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"PHYTOCHEMICAL INVESTIGATION AND PHARMACOLOGICAL EVALUATION OF *LIMONIA ACIDISSIMA* LINN. STEM BARK EXTRACTS FOR ANTI-ANXIETY ACTIVITY"

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Abstract -The stem bark of *Limonia acidissima* Linn. were collected, processed and standardized as per official methods. Two extracts of *Limonia acidissima* Linn. stem bark (ethanol & acetone) were studied to detect the chemical compounds present, to evaluate acute oral toxicity & anti-anxiety activity. Phytochemical testing of extracts revealed presence of glycosides, flavonoids , carbohydrates etc. the total phenolic and flavonoid content of plant extracts was expressed as gallic acid equivalents and as rutin equivalents respectively. The result of acute oral toxicity studies of plant extracts as per standard references revealed median lethal dose (LD_{50}) could be greater than 2000 mg/kg body weight in rats. Accordingly safe experimental dose was calculated as 100 and 200 mg/kg .From the acute toxicity studies as per reference standard and on the basis of literature survey for acetone and ethanol extract of *Limonia acidissima* Linn. stem bark maximum and minimum therapeutic experimental safe dose was found to be 200 mg/kg and 100 mg/kg respectively. The evaluation of *in-vitro* anti-anxiety activity in rats was done using various experimental models. Light-dark test and Elevated plus Maze test were performed for the evaluation of anti-anxiety activity in the ethanol test rats showed the increased anti-anxiety activity at a concentration of 200mg/kg and 100mg/kg compared acetone and control group.

Key words: Anti-anxiety, *Limonia acidissima* Linn. , elevated plus maze, light & dark

1. INTRODUCTION

Anxiety is an emotional state, unpleasant in nature, associated with uneasiness, discomfort and concern or fear about some defined or undefined future threat. Some degree of anxiety is a part of normal life. Treatment is needed when it is disproportionate to the situation and excessive (K. D. Tripathi, 2013). Anxiety is an adaptive response to stress which helps one to cope with stressful situation. It involved an unwanted perception of insecurity accompanied with apprehension and fear (McNaughton, 2011).

Limonia acidissima Linn belongs to Rutaceae family. It is commonly known as 'wood apple '. It is an important member of the genus *Limonia L***.** It is ordinarily deciduous and commonly referred to as "Kovit". *Limonia acidissima* Linn. is native to India and also cultivated in Bangladesh ,Pakistan and Srilanka. The wood apple is native and common in dry plains. It prefers a mansoon climate with a distinct dry season.

The tree of *Limonia acidissima* Linn. is a moderate sized, deciduous, erect tree with a few upward reaching branches bending outward near the summit where they are subdivided into slender, branchlets drooping at the tips throughout India . It is a slow growing tree up to 9m tall, grows all over India in dry and warm areas up to 450m elevation, Often tree with rough, spiny bark. The spines are axillary, short, straight, 2-5 cm long on some of the zigzag twigs.

2. MATERIALS AND METHODS

2.1 Processing of crude drug-

The collected dried roots of plant were powdered and the powder was passed through appropriate sieve and subjected to extraction. Powdered drug is stored in air tight container for further use.

image no.1 - *limonia acidissima* linn. stem bark powder

2.2. Extraction of plant material

Selection of Solvent -Successive solvent extractions (soxhlet extraction) were employed for extraction. Solvents may be selected from non-polar to polar nature like Petroleum ether, Acetone and Ethanol etc. Solvent was selected by considering nature of phytoconstituents present in plant material. As per literature review and the nature of phytochemicals present in drug as well as on the basis of their polarity, the solvents were selected for the extraction of the stem bark of *Limonia acidissima* Linn. were petroleum ether, acetone and ethanol. On the basis of literature survey the extraction method was selected for extraction of the stem bark of plant of *Limonia acidissima* Linn. Continuous hot extraction method using soxhlet apparatus for petroleum ether, acetone and ethanol solvent.

