:: Hygeia
Journal for drugs and medicines
April 2012-September2012
OPEN ACCESS
www.hygeiajournal.com

A half yearly scientific, international, open access journal for drugs and medicines
Research article section: Pharmacognosy and Phytochemistry

Preliminary Phytochemical, antimicrobial and toxicity studies on *Clerodendrum paniculatum Linn* leaves

Praveen.M[1], Radha K[1*], Hari Kumar R[1], Padmaja V[1], Anne Mathew[1], and Ajith Kumar P[2]

1. College of Pharmaceutical Sciences, Medical College, Trivandrum, Kerala, India.
2. Sree Narayana Guru Institute of Medical Sciences, Ernakulum, Kerala, India.

Article history: Received: 29 November, 2011, revised: 20 December 2011, accepted: 9 January 2012, available online: 5 April 2012

Abstract

***Plan**: The present study was undertaken for investigating and scientifically validating the traditional claims of Clerodendrum paniculatum Linn for its therapeutic activity.*

***Methodology**: Phytochemical screening demonstrated the presence of carbohydrates, glycosides, and–tannins and Phenolic compounds in methanolic and aqueous extracts and sterols in all the extracts used. The brine shrimp toxicity assay was carried out on Petroleum ether, Chloroform, Ethyl acetate, Methanol and aqueous extracts using standard procedure. Brine shrimp assay (BSA) test showed LC_{50} of 400, 1600, 1700, and 1700 µg for the Petroleum ether, Chloroform, Ethyl acetate, Methanol extracts respectively. The degree of lethality was found to be directly proportional to the concentration of the extract. Maximum mortality rate of 100% was observed in the petroleum ether extract at 1600µg/ml while least mortality was observed in methanolic extract of 100 and 200µg/ml and 10% at 400 and 800µg/ml .*

***Outcome**: In summary petroleum ether extract has the highest mortality rate than compared to methanolic extract. The methanolic and chloroform extracts of the leaves of C.paniculatum showed effective antimicrobial activity against Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans.*

***Keywords:** Clerodendrum paniculatum leaves Artemia salina*, BSA method, antimicrobial activity, antifungal activity

1. Introduction

Ethnomedical importance of various species of *Clerodendrum* genus has been reported in various indigenous systems of medicines and folk medicines. The plant is being used in therapeutics, specifically in Indian, Chinese, Thai, Korean, Japanese systems of medicine for the treatment of various life threatening diseases such as HIV, syphilis, typhoid, cancer, jaundice and hypertension.[1.].The powder/paste form and the various extracts of root, stem and leaves are reported to be used as medicine for the treatment of asthma, pyreticosis, cataract, malaria, and diseases of blood, skin and lung. To prove these ethno-medical claims, some of these species are being extensively studied for their biological activities using various animal models.

For correspondence: bhatkradhajith@yahoo.co.in
Contact: +919446827686

Along with biological studies, isolation and identification studies of chemical constituents and its correlation with the biological activities of the genus has also been studied. The major chemical components reported from the genus are phenolic, steroids, di- and triterpenes, flavonoids, volatile oils, etc diseases . Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity . It facilitates pharmacological studies and leads to the synthesis of pure and potent compounds with decreased toxicity. Furthermore, the active components of herbal remedies have the advantage of being combined with many other substances that may be contraindicated with conventional foods or drugs[2].

Brine shrimp cytotoxicity assay[3]was considered an important tool for preliminary assessment of toxicity and have been used for detection of fungal toxins ,plant extract toxicity, heavy metals pesticide and cytotoxicity of dental metals[4]. It is also considered a veritable tool for the isolation of bioactive compounds from plant extracts. The method is simple ,detects small amount of toxins and can be performed in micro cells scale[5]. *Clerodendrum paniculatum* Linn is a shrub having 3-6 feet in height with large ever green leaves and huge showy clusters of orange red flowers held above the foliage, commonly known as Pagoda flower. The present study aimed primarily to identify the Phytochemical constituents, antimicrobial activities and cytotoxicity of the leaves of *Clerodendrum paniculatum* Linn. *(*Verbenaceae)

2. Materials and Methods

2.1. Plant material

Clerodendrum paniculatum Linn leaves were collected from localities of Medical College, Thiruvananthapuram. The Collected plants were carefully examined and authenticated by Dr. G. Valsaladevi, Curator, Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram. A voucher specimen (Voucher No. 5793) has been deposited for future reference.

