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ETHNOPHARMACOLOGICAL EVALUATION OF CALOTROPIS GIGANTEA

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ABSTRACT

Calotropis gigantea, commonly known as milkweed or swallow-wort, is a common wasteland weed (Singh et al. 1996). *Calotropis* belongs to Asclepiadaceae or Milkweed or Ak family which includes 280 genera and 2,000 species of world-wide distribution but most abundant in the sub-tropics and tropics, and rare in cold countries. *Calotropis gigantea* Linn. (Asclepiadaceae) a widely growing plant has been reported to possess number of medicinal properties. The aim of the present study was to screen leaves of *Calotropis gigantea to study & compare* Antioxidant, Antibacterial, Anti-inflammatory & wound healing Activity of *Calotropis gigantea* Linn. Calotropis gigantea, with ethanolic extract. It has been reported as a traditional folk medicine for a variety of aliments. The stages of wound healing are inflammatory phase, proliferation phase, fibroblastic phase and maturation phase. Extract treated animals exhibit 83.42 % shows the Anti-inflammatory area when compared to controls which was 76.22 %. The extract treated wounds are found to epithelize faster as compared to controls. The plant *C. gigantea* is also used in some parts of India for wound healing in combination with other plants. However there are no scientific reports on wound healing activity of the plant *C.gigantea*. The oral administration of 400mg/kg of *C.gigantea* and 300 mg/kg of *C.gigantea* were showed significant anti-inflammatory activity more than that of 100mg/kg of Ibuprofen. This study also proved the greater anti-inflammatory action due to the effect of *C. gigantea* with Acetyl Choline than Acetyl Choline alone.

KEY WORDS: Antioxidant, Antibacterial, Muscle Relaxant Activities, Calotropis gigantea, Eethanolic extract, Acetylcholine.

INTRODUCTION

Medicinal plants are usually of medicine for the treatment of leprosy, ulcers, tumors, used for Ayuerbedic, Unani and other rural areas. Recent discovery shows that these plants have fewer side effects than the Allopathic medicine. So, herbal medicine becoming popular for medication among whole over the world. The number of plants with medicinal properties included in the Materia Medica of traditional medicine in this subcontinentat present stands at about 2000 [1]. More than 500 of such medicinal plants have so far been enlisted as growing in Bangladesh [2]. Thus the human race started using plants as a means of treatment of diseases and injuries from the early days of civilization on earth and its long journey from ancient time to modern age the human race has successfully used plants products as effective therapeutic tools for fitting against diseases and various other health hazards [3]. *C. gigantea* is a common wasteland weed found abundant throughout India right from Himalayas to southern India. *C. gigantea* was regarded as a useful medicinal plant and used in folk medicine1, 2. Traditionally it is used for the treatment of different aliments in ayurvedic and unani systems of medicines. The plant has been known as “Vegetable Mercury” since it is used as a remedy for syphilitic affections, also advocated for a variety of diseased conditions including leprosy, ulcers, tumours and piles. The plant is reported to have diverse pharmacological actions like antifertility, cardiotonic, antimicrobial activities4. The Ethenolic extract of the root has been shown to exhibit protective activity against carbon tetrachloride induced liver damage [10]. Methanol extract possess antioxidant activity in Trema orientalis [11] and Senna tora[12]. So, the present work was designated to investigate the antioxidant and antibacterial activities of of Calotropis procera Linn to know the scientific basis of ascorbic acid were weighed three times and dissolved in ethanol to make the required concentration by dilution technique. Here ascorbic acid was taken as standard. DPPH was weighed and dissolved in ethanol to make 0.005% (w/v) solution. To dissolve homogeneously magnetic stirrer was used. After making the desired concentration 4 ml of 0.004 DPPH solution was applied on each test tube by pipette. The room temperature was recorded and kept the test tubes for 30 mins in light to complete the reactions. DPPH was also applied on the blank test tube at the same time where only ethanol taken as blank. [13]

MATERIALS AND METHODS

PLANT MATERIALS

Fresh leaves of *Calotropis gigantea* were collected from Lucknow local area of 2011. The

plant was identified by the expert of National Botanical Research Institute (NBRI), Lucknow and a voucher specimen was kept for future reference. The dried leaves of *Calotropis gigantea* were ground into a fine powder with the help of suitable grinder. About 400 g of powder grinded material was extracted by soxhlet apparatus with 90% methanol at 55°C temperature.