2.3. Determination of Total phenolic & Flavonoid Content Total Phenolic Content-

2.3.1. Total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu reagent assay. An aliquot (1ml) of extracts or standard solution of gallic acid was added to 10ml volumetric flask containing 9 ml of distilled water. A reagent blank using distilled water was prepared. 0.5 ml of Folin-Ciocalteu phenol reagent was added the mixture and shaken. After 5 min 1.5 ml of 7% Na2CO3 solution was added to the mixture. The volume was then made up to the mark. After incubation for the 90 minutes at room temperature the absorbance against the reagent blank was determined at 637 nm with an UV-Visible spectrophotometer. Total phenolic content was expressed as mg Gallic Acid Equivalents (GAE).

2.3.2. Total Flavonoid Content

Total Flavonoid content was measured by the Aluminium chloride colorimetric assay. An aliquot (l ml) of extract or standard solution of Rutin was added to 10 ml volumetric flask containing 4 ml of distilled water. To the flask added 0.30 ml 5% NaNO2, after 5 minutes 0.3 ml 10% AlCl3 was added. After 5 min 2 ml 1M NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510nm. The total flavonoid content was expressed as mg Rutin Equivalent (QE).

2.4. Evaluation of anti-anxiety activity

Evaluation of **Anti-anxiety** activity was done by using following models;

2.4.1. Elevated plus maze test:

Principle: This model is based on natural behavior of rodents for open spaces and fear of height. Rodents always tend to avoid the open areas and stay in darker areas, more enclosed spaces. When animal is placed on EPM anxious animals spend more time in enclosed arms and non-anxious animals explore and spend more time on open arms. Anxiolytic compounds by decreasing anxiety, increases the open arm exploration time.

Animal Grouping

Group A - Control: DMSO (0.5%)
Group B - Standard: DIAZEPAM (2 mg/kg)
Group C - Test Dose 1: LAACE (100mg/kg)
Group D – Test Dose 2: LAACE (200mg/kg)
Group E – Test Dose 1: LAEEE (100mg/kg)
Group F – Test Dose 2: LAEEE (200mg/kg)

Procedure: The plus maze consist of two open arms, 50-10-40cm, and two enclosed arms, 50-10-40 cm, with an open roof, arranged so that the two arms were opposite to each other. The maze elevated to height of 50 cm. The rats (200-500 g body weight) were housed in pairs for 10 days prior to testing in the apparatus. During this time the rats were handled by investigator on alternate days to reduce stress. Group consisted of 6 rats for each dose. Thirty min after i.p administration of the test drug or the standard, the rat were placed in the center of the maze, facing one of the enclosed arms. During 5 min test period the following measures were taken: the number of entries and time spent in the open and enclosed arms; the total number of arms entries. The procedure was conducted in a sound attenuated room, with observations made from an adjacent room via a remote control camera (Gerhard Vogel, 2002).

Image no.2 -Elevated plus maze model

Evaluation : Evaluation of anti-anxiety activity was done by observing the parameters like number of entries in open and closed arm, time spent by the rats in open and closed arms and comparing these parameters with that of control group.

2.4.2. Light and Dark Model

Principle:

In a two chambered system, where the animals can freely move between a brightly-lit open field and a dark corner, they show more crossings between the two chambers and more loco motor activity after treatment with anxiolytics.

The numbers of crossings between the light and dark sites were recorded

Animal Grouping:

Group A - Control: DMSO (0.5% in water)

Group B- Standard: DIAZEPAM (2 mg/kg)

Group C - Test Dose-I: LAACE (100 mg/kg)

Group D- Test Dose-II: LAACE (200 mg/kg)

Group E- Test Dose -III: LAEEE (100mg/kg)

Group F- Test Dose -IV: LAEEE (200mg/kg)

Image no.3: Light-Dark Model

Procedure:

The testing apparatus consists of a light and a dark chamber divided by a photocell-equipped zone. A polypropylene animal cage, 44 × 21 × 21 cm, was darkened with black spray over one third of its surface. A partition containing a 13 cm long × 5 cm high opening separates the dark one third from the bright two thirds of the cage. The cage rests on an Animex® activity monitor which counts total locomotor activity. An electronic system using four sets of photocells across the partition automatically counts movements through the partition and clocks the time spent in the light and dark compartments. Naive male mice or rats were placed into the cage. The animals were treated 30 min before the experiment with the test drugs or the vehicle intraperitoneally and were then observed for 10 min. Groups of 6–8 animals were used for each dose. (H. Gerhard Vogel 2002)

Evaluation: Dose-response curves were obtained and the number of crossings through the partition between the light and the dark chamber was compared with total activity counts during the 5min.

3. Observation and results

table no. 3.1 physicochemical parameters of *limonia acidissima* stem bark

Sr. no	Standardization Parameters	Results (%w/w)
1	Ash value	8.5%
2	Acid insoluble ash	5.5%
3	Water soluble ash	6%
4	LOD	10%

3.1. Physicochemical parameters

table no 3.2 determination of extractive value of *limonia acidissima* linn.stem bark extracts

Sr.no.	Solvent	% values
1	Pet. ether	4%
2	Ethyl acetate	7.5%
3	**Acetone**	**10%**
4	Methanol	3%
5	**Ethanol**	**3.5%**
6	chloroform	5%

Result: Above observation table revels that acetone extract and ethanol extract have higher extractive value 10% and 3.5% respectively.

table no. 3.3determination of extractive yield of *limonia acidissima* linn. stem bark extract

Sr. no	Drug taken (g)	Solvent used (ml)	Consistency	Colour of extract	Yield (g)	% yield
1	250	Petroleum Ether 1200ml	Sticky	Dark brown	1.34	1.34%
2	250	Acetone 1200ml	Sticky	Brown	4.42	1.76%
3	250	Ethanol 1200ml	Sticky	Dark brown	5.5	2.2%

table no .3.4 total phenolic content of *limonia acidissima* linn.

Sr no.	Extract	Conc. (µg/ml)	Absorbance	TPC of test (mg GAE/gm)
1	Acetone	100	0.062±0.0023	10.3
2	Ethanol	100	0.084±0.0011	14

Result: Above table indicates that the ethanol extract of *Limonia acidissima* Linn. Contains more quantity of phenolic compounds 14 mg GAE/gm of dry weight of extract than acetone extract contains 10.3 mg GAE/gm of dry weight of extract of *Limonia acidissima* Linn. respectively equivalent to gallic acid.

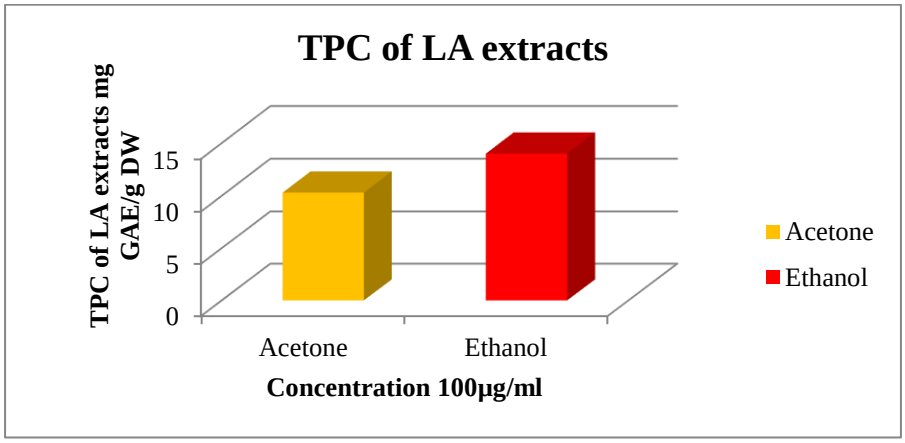

chart no.1-total phenolic content of *limonia acidissima*

3.5 Total flavonoid content of *Limonia acidissima* Linn.

Sr no.	Extract	Conc. (µg/ml)	Absorbance	TFC of LA (mg RU/gm)
1	Acetone	100	0.468	18.7
2	Ethanol	100	0.773	31.04

Result: Above table indicate that the ethanol extract of *Limonia acidissima* Linn. contains more quantity of flavonoid 31.04 mg RU/gm of dry weight of extract than acetone extract contains 18.07 mg RU/gm of dry weight of extract of *Limonia acidissima* Linn. respectively equivalent to rutin.