2.2. Preliminary Phytochemical screening

2.3. Preparation of plant extracts [6]

Shade dried, powdered (40mesh size) leaves of *Clerodendrum paniculatum* Linn were successively macerated with Petroleum ether, Chloroform, Ethyl acetate, Methanol and Distilled water. All those extracts were then evaporated to complete dryness under vacuum. The extract was then weighed and calculated the percentage yield in terms of air-dried crude material.The percentage yield of various extracts of powdered leaves of *Clerodendrum paniculatum.Linn* were tabulated in Table No .1

2.4. Phytochemical Screening [6,7,8,9]

All the extracts obtained by successive maceration of the powdered leaves of *Clerodendrum paniculatum.* Linn (*with Petroleum ether, Chloroform, Ethyl acetate, Methanol and Chloroform water*) were subjected to various qualitative tests for the identification of different plant constituents present using the standard methods [7]

3. Determination of antimicrobial action

3.1. Preparation of Extract

For microbiological studies the dried aqueous extract was dissolved in water so that a concentration of 2000µg/ml was obtained. Likewise Methanolic extract was dissolved in 70% Methanol, Chloroform extract was dissolved in chloroform and Petroleum ether extract was dissolved in petroleum ether and all those solutions were adjusted to a concentration of 2000µg/ml. Control was also used for each solution with the solvent alone.

3.2. Microorganisms used

The test organisms used were *Staphylococcus aureus(*ATCC29737), *Pseudomonas aeruginosa*(ATCC9027), *Candida albicans*(ATCC10231)

Inoculum

The microorganisms were inoculated into SBCB and incubated at 35 ± 2°C for 4 h. The turbidity of the resulting suspension was diluted with SBCB to match with 1 McFarland turbidity standard. This level of turbidity is equivalent to approximately 3.0×10^8 CFU/ml.

4. Antimicrobial Activity.

4.1. Agar diffusion assay [10, 11]

Nutrient agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking 2 more times, rotating the plate approximately 60° each time to ensure even distribution of the inoculum. As a final step, the rim of the agar was also swabbed. After allowing the inoculum to dry at room temperature, 6-mm-diameter wells were bored in the agar .Each extract was checked for antimicrobial activity by introducing 100 µL of 2000µg/ml concentration into wells. Simultaneously, amikacin (*S. aureus and P. aeruginosa*), and clotrimazole (*C. albicans*) were used as positive controls at a concentration of 100.0 µg/ml and the dilution medium for the positive controls was methanol and chloroform. The plates were allowed to stand at room temperature for1hr for extract to diffuse into the agar and then they were incubated at 35 ± 2°C for 24 h, except for *C. albicans* which was incubated at 29 ± 2°C.

4.2. Brine shrimp Hatchability bioassay[12]

The brine shrimp Hatchability bioassay was carried out on Petroleum ether, Chloroform, Ethyl acetate, Methanol and Distilled water extracts using standard procedure. The cysts were hatched in seawater (1 g cyst per liter) at 28°C, under conditions of continuous illumination and strong aeration. After 2 h aliquots measuring 250 µl were placed in each tube where the extracts of various concentrations had previously been deposited, and they were incubated at the same conditions of temperature and illumination under gentle shaking.

After 12, 18, 24 and 48 h of exposure the free nauplii were counted under a stereoscopic microscope. The percentages of hatchability were calculated by comparing the number of free nauplii in each treatment with the number of free nauplii in the control.

The percentage of hatch inhibition (%HI) = % hatchability in the control - % hatchability in each treatment.

5. Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was carried out on Petroleum ether, Chloroform, Ethyl acetate, Methanol and Distilled water extracts using standard procedure.

