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EVALUATION OF ISOLATED PHYTOSTEROL FROM LEAVES OF *HOLOPTELEA INTEGRIFOLIA* (ROXB.) PLANCH FOR IT'S ANTIDEPRESSANT ACTIVITY IN EXPERIMENTAL ANIMALS

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ABSTRACT

Background: Majority of scientific documentation suggested prominent role of Phytosterols towards Antidepressant activity. The main objective of the work was to evaluate Antidepressant activity of *Holoptelea integrifolia* isolated Phytosterol (HIIP) from petroleum ether extract (PEHI) of leaves of *Holoptelea integrifolia* (Roxb) Planch. Methods: The Antidepressant activity of different doses of HIIP (10 and 30 mg/kg-p.o.) was evaluated using Forced Swim Test (FST) in mice. Results: HIIP-30 mg/kg was more potent than HIIP-10 mg/kg for showing Antidepressant activity. Conclusions: The results indicate that HIIP shows Antidepressant activity which was dose dependent.

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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 antidepressant 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 antidepressant 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 Antidepressant activity.

MATERIALS AND METHODS

Chemicals and Drugs

Standard drug *fluoxetine* was obtained from Crescent Therapeutics limited, Himachal Pradesh. The test drug HIIP was prepared individually as suspension in distilled water with tragacanth (1%w/v) as a suspending agent. A gastric catheter was used for oral drug administration. All the solvents used for the extraction were of AR grade.

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.

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

Fig. No. 1 : *Holoptelea integrifolia* (Roxb.) Planch tree.

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°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].

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.

Animals

Albino wistar mice of either sex weighing between 20-30g were procured from Central Animal House, Rajah Muthiah Medical College & Hospital, Faculty of Medicine, Annamalai University, Annamalai Nagar 608002, TamilNadu, 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, TamilNadu, India (CPCSEA registration number-160/1999 /IAEC/CPCSEA, Proposal no:1029). All experiments were carried out between 12:00- 16:00 h

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 it 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 500 mg/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.

Evaluation of antidepressant activity of HIIP using Forced Swim Test (FST) model in mice

On the 14th day immediately after administration of last dose, each mice was individually allowed to swim freely in a transparent glass vessel (25 cm high, 10 cm diameter) filled with 10 cm of water at room temperature for a period of 05 minutes as a pre test session without any recording for any parameters. 24 hours later Forced Swim Test was conducted in the same cylinder for 05 minutes wherein

- Swimming (Active movements of extremities and circling in the cylinder)
- Climbing/Trashing (Active upward directed movements of forelimbs on the container wall)
- Immobility (floating in water without swimming i.e. mice made no further attempts to escape except the movements necessary to keep its head above the water were noted down using stop watch as an indices of depression).

The increase in active responses, such as climbing/trashing as well as swimming and reduction in immobility were considered as behavioral profiles that indicated antidepressant like action.

Group 1- Control group of mice treated with vehicle 10 ml/kg/p.o/b.wt for 14 days
Group 2- Test group of mice treated with low dose of HIIP-10 mg/kg/p.o/b.wt for 14 days
Group 3- Test group of mice treated with high dose of HIIP-30 mg/kg/p.o/b.wt for 14 days
Group 4- Test group of mice treated with (std.) Fluoxetine-10 mg/kg/p.o/b.wt for 14 days

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.