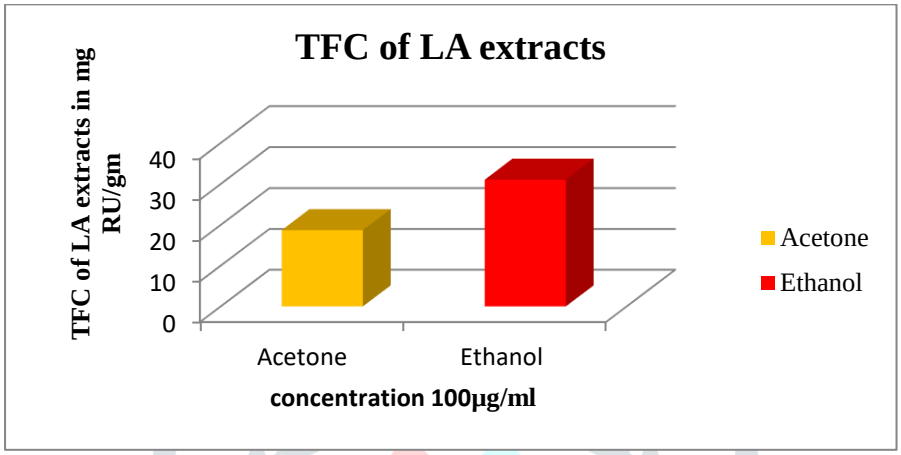

chart no.2 -total flavonoid content of different extracts of *limonia acidissima* linn.

table no.3.6 : *limonia acidissima* linn. stem bark extracts average readings from elevated plus maze model

Treatment	Time spent in (sec)			Number of entries	
	Open arm	Enclosed arm	Central zone	Open arm	Enclosed arm
Control	106.66±2.29	170.16±2.48	17.83±1.80	2.5±0.41	5.73±0.47
DIAZEPAM (2mg/kg)	209.83±1.56**	69.5±1.18**	22.66±0.85**	29.33±0.73**	9.3±0.42**
LAACE (100mg/kg)	165±1.98*	145±2.38*	19.33±0.86*	15.16±0.68*	3.14±0.47*
LAACE (200mg/kg)	185.16±2.67**	79.33±2.74**	22.5±0.74**	21.5±0.73**	3.63±0.33**
LAEEE (100mg/kg)	167±1.61**	138.5±1.44**	13.5±0.76**	14.5±0.40**	6.33±0.33**
LAEEE (200mg/kg)	197.66±1.33**#	72.83±1.60**#	20.5±0.74**#	22.33±0.82**#	7±0.36**#

Values are expressed as mean ± SEM (n = 6).**p <0.001,*p<0.05 v/s vehicle (One way ANOVA followed by Tukey's test.) #p>0.05 non-significant difference when compared with standard.The vehicle treated rat spent less time in open arm (106.66±2.29 s) and more time in enclosed arm (170.16±2.58 s) with 2.5±0.41 entries in open arm and 5.73±0.47 entries in enclosed arm. The LAACE (200mg/kg) & LAEEE (200mg/kg) show highly significant decrease in time spent in enclosed arm. Administration of LAACE(200mg/kg), LAACE (100mg/kg) & LAEEE (200mg/kg) & Diazepam (2mg/kg) show significant increase in number of entries in open arm. The LAACE & LAEEE (200mg/kg) and Diazepam (2mg/kg) show significant (p<0.001) increase in the occupancy in open arm indicating anti-anxiety activity of LAACE (200mg/kg) & LAEEE (200mg/kg) extract as compared to LAACE (100mg/kg) & LAEEE (100mg/kg) extract.