Dried cysts were hatched (1 g cyst per litre) in a hatcher at 28–30°C with strong aeration, under a continuous light regime. Approximately 12 h after hatching the phototropic Artemia nauplii were collected with a pipette from the lighted side and concentrated in a small vial. Ten brine shrimp were transferred to each well using adequate pipette. Each test consisted of exposing groups of 10 Artemia nauplii aged 12 h to various concentrations of the drug extract. The toxicity was determined after 12 h, 24 h and 48 h of exposure. The numbers of survivors were counted and percentages of deaths were calculated.

Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation.

The percentage of mortality (% M) = percentage of survival in the control - percentage of survival in the treatment.

From all those extracts of various concentrations were prepared by serial dilution using DMSO as solvent. Each concentration was tested in triplicate, giving a total of 15 test-tubes. A control containing 5 ml of DMSO alone was used. The final volume of the solution in each test-tube were made up to 5 ml with sea water immediately after adding shrimp larvae.

5.1. Lethality concentration determination

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality. The number of dead and live napulii in each well was counted using a stereomicroscope. If deaths occurred in the solvent controls at the end of the treatment, the percentage of deaths were corrected using Abbott's formula.

$$\textit{Corrected mortality percentage} = (m\text{-}M) / S \times 100.$$

Where, m = mean percentage of dead larvae in treated tubes, M = mean percentage of dead larvae in solvent controls, S = mean percentage of living larvae in solvent controls.

Correction factor is applied to 0 and 100 percent mortality group. The percent mortality values are converted to probit values. LD 50 value can also be obtained from the best-fit line plotted between log of concentration and probit scale values.
Correction factor for 0% dead = 100 (0.25/n) and 100% dead = 100 (n – 0.25/n).

LD_{50} were expressed rate of mortality reflecting the fold decrease (up to 50% evidence of mortality in the nauplii test population) compared to control.

6. Results and Discussion

Preliminary Phytochemical screening

Table No.2 demonstrates Qualitative Phytochemical screening of various extracts of leaves of *Clerodendrum paniculatum.* Linn carried out and indicates the presence of carbohydrates, glycosides and tannins and phenolic compounds in methanolic and aqueous extract and sterols in all the extracts used.

Antimicrobial activity

Table No 3 demonstrates the anti microbial activity of various extracts of *Cleodandrum paniculatum* against some pathogenic micro organism namely Pseudomonas *aeruginosa, Staphycoccus aureus and Candida albicans.*

Antibacterial Activity

The chloroform extract had great in vitro potential for anti microbial activity against tested micro-organism used during the analysis. Amikacin at the concentration of 100 µg/ml was used as control for antibacterial.

Antifungal Activity

Antifungal activities against *Candida albicans*. The results were compared with standard drug clotrimazole at a concentration of 100µg/ml. the aqueous and Pet. Ether extracts does not possess any antifungal activities. Antifungal activity studies on various extracts shows that Methanolic and Chloroform extracts possess significant antifungal activity.

In the current research, the antimicrobial and general toxicity with brine shrimp toxicity assay of various extract of leaves of *Clerodendrum paniculatum*.Linn (Verbenaceae) was evaluated.

Brine shrimp Hatchability bioassay

Table No.4 demonstrates Brine shrimp *[Artema salina]* Hatchability Assay of petroleum ether chloroform, ethyl acetate, methanolic and aqueous extract showed no strong hatch inhibition.The result indicates that the leaves of *Cleodandrum paniculatum* in various solvents of extract used is non-toxic. The degree of lethality was found to be directly proportional to the concentration of the extract.

Maximum mortality rate of 100% was observed with 1600 µg/ml of Petroleum Ether extract and minimum or least mortality rate was recorded for 400 µg/ml with mortality rate of 10 for methanolic and ethylated extract respectively and no mortality was recorded for 100 µg/ml of various solvent extract used. In summary, petroleum ether extract has highest mortality rate when compared to aqueous and methanolic extract.

Brine shrimp lethality bioassay

Table 5 demonstrates the result of activity for the analyzed petroleum ether, chloroform, ethyl acetate, methanolic and aqueous extract against brine shrimp larvae. The brine shrimp lethality results are interpreted as follows.

