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Screening of isolated phytosterol from leaves of *Holoptelea integrifolia* (Roxb.) Planch for it's 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 anticonvu ant activity of *Holoptelea integrifolia* isolated Phytosterol (HIIP) from petroleum ether extract (PEHI) of leaves of *H oloptelea 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 integrifoila* Isolated Phytosterol

1. Introduction
In traditional system of medicine, bark and leaves of *Holoptelea integrifoila* (HI) used as bitter, astringent, acrid, 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 leaf 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, Anchrom Enterprises (I) Pvt. Ltd, Mumbai, consisting of sample applicator (Linomat 5), Twin trough chamber with lid (20×10 cm, CAMAG, Muttenz, Switzerland), UV cabinet (Aetron, Mumbai) with dual wavelength (254/366 nm) and the HPTLC photo documentation (Aetron, 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. 60-80ºC) 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 ^{0}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 compostion 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 ºC. 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].

Fig 1: *Holoptelea integrifolia* (Roxb.) Planch tree

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 mice of either sex weighing between 20-30g and 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 for1 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 *Pentylenete trazole* (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-epilepticus

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/p.o/b.wt

Group 3: Test group of rats treated with high dose of HIIP-30 mg/kg/p.o/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't' test, **P*<0.05, ***P*<0.01, ****P*<0.001.

3. Results and discussion

3.1 Acute toxicity study

Table 1: LD50 Values of isolated compound HIIP

Isolated Compound	Up and down doses in mg/kg/p.o/b.wt				
HIIP	100	200	300	400	500
	5/5	4/5	3/5	0/5	-

(-) not performed

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.

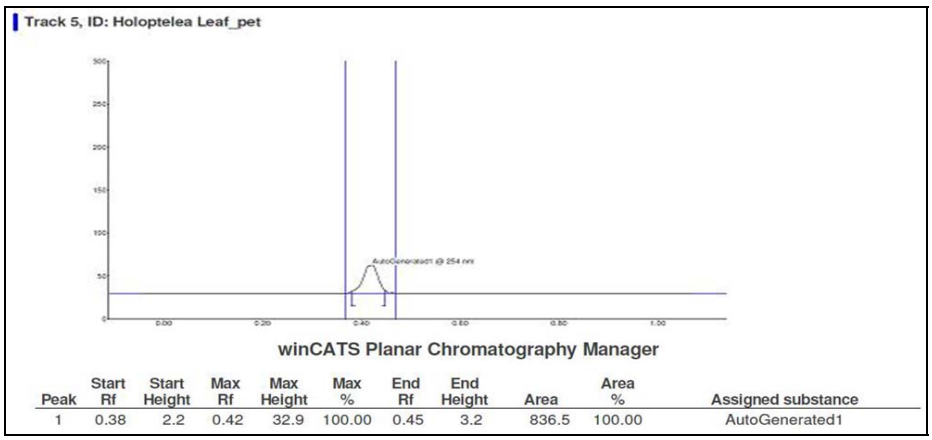


Fig 2: HPTLC chromatogram of a new phytosterol (HIIP) from petroleum ether extract of leaves of *Holoptelea integrifolia* by preparative TLC (Rf: 0.42)

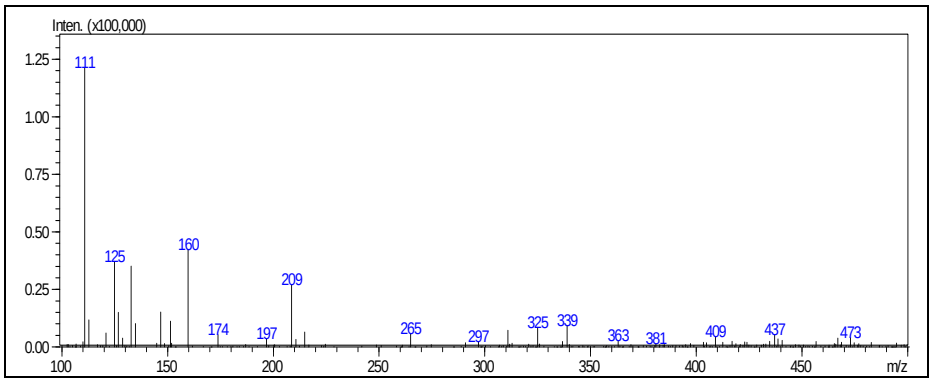


Fig 3: Liquid Chromatography/ Mass Spectrometry (LC/MS) of isolated new phytosterol (HIIP) (R_f : 0.42)