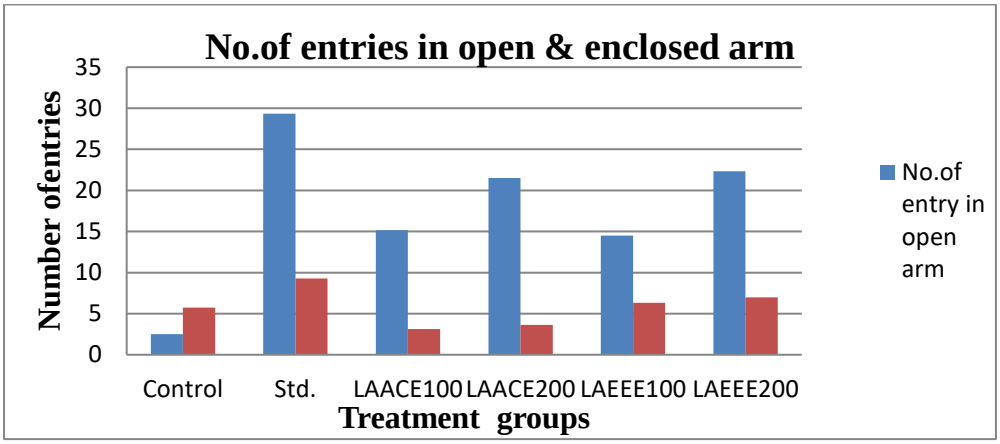

chart no. 3: total number of entries in open & enclosed arm in elevated plus maze test.

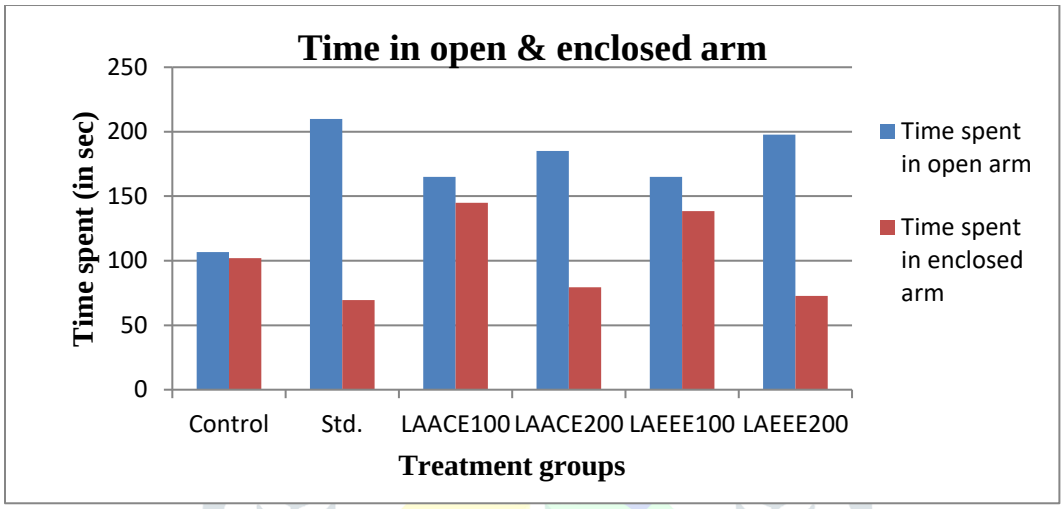

chart no. 4: time spent in open arm (in sec) in elevated plus maze test.