LC_{50} < 1.0µg/ml –highly toxic; LC_{50} 1.0-10µg/ml toxic; LC_{50} 10-30 µg/ml moderately toxic;

LC_{50} >30<100 µg /ml mildly toxic and LC_{50} >100 µg/ml as non toxic[12,13].

The LC_{50} was calculated from the dose response curve of concentration of the drug vs. percentage mortality.The LD_{50} of the extract was determined from log dose response curve of log dose Vs.probit values. The degree of lethality was found to be directly proportional to the concentration of the extract. Maximum mortality rate of 100% was observed in the petroleum ether extract 1600µg/ml while least mortality was observed in methanolic extract of 100 and 200µg/ml and 10% at 400 and 800µg/ml.

In summary petroleum ether extract has the highest mortality rate than compared to methanolic extract. The brine shrimp lethality assay indicates various degrees of toxicity.The result of these study revealed that the toxic activity of the plant extract decreases with increase in polarity of solvent. This can be seen in terms of the polarity of compound extracted by each solvent in addition to their intrinsic bioactivity and by ability to dissolve or diffuse in different solvent media used in assay.

The Methanolic and chloroform extract showed great potential for antimicrobial activity against gram positive and gram negative organism used. The Methanolic and chloroform extract possess significant antifungal activities against *Candida albicans* compared with standard drug clotrimazole.Also result of Phytochemical screening showed the presence of sterols, tannins, sugars, glycosides etc. in the solvents used. The presence of these secondary metabolites confirms plant use for pharmaceutical manufacturing and drug discovery.

Conclusion

While the brine shrimp assay may be inadequate for elucidation of mechanism of action, yet is a convenient way of monitoring biological activities of plant used in traditional medicines. The result of this study justifies the use of *Clerodendrum paniculatum* Linn as traditional medicine. Our results indicate that, the plant extract is non toxic. There is a need for further studies on this plant to ascertain the active compounds so as to maximize the widely used medicinal plant in the development of antimicrobial drugs.

Table No: 1The color, nature and percentage yield of various extract of Powdered Leaves of *C. paniculatum.* Linn.

Sl. no	*Extracts*	*Colour*	*Nature*	*Percentage yield (%w/w)*
1	Petroleum Ether	Yellow green	Sticky	1.7
2	Chloroform	Dark green	Amorphous	2.15
3	Ethyl acetate	Green	Sticky	1.28
4	Methanolic	Brown green	Sticky	2.22
5	Aqueous	Reddish brown	Sticky	2.26

Table No:2 Qualitative Phytochemical Screening of Various Extracts of Leaves of *C. paniculatum.* Linn

Sl.no	*Phytoconstituents*	*Petroleum ether extract*	*Chloroform extract*	*Ethyl acetate extract*	*Methanolic extract*	*Aqueous extract*
1	Alkaloids	-	-	-	-	-
2	Carbohydrates and Glycosides	-	-	-	+	+
3	Proteins and amino acids	-	-	-	-	-
4	Sterols	+	+	+	+	+
5	Fixed oils and fat	-	-	-	-	-
6	Tannins and phenols	-	-	-	+	+
7	Triterpenoids	-	-	-	-	-
8	Saponins	-	-	-	-	-
9	Gums and mucilages	-	-	-	-	-
10	Flavones and flavanoids	-	-	-	-	-

+ = Positive ; -- = negative

Table No.3 .Antibacterial activity studies on the various solvent extracts of the Leaves of *C. paniculatum*

Group	*Pseudomonos auerignosa*	*Staphylococcus aureus*	*Candida albicans*
Control	14. 96 ± 0. 33	14. 96±0.33	12. 0± 0. 45
Standard	18. 8± 0. 45	18. 8± 0. 45	17. 0± 0. 8
Chloroform(5%)	16.7± 0.23	15. 3± 0. 32	14.3± 1. 5
Chloroform (10%)	17.7± 0. 22	16. 4± 0. 4	15. 2±0.1
Methanol (5%)	18. 1 ± 0. 8	8. 5± 0. 5	16. 2± 0. 5
Methanol (10%)	16. 2 ± 0. 5	9. 3± 0. 4	16.2±0. 5