PROCESSING OF THE PLANT

Plant leaves were collected and washed properly with distilled water. The leaves were shade dried at room temperature. Dried leaves were uniformly grinded using mechanical grinder. The leaves powder was extracted in distilled water.

Fig 1. crude Leaves and Dried Powder of *Calotropis gigantean*

Ten gram of plant powder was soaked in 100 ml of distilled water in a conical flask and loaded on an orbit shaker at a speed of 120 rpm for 24 hours. The mixture was filtered using Whatman filter paper number 1. The filtrate was concentrated using rotary evaporator and dried using lyophilizer. Dried extract was collected in an air tight container and stored at 4°C. The extracted powder was dissolved in sterilized distilled water to make 1000 µg/ml solution. This mixture was used to perform antibacterial assay.

TEST MICROORGANISM

The following six clinical isolates of bacteria were used for the study: *S. aureus, K. pneumoniae, B. cereus, P. aeruginosa, M. luteus* and *E. coli*. All these cultures were maintained on nutrient agar plates at 4°C.

ANTIOXIDANT ACTIVITIES

Antioxidant potential of the Ethanolic extract was determined on the basis of their scavenging activity of the stable 1,1- diphenyl-2-picryl hydrazyl free radical. DPPH method is most widely used and easiest method to determine Antioxidant activity DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. [13] The aliquots of the different concentrations (1-500 µg/ml) of the extract was added to 3 ml of a 0.004 %w/v solution of DPPH. Absorbance at 517 nm was determined after 30 min, and IC50 (Inhibitory concentration 50%) was determined. IC50 value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals. At first 6 test tubes were taken to make aliquots of 6 conc.(1,5,10,50,100,500 µg/ml).Plant extract and ascorbic acid were weighed 3 times and dissolved in ethanol to make the required concentration by dilution technique. Here ascorbic acid was taken as standard. DPPH was weighed and dissolved in ethanol to make 0.004% (w/v) solution. To dissolve homogeneously magnetic stirrer was used. After making the desired concentration 4 ml of 0.004 DPPH solution was applied on each test tube by pipette. The room temperature was recorded and kept the test tubes for 30 mins in light to complete the reactions. DPPH was also applied on the blank test tube at the same time where only ethanol taken as blank. After 30 mins, absorbance of each test tubes were determined by UV spectrophotometer. IC50 was determined from % inhibition vs. concentration graph. [14,15]

ANTIMICROBIAL ACTIVITY

DISC DIFFUSION METHOD

The extracts of C.gigantea leaf obtaind by maceration process by using water as a solvent. Extracts were screened for antimicrobial activity using by disc diffusion method . A suspension of organism was added to sufficient quantity of nutrient agar at 45C. The mixture was aseptically transferred to sterile petri dish and allowed to solidify. The overnight culture grown in broth was used for inoculation. The plant extracts to be tested were prepared in various concentrations i.e. 25%, 50%, 75% and 100%. The sterile impregnated discs with plant extracts were placed on the agar surface with framed forceps and gently pressed down to ensure complete contact of the disc with agar and dextrose surfaces. Positive control discs were also prepared in the same manner using Ampicillin, a bactericide. But it was not used for fungi. The prepared control discs were placed using respective solvents. [16,17]

All the plates including control plates were incubated at 37°C for 24 hours. After incubation, the size (diameter) of the inhibition zones was measured. Triplicates were maintained for each sample of the extract respectively. The results were expressed in terms of the diameter of the inhibition zone: <9 mm - inactive; 9-12mm - partially active; 13-18mm - active; >18mm - very active . After the confermation of antibacterial activity with 100mg/kg dose the experiment was carried out in triplicate and average values were taken into consideration. Similar procedure was carried out with standard drug Ampicillin 100mg/ml. And the zone of inhibition was compared with test sample and control and the percentage of inhibition was calculated which Are given below in table. [16,18]