B) Light & Dark test

table no.21*: limonia acidissima* linn. stem bark extracts average readings from light & dark test

Treatment	No. of crossings	Time spent in (sec)		Transfer latency
		Dark zone	light zone	
Control	12 ± 2.8	190.5 ± 4.6	105.1 ± 3.6	14.8 ± 3.3
DIAZEPAM(2mg/kg)	31.33 ±2.2**	85.6 ± 4.17**	203.1 ± 3.6**	25.5± 1.5*
LAACE(100mg/kg)	20 ± 1.4*	160.2 ± 5.5*	131.1 ± 4*	19.3 ± 2.3*
LAACE(200mg/kg)	21.8± 1.3**	159.5 ± 4.8**	143.16± 4.2**	22 ± 3.2**
LAEEE(100mg/kg)	26 ± 3.3*	170.6± 3.3**	178.6 ± 4.3**	20.1 ± 1.1**
LAEEE(200mg/kg)	30.1 ±3.1**#	130.6 ± 5.5**#	196.2 ± 5.2**#	25± 1.3**#

Values are expressed as mean ± SEM (n = 6).**p <0.001,*p<0.05 v/s Vehicle (One-way ANOVA followed by Tukey's test.) The animal treated with LAACE (200mg/kg) & Diazepam (2mg/kg) show highly significant (p <0.001) and LAEEE (200mg/kg) show significant increase in time spent in light zone & decrease in time spent in dark zone. Administration of LAACE (200mg/kg) & LAEEE (200mg/kg) show significant decrease in time spent in dark zone as compared to the vehicle group. Animal treated with LAACE (200mg/kg) show increase in no. of crossing & transfer latency as compared to vehicle group & LAEEE (200mg/kg) in light & dark test indicating the anti-anxiety activity of LAACE (200mg/kg) & LAACE (100mg/kg) extract as compared to LAEEE (200mg/kg) & LAEEE (100mg/kg) extract.