RESULTS AND DISCUSSION

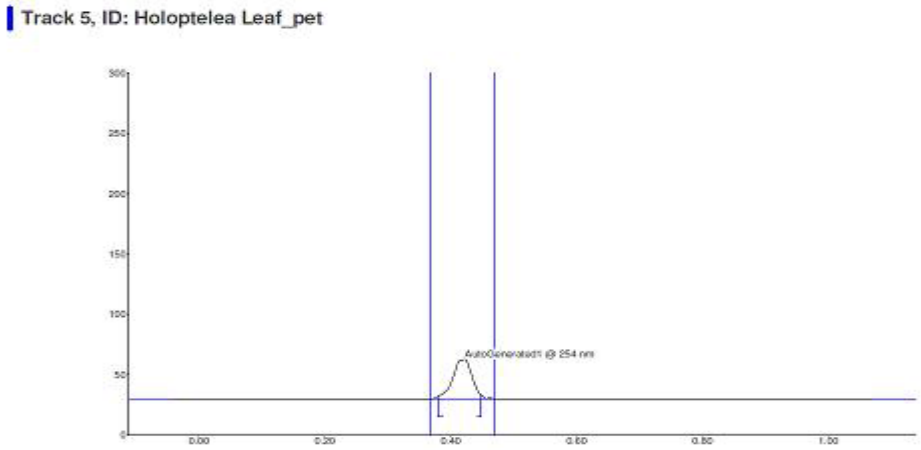
Acute toxicity study

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.



winCATS Planar Chromatography Manager

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.38	2.2	0.42	32.9	100.00	0.45	3.2	836.5	100.00	AutoGenerated1

Fig.No.2: HPTLC chromatogram of a new phytosterol (HIIP) from petroleum ether extract of leaves of *Holoptelea integrifolia* by preparativ TLC (Rf : 0.42).

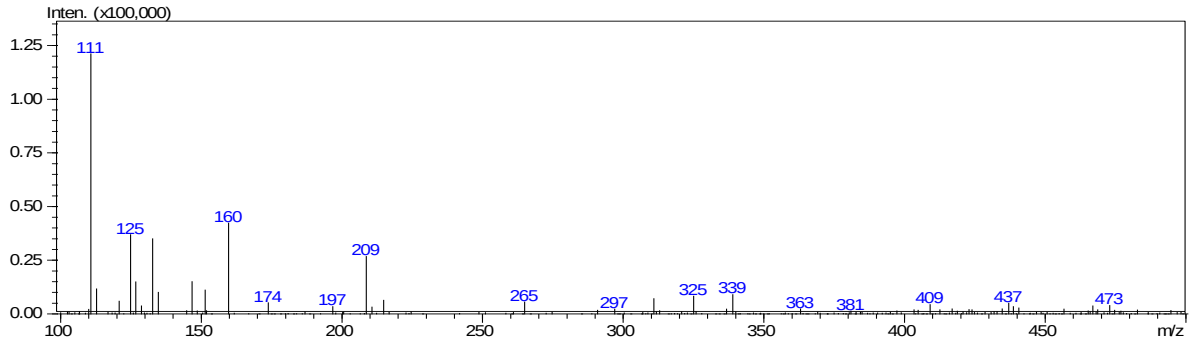
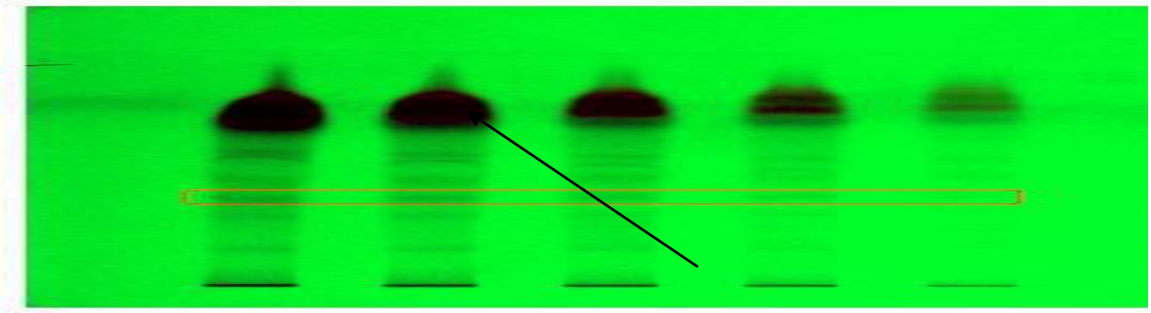
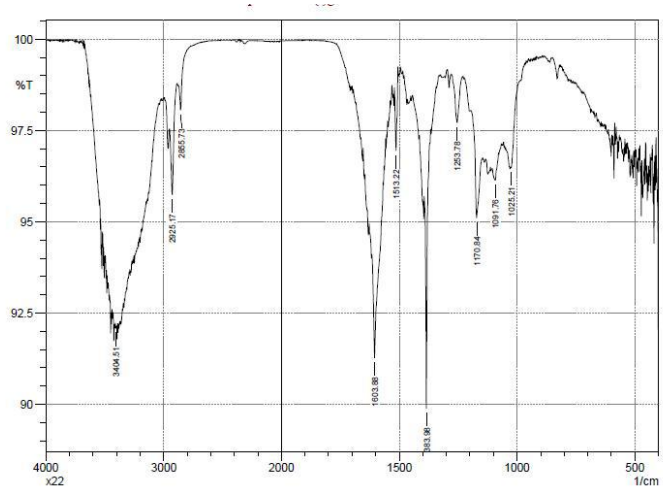