PHARMACOKINETIC PARAMETER OF ANTIMICROBIAL ACTIVITY

MINIMUM BACTERICIDAL CONCENTRATION

The Minimum Bactericidal Concentration (MBC) is the lowest concentration of antibiotic required to kill the germ not as commonly seen as the Minimum inhibitory concentration (MIC). It can be determined from broth dilution MIC tests by sub culturing to agar media without antibiotics. The minimum bactericidal concentration (MBC) is the lowest dilution where the culture has been completely sterilized. It is not routinely determined. Treatment decisions are made related to MICs, and more specifically, the breakpoint MICs.[19]

MINIMUM INHIBITORY CONCENTRATION

The minimum inhibitory concentration (MIC) is the concentration required to inhibit growth of a specific isolate in vitro under standardized conditions. It is determined by finding the lowest dilution without visible growth during serial dilution testing. In microbiology, is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. An MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. [19.20]

DETERMINATION OF RELATIVE PERCENTAGE INHIBITION

The relative percentage inhibition of the test extract with respect to positive control was calculated by using the following formula 6, 11.

Relative percentage inhibition of the test extract =

$$\frac{100 \times (x-y)}{(z-y)}$$

Where,

x: total area of inhibition of the test extract

y: total area of inhibition of the solvent

z: total area of inhibition of the standard drug

The total area of the inhibition was calculated by using

area = πr2; where, r = radius of zone of inhibition. [16,17]

EFFECT OF EXTRACT FROM *CALOTROPIS GIGANTEA* ON THE SKELETAL MUSCLE OF THE RAT

Since the antimigraine drugs were reported to have muscle relaxant activity, so this experiment was attempted to assess the effect of extract from *C. gigantea* on the rat rectus abdominis muscle preparation. The experiment was carried as per the method described by Kulkarni 5. rat weighing 20-25 g were used in this study. The rat was stunned and decapitated and the spinal cord was destroyed.

A rat was pithed and the skin of the anterior and abdominal wall was cut by a midline incision and then it was cut laterally to expose the anterior abdominal wall. [18,19]

The two rectus were seen running from the base of sternum. The muscles were cut across just above the sternum at its base and the pair of muscles attached to it were dissected and transferred to a dish containing frog ringer solution at room temperature. The muscles were then carefully cleaned and one of them was trimmed to the desired size and mounted in an organ bath filled with ringer solution at room temperature and aerated by stream of fine bubbles emerging near the bottom of the bath. Isotonic contractions were recorded using gimbel lever with a sideways writing point. The lever was balanced for a tension of approximately 2-5g. An extra load of approximately 1g on the long arm was supplied because sometime the lever may not return to the base line after washing. [18, 20]

The drug period allowed for stabilization was 30 minutes during which the muscle was subjected to 1g stretch. At 0th min - the kymograph was started after raising the extra load; in the 1st min- the drug was added and in the 2nd min- the kymograph was stopped. The tissue

was washed and allowed to relax by applying an extra load. At the 5th min- the lever point was brought to the base line and the next cycle was started. After recording the graded responses to different log dose of acetylcholine, the test drug (Extract) was added and their effects upon acetylcholine induced contractions as well as the effect of its own in the tissue was studied. [21]

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MICs can be determined by agar or broth dilution methods usually following the guidelines of a reference body such as the CLSI, BSAC or EUCAST. There are several commercial methods available, including the well established Etest strips and the recently launched Oxoid MIC Evaluator method.

The Etest system comprises a predefined and continuous concentration gradient of different antimicrobial agents, which when applied to inoculated agar plates and incubated, create ellipses of microbial inhibition. The MIC is determined where the ellipse of inhibition intersects the strip, nand is easily read off the MIC reading scale on the strip. [20.21]

RESULTS AND DISCUSSION

FOR ANTIOXIDANT ACTIVITIES TEST

In the present study, methanol extracts of the leaves of *C. procera* showed potential free-radical scavenging activity but aqueous extract showed very little free-radical scavenging activity.