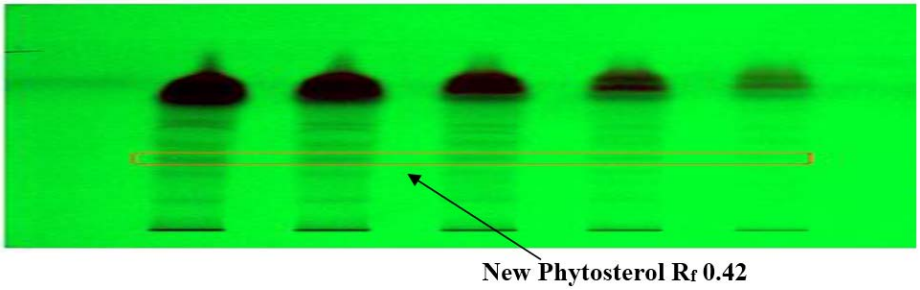
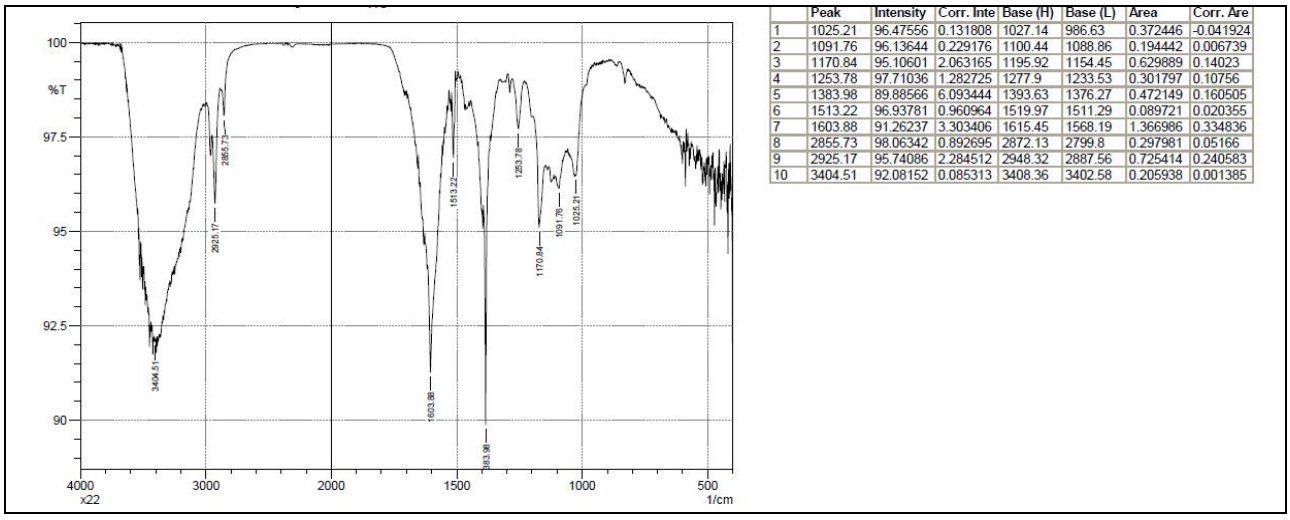


Fig 4: UV Spectra of a new phytosterol (HIIP) at 254 nm, isolated from petroleum ether extract of leaves of *Holoptelea integrifolia* using preparative TLC



	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	1025.21	96.47556	0.131808	1027.14	986.63	0.372446	-0.041924
2	1091.76	96.13644	0.229176	1100.44	1088.86	0.194442	0.006739
3	1170.84	95.10601	2.063165	1195.92	1154.45	0.629889	0.14023
4	1253.78	97.71036	1.282725	1277.9	1233.53	0.301797	0.10756
5	1383.98	89.88566	6.093444	1393.63	1376.27	0.472149	0.160505
6	1513.22	96.93781	0.960964	1519.97	1511.29	0.089721	0.020355
7	1603.88	91.26237	3.303406	1615.45	1568.19	1.366986	0.334836
8	2855.73	98.06342	0.892695	2872.13	2799.8	0.297981	0.05166
9	2925.17	95.74086	2.284512	2948.32	2887.56	0.725414	0.240583
10	3404.51	92.08152	0.085313	3408.36	3402.58	0.205938	0.001385

Fig 5: IR Spectrum of isolated new phytosterol (HIIP) (R_f : 0.42)

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 2: Effect of HIIP on PTZ (80 mg/kg-s.c.) induced convulsions in mice

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

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 f or 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 3: Effect of HIIP on Lithium-pilocarpine induced status-epilepticus in rats

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

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 *pentylenetetrazole* 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* administred 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 [15]. 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 rol e of phytosterols towards anticonvulsant activity [19, 20]. This is again proved from the anticonvulsant activity exhibited by the isolated phytosterol compound, HIIP.

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