Mean±SD (n=3)Zone of inhibition of test organism (mm)

Table No.4: Brine Shrimp Hatchability Bioassay

Plant extract	*Conc. (µg/ml)*	*Number of brine shrimp per test*				*% Hatchability*				*% Hatchability Inhibition*			
		Time in hours				*Time in hours*				*Time in hours*			
Control	5	12	18	24	48	12	18	24	48	12	18	24	48
		0	20	20	20	0	100	100	100	0	0	0	0
	100	0	12	10	8	0	60	50	40	0	40	50	60
	200	0	10	9	6	0	50	45	30	0	0	50	70
	400	0	8	6	5	0	40	30	25	0	60	70	75
Petroleum ether extract	800	0	6	5	2	0	30	25	10	0	70	75	90
	1600	0	0	0	0	0	0	0	0	0	100	100	100
	100	0	15	15	12	0	75	75	60	0	25	25	40
	200	0	12	10	10	0	60	50	50	0	40	50	60
	400	0	10	10	8	0	50	50	40	0	50	50	60
Chloroform extract	800	0	10	8	7	0	50	40	35	0	50	60	65
	1600	0	5	0	0	0	25	0	0	0	75	100	100
	100	0	12	10	10	0	60	50	50	0	40	50	50
	200	0	10	10	7	0	50	50	40	0	50	50	60
	400	0	8	8	8	0	40	40	35	0	60	60	65
Ethyl acetate extract	800	0	7	6	4	0	35	30	20	0	65	70	80
	1600	0	65	70	80	0	0	0	0	0	100	100	100
	100	0	20	20	16	0	100	100	80	0	0	0	20
	200	0	15	15	12	0	75	75	60	0	25	25	40
‘	400	0	12	10	10	0	60	50	50	0	40	50	50
Methanolicextract	800	0	8	4	0	0	50	50	40	0	50	50	60
	1600	0	8	6	0	0	40	20	0	0	60	80	100
	100	0	20	20	20	0	100	100	100	0	0	0	0
	200	0	20	20	18	0	100	100	90	0	0	0	10
	400	0	18	17	15	0	90	85	75	0	10	15	25
Aqueous extract	800	0	14	12	10	0	70	60	50	0	30	40	50
	1600	0	8	6	0	0	40	30	0	0	60	70	10

Table No.5 Brine Shrimp Lethality Bioassay

Plant Extract	*Conc. (µg/ml)*	*Log Conc.*	*Initial No. of larvae*	*Total Death*	*Total Survivors*	*% mortality (mean)*	*Corrected Percentage*	*Probit Value*
				1 2 3	1 2 3			
	100	2	10	10 9 10	0 0 0	0	0.25	3.04
Petroleum Ether	200	2.3	10	8 8 7	2 2 2	20	20	4.16
	400	2.6	10	5 5 6	5 6 5	50	50	5.00
	800	2.9	10	2 3 2	8 8 9	80	80	5.84
	1600	3.2	10	0 0 0	10 10 9	100	100	6.96
	100	2	10	10 10 9	0 0 0	0	0	3.04
Chloroform	200	2.3	10	10 10 10	0 0 0	0	0	3.04
Extract	400	2.6	10	8 8 9	2 2 2	20	0	4.16
	800	2.9	10	7 7 8	3 3 3	30	0	4.48
	1600	3.2	10	5 5 6	5 4 5	50	0	5.00
	100	2	10	10 9 10	0 0 0	0	0	3.04
Ethyl acetate	200	2.3	10	10 10 9	0 0 0	10	0	3.04
Extract	400\]	2.6	10	9 9 10	1 1 0	30	10	3.72
	800	2.9	10	7 8 7	3 2 3	40	30	4.48
	1600	3.2	10	6 7 8	4 4 3	0	40	4.75
	100	2	10	0 0 0	0 0 0	0	0	3.04
Methanolic	200	2.3	10	0 0 0	0 0 0	0	0	3.04
Extract	400	2.6	10	9 9 8	1 2 2	10	10	3.72
	800	2.9	10	9 9 8	1 2 2	10	10	3.72
	1600	3.2	10	6 7 6	4 3 2	40	40	4.75