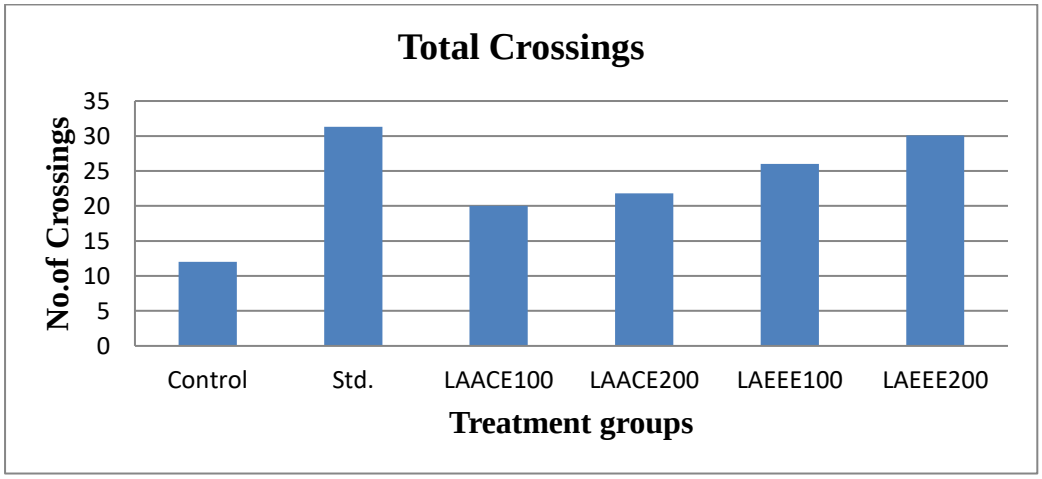

chart no.5: total crossings in light & dark test

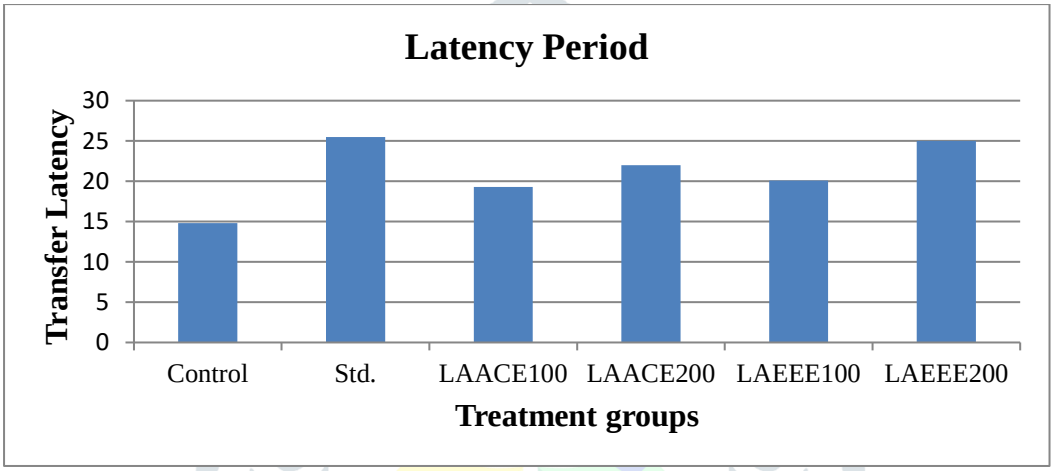

chart no 6: latency period in light & dark test

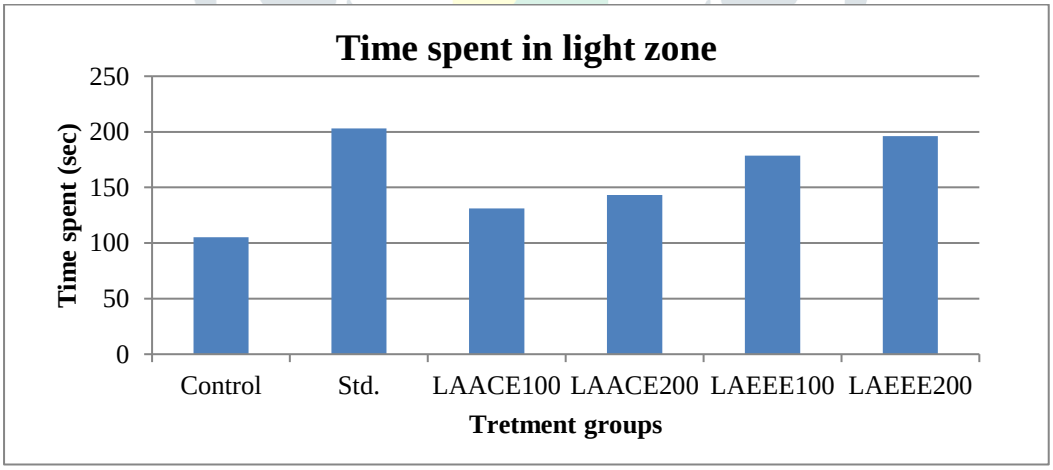

chart no 7: time spent in light zone

4. Result and conclusion :

- Extractive value of *Limonia acidissma* L.stem bark powder is more in acetone and ethanol than other solvent.
- Total Phenolic content of Ethanol extract of *Limonia acidissima* L.is higher than Acetone & Pet ether extract.
- Total flavonoid content of Ethanol extract of *Limonia acidissima* is higher than Acetone & Pet ether extract .
- Acetone and ethanol extract at 200 mg/kg concentration showed increased open exploratory time in rats as compared to control group. But when compared with standard drug diazepam acetone and ethanol extract at 200

mg/kg dose have very less difference. That is the acetone and ethanol extracts at 200 mg/kg shows near similar activity like diazepam in elevated plus maze model

- The ethanolic extract showed maximum locomotors activity at concentration of 200mg/kg . Acetone and ethanol extract at 200 mg/kg concentration showed increased light exploratory time in rats as compared to control group. But when compared with standard drug diazepam acetone and ethanol extract at 200 mg/kg dose have very less difference. That is the acetone and ethanol extracts at 200 mg/kg shows near similar activity like diazepam in Light & Dark test.

5. Acknowledgement

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