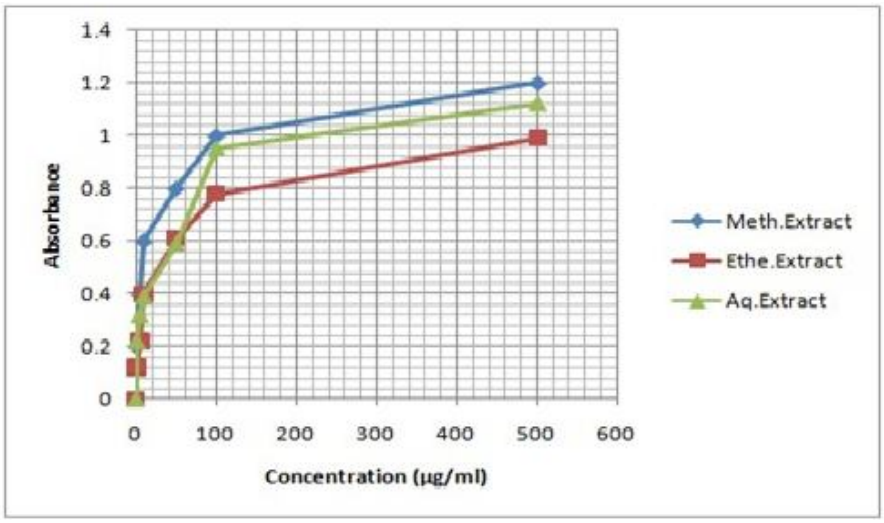

Fig. 2 Comparison of Antioxidant activity of Various Extract

FOR ANTIMICROBIAL ACTIVITIES TEST

The bacterial suspensions were seeded on MHA plates using a sterilized cotton swab. In each of these plates four wells were cut out using a standard cork borer (7 mm). Using a micropipette, 100 µl of each dilution was added in to wells. All the plates were incubated at 37ºC for 24 hours. Antimicrobial activity of the leaf extract was evaluated by measuring the zone of inhibition. Experiment was carried out in triplicates for each test organism.

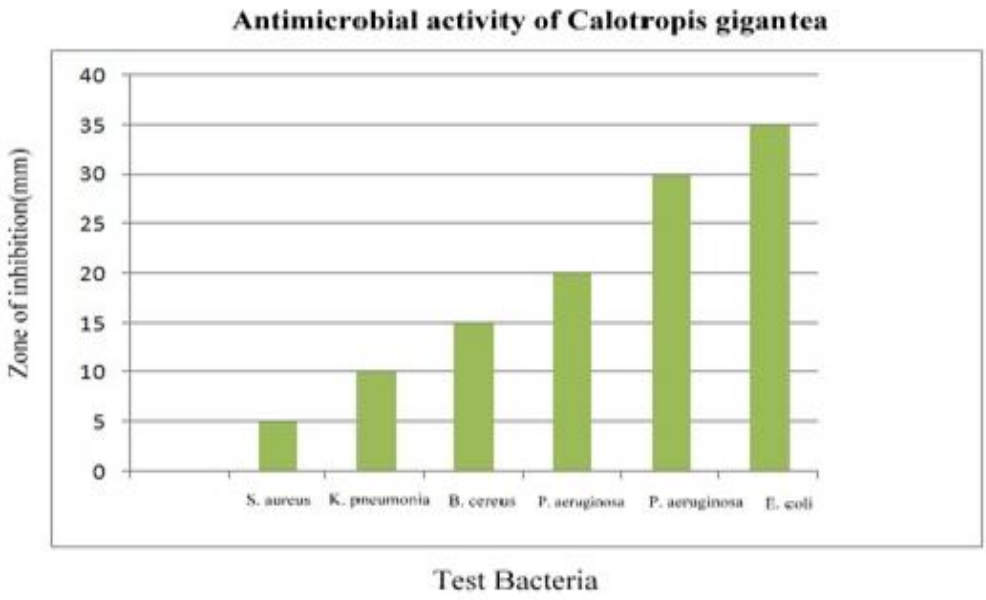

Figure 3: Antimicrobial activity of *Calotropis gigantean*

Table 1: Test organisms Relative percentage inhibition (%)

Test organisms	Relative percentage inhibition (%)
Staphylococcus aureus	48.05
Klebsiella pneumoniae	75.64
Bacillus cereus	175.36
Pseudomonas aeruginosa	108.16
Micrococcus luteus	26.67
Escherichia coli	155.89