Figure 1: LC 50 value of Pet. Ether extract = 400 µg/ml

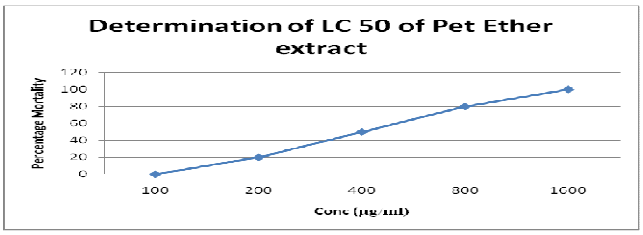


Figure 2: LC 50 value of Chloroform extract = 1600 µg /ml

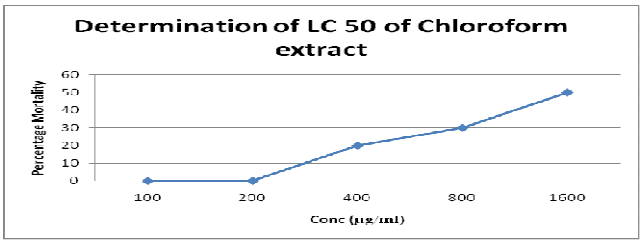


Figure 3 : LC 50 value of Ethyl acetate Extract– 1700 µg/ml

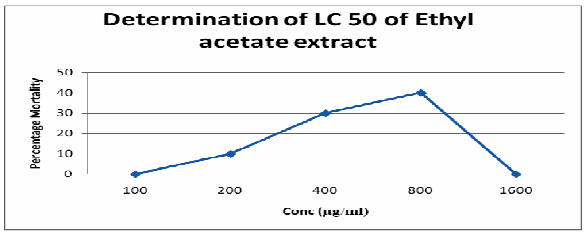


Figure 4: LC 50 value of Methanol Extract– 1700 µg/ml

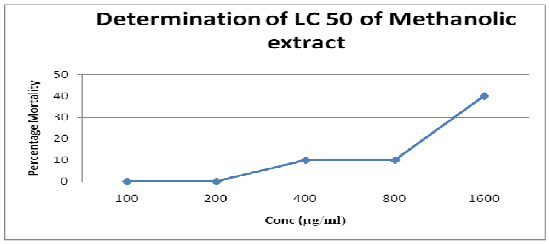


Figure 5: LD 50 value of Pet. Ether extract = 400 µg

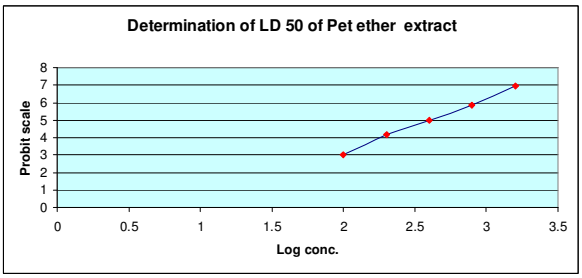


Figure 6:LD 50 value of Chloroform extract = 1600 µg

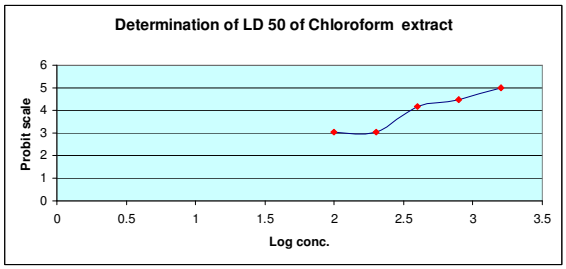


Figure 7: LD 50 value of Ethyl acetate Extract– 1700 µg

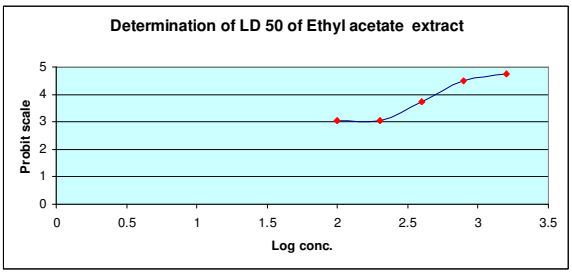
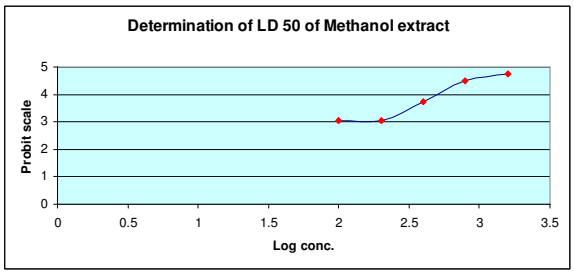


Figure 8:LD 50 value of Methanol Extract– 1700 µg



Acknowledgment

We sincerely thank Mrs.Leena Joy, Sree Narayana Guru Institute of Medical Sciences, Ernakulum, for all her assistance in editing this article.

References

1. Neeta Shrivastava, Tejas Patel, *Clerodendrum* and Heathcare: An Overview, *Medicinal and Aromatic Plant Science and Biotechnology* Global Science Books 1(1), **2007**,142-150.
2. Alfred Byrd Graf, Tropica, Color Cyclopedia of exotic plants and trees, II edition, Roehrs Company Publishers, New Jersey,USA. **1981**. pp- 913, 917.
3. Mukhtar MD, Sani A, Yakasai AA. Cytotoxicity of fractions of *Pista sreatiotes* on larvae of Culex mosquito and *A. salina*. *Animal Research International*, 2004, (2):95-9.
4. Bonjar GH. Antibacterial screening of plants used by Iranian folkoric medicine. *Fitoterapia,***2004**,75:231-35.
