p/b.wt

**Group 4:** Test group of rats treated with (std.) Diazepam- 1 mg/kg i.p/b.wt

**Statistical analysis**

Comparison was made against the vehicle treated control group. All the data was presented as Mean ± SEM. The data was analyzed by one way analysis of variance (ANOVA) followed by Dunnett’s’ test, *P<0.05, **P<0.01, ***P<0.001.

3. Results and discussion

3.1 Acute toxicity study

<table>
<thead>
<tr>
<th>Isolated Compound</th>
<th>Up and down doses in mg/kg/p.o/b.wt</th>
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</thead>
<tbody>
<tr>
<td>HIIP</td>
<td>100 200 300 400 500 (-) not performed</td>
</tr>
</tbody>
</table>

The LD50 of HIIP, was performed at the dose level of 100 to 300 mg/kg/p.o/b.wt. At 300 mg/kg/p.o/b.wt two mice were dead. And hence, the LD50 dose of HIIP, was fixed at 300 mg/kg/p.o/b.wt.

**Fig 2:** HPTLC chromatogram of a new phytosterol (HIIP) from petroleum ether extract of leaves of *Holoptelea integrifolia* by preparative TLC (Rf: 0.42)

**Fig 3:** Liquid Chromatography/ Mass Spectrometry (LC/MS) of isolated new phytosterol (HIIP) (Rf: 0.42)

**Fig 4:** UV Spectra of a new phytosterol (HIIP) at 254 nm, isolated from petroleum ether extract of leaves of *Holoptelea integrifolia* using preparative TLC

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3.2 Assessment of Anticonvulsant Activity of HIIP

**PTZ**-induced seizures

HIIP was screened for anticonvulsant activity using PTZ induced convulsion model in mice. Study was conducted using low and high doses of HIIP (10 & 30 mg/kg-p.o. respectively). The above mentioned doses were administered as mentioned earlier. HIIP 30 mg/kg was more potent than HIIP 10 mg/kg for showing anticonvulsant activity. It was observed that the anticonvulsant activity of HIIP was dose dependent. The standard drug clonazepam (0.1 mg/kg-i.p.) exhibited a significant anticonvulsant activity and offered 100% protection. The observations are given in Table 2.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Onset of first clonus (second)</th>
<th>No. of animals survived/used</th>
<th>Percent mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle Control (10 ml/kg)</td>
<td>211.14 ± 07.57</td>
<td>0/6</td>
<td>100 %</td>
</tr>
<tr>
<td>HIIP-10 mg/kg /p.o/b.wt</td>
<td>240.9 ± 05.20*</td>
<td>3/6</td>
<td>50 %</td>
</tr>
<tr>
<td>HIIP-30 mg/kg /p.o/b.wt</td>
<td>276.8 ± 06.085**</td>
<td>4/6</td>
<td>33.33 %</td>
</tr>
<tr>
<td>Clonazepam -0.1mg/kg/ i.p./b.wt</td>
<td>Nil</td>
<td>6/6</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n=6; Statistical Analysis- one way ANOVA followed by Dunnetts’t test *P<0.05, **P<0.01, ***P<0.001, HIIP: Holoptelea integrifolia isolated phytosterol

**Lithium pilocarpine** induced status-epilepticus

HIIP was screened for anticonvulsant activity using Lithium pilocarpine induced status epilepticus model in rat. In vehicle treated group latency to forelimb clonus was observed at 23.5±0.4282 min after pilocarpine. Study was conducted using low, and high doses of HIIP (10 & 30 mg/kg-p.o. respectively). The above mentioned doses were administered as mentioned earlier. It was observed that both low and high doses of HIIP exhibited a significant anticonvulsant effect by showing significant delay in latency to rearing with forelimb clonus when compared to control group. HIIP-30 mg/kg was more potent than HIIP-10 mg/kg if showing anticonvulsant activity.

It was observed that the anticonvulsant activity of HIIP was dose dependent. The standard drug diazepam (1mg/kg-i.p.) exhibited a significant anticonvulsant activity. The animals were normal in behaviour after 180 min. The observations are given in Table 3.

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Latency to rearing with forelimb clonus (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control-10 ml/kg/p.o/b.wt</td>
<td>23.5±0.4282</td>
</tr>
<tr>
<td>HIIP-10 mg/kg/p.o/b.wt</td>
<td>39.5±2.078*</td>
</tr>
<tr>
<td>HIIP-30 mg/kg/p.o/b.wt</td>
<td>72.00±1.461**</td>
</tr>
<tr>
<td>Diazepam -1mg/kg/i.p/b.wt</td>
<td>78.33±0.7149**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; n=6; One way analysis of variance (ANOVA) followed by Dunnetts test. Where, *P<0.05, **P<0.01, ***P<0.001 HIIP: Holoptelea integrifolia isolated phytosterol

From the above data it is concluded that HIIP possesses significant anticonvulsant activity against pentylentetrazole induced seizures and Lithium-pilocarpine induced status-epilepticus. GABA is the primary inhibitory neurotransmitter in the central nervous system (CNS). Diminution of brain GABA level has been reported after PTZ. Diminution of brain GABA level has been reported after subconvulsive dose of PTZ [16]. Many plants having anticonvulsant activity are known to inhibit GABA transaminase activity thereby increasing brain contents of GABA. Pretreatment of lithium initiates limbic seizures after administration of subconvulsant doses of pilocarpine and other cholinergic agonist; Still lithium does not have proconvulsant activities [17]. If lithium and pilocarpine administered concurrently it results in an accumulation of inositol monophosphate and reduction in cortical inositol that are about 10 times greater than the effects obtained with either drugs alone [17, 18]. Lithium-pilocarpine was found useful in status epilepticus [19]. On observation and reference to reported data from Phytochemical tests, it was
clear that, HIIP, isolated from Petroleum ether extract of *Holoptelea integrifolia* (Roxb) Planch leaves showed the presence of flavonoids, steroids, triterpenoids. Flavonoids, sterols and terpenoids in preliminary phytochemical screening, these phytochemicals have been implicated in various pharmacological actions on central nervous system including anticonvulsant and anxiolytic activity [19, 20].

### 4. Conclusion

Majority of scientific documentation suggested prominent role of phytosterols towards anticonvulsant activity [19, 20]. This is again proved from the anticonvulsant activity exhibited by the isolated phytosterol compound, HIIP.

### 5. References