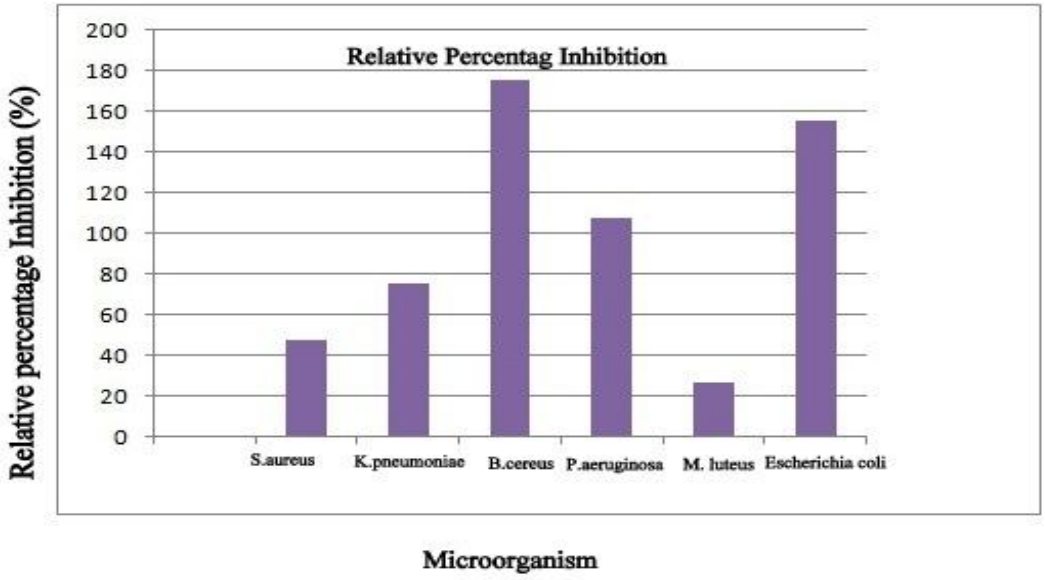

Figure 4: Relative percentage inhibition of *Calotropis gigantean*

Table 2: MIC values of methanol and aqueous extracts of *Calotropis gigantea* on test organisms

MIC in mg/ml					
S. aureus	***K. pneumonia***	***B. cereus***	***P. aeruginosa***	***M. luteus***	***E. coli***
52	23	5.25	4.1	1.7	11.65

Table -3 Antimicrobial activity of *Calotropis gigantea*

Test organisms	**Inhibition zone diameter (mm)**		
	AE	**PC**	**NC**
Staphylococcus aureus	14.3±1.15	18.6±1.52	0
Klebsiella pneumoniae	13.6±1.52	13.3±0.57	0
Bacillus cereus	16.3±1.52	15.6±1.15	0
Pseudomonas aeruginosa	14.0±1.73	16.6±1.52	0
Micrococcus luteus	16.6±1.52	33.3±1.52	0
Escherichia coli	18.6±1.15	16.6±2.08	0

Results of MIC are reported in Table . The crude extract showed 52, 23, 11.65, 5.25, 4.1 and 1.7 mg/ml MIC values for *S. aureus, K. pneumoniae, E. coli, B. subtilis, P. aeruginosa* and *M. luteus* respectively.

Aqueous extract of *Calotropis gigantean* showed high inhibitory activity followed by methanol extract, where as ethanol and petroleum ether extracts showed low activity 6. Previous studies report the presence of phytochemicals like cardenolides, flavonoids, terpenes, pregnanes, nonprotein amino acid and cardiac glycoside as major constituents in *C. gigantean* may acknowledge the medicinal property of this plant.

FOR MUSCLE RELAXANT ACTIVITIES TEST

The Aqueous and Ethanolic Extract of *C. gigantea* was found to have skeletal muscle relaxant property at T1 (1:100), T2 (1:500) and T3 (1:1000), when tested along with acetylcholine. When the relaxant property was compared with the standard drug acetylcholine, Ethanolic Extract tested along with the acetylcholine produces more relaxant property than the standard drug acetylcholine (Table 1 & Fig. 1-2). Lesser the concentration of the test drug (Ethanolic Extract) increases the responses of the muscle relaxant property. Maximum relaxant effect in T1 and T3 was found i.e. 22mm and 23mm at the dose of 16µg and T2 was 13mm at 2µg. Earlier studies have proved that chloroform extract of *Ervatamia crispa* revealed skeletal muscle relaxant effect on an isolated rat rectus abdominis muscle preparation[16].