5. Alluri V, Krishnaraju TN, Rao D, *et al*. Assessment of bioactivity of Indian medicinal Plants using brine shrimp (*Artemia salina*) lethality assay. *International Journal of Applied Sciences and Engineering*, **2005**, 3(2)**:**125-34.
6. J.B. Harborne, Phytochemical methods- A guide to Modern techniques of plant analysis, 3 ed. Springer, New Delhi.1- 32.
7. Kokate CK. Practical Pharmacognosy; fourth edition; reprint **2001**; Vallabh Prakasan, Delhi, p. 107-111, 123-125, 130.
8. Patra Arjun, Jha Shivesh, Narasimha Murthy. P, Pharmacognostical standardization of leaves of *Hygrophila Spinosa* T.Anders. *Phcog j,* **2009***,* Vol.1, 2, p 82-87.
9. Khandelwal.K. R, Practical Pharmacognosy- Techniques and Experiments, Nirali Prakasan, Pune. **2003**, p.149-156.
10. Chenthurpandy. P, Kalidass C, Mohan V.R, (2009), Pharmacognostical investigation of Hiptage benhalensis (L.) Kurz. (Malpighiaceae), *Phcog j*, **2009**,Vol.1, 2, p 103-105.
11. Mariela González, Bernardo Guzmán 2, Roxana Rudyk 1,Elida Romano 1 & María A.A. Molina, Spectrophotometric Determination of Phenolic Compounds in Propolis, *Lat. Am. J. Pharm.***2003***,* 22 (3): 243-8121.
12. Jose Luis Carballo, Zaira L Hernandez-Inda, Pilar Perez and Maria D Garcia-Gravalos, 2002, Methodology article-A comparison between two brine shrimp assays to detect *in vitro*cytotoxicity in marine natural products, *BMC Biotechnology,* **2002**, 2:17
13. Abdullahi Shafiu Kamba1 and Lawal Gusau Hassan, Antibacterial Screening and Brine Shrimp (*Artemia salina*)Toxicity of *Securidaca longepedunculata* (Polygalaceae) Root Bark ,*African Journal of Pharmaceutical Sciences and Pharmacy*;**2010**,1:1:85-95.