Fig. No. 3 : Liquid Chromatography/ Mass Spectrometry (LC/MS) of isolated new phytosterol (HIIP) (R_f : 0.42).



New Phytosterol R_f 0.42

Fig. No. 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. No. 5: IR Spectrum of isolated new phytosterol (HIIP) (R_f : 0.42).

Assessment of Antidepressant Activity of HIIP

Effect of HIIP on FST induced depression in mice

HIIP was screened for antidepressant activity using FST induced depression 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 antidepressant activity. It was observed that the antidepressant activity of HIIP was dose dependent. The standard drug *fluoxetine* (10 mg/kg-i.p.) exhibited a significant antidepressant activity.

The observations are given in Table 2

Table 2: Effect of HIIP on FST induced depression in mice.

Treatment (mg/kg)	Immobility (second)	Climbing (second)	Swimming (second)
Vehicle control-10 ml/kg/p.o/b.wt for 14 days	107.9± 4.535	92.43± 2.776	99.28±6.841
HIIP 10 mg/kg/p.o/ b.wt for 14 days	87.24± 2.444*	101.4±1.456*	105.2±4.236
HIIP-30mg/kg/p.o/ b.wt for 14 days	78.75± 2.285**	105.7±2.349**	112.6±2.897
Fluoxetine-10 mg/kg/p.o/b.wt for 14 days	78.21± 2.484**	106.1±0.9667**	115.4±3.113

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.

In FST, mice were forced to swim in a restricted space from which there was no escape, and will, after periods of agitation, cease attempts to escape and become immobile. It is accepted that immobility is seen in rodents during swimming reflects behaviour despair as seen in human depression and that the antidepressant drugs are able to reduce the immobility time in mice [12]. The chronic treatment of the extracts significantly reduced the immobility time however it didn't show any effect on swimming suggesting role of nor adrenergic transmission in its antidepressant activity. This shows that the petroleum ether and methanolic extract of the holoptelea integrifolia possesses antidepressant activity and its specificity towards particular behaviour may depend on the dose of the extracts. There are reports to indicate that immobility, swimming and climbing behaviours are enhanced by different groups of antidepressant drugs[13]. The NE-selective uptake inhibitors like desipramine (DMI) and maprotiline (MAP) enhances the climbing behaviour where as the serotonin specific reuptake inhibitors (SSRIs) like fluoxetine (FLX), sertraline (SRT) and paroxetine (PRX) enhance swimming but not climbing behavior. However, both the types of antidepressants reduce immobility behaviour. Recent studies have shown that the dopaminergic activation is also involved in struggling (climbing) behaviour [14,15,16]

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, these phytosterols have been implicated in various pharmacological actions on central nervous system including antidepressant and anxiolytic activity [15, 16].

CONCLUSION

Majority of scientific documentation suggested prominent role of phytosterols towards antidepressant activity[15,16]. From the above data it is concluded that the isolated phytosterol compound, HIIP possesses significant antidepressant activity against Forced Swim Test (FST) induced depression in mice.

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