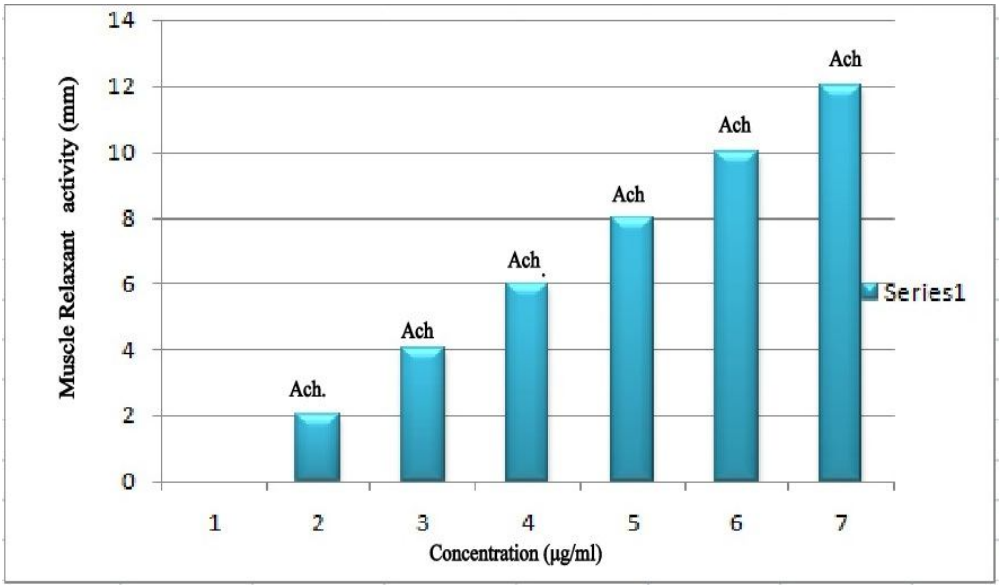

Fig-5: Muscle Relaxant Activity of *Calotropis gigantea*

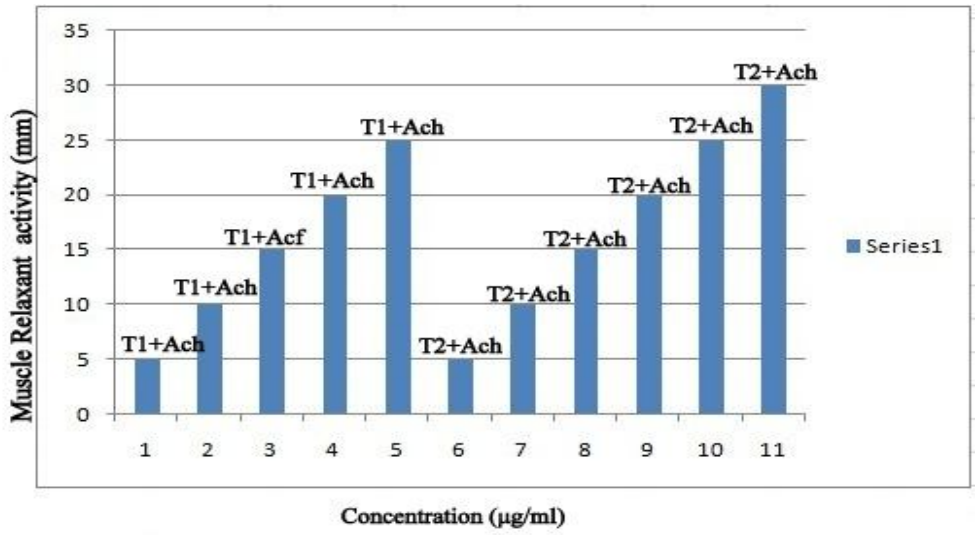

Fig-6

Fig. 5-6.Comparison of Muscle Relaxant activity of acetylcholine and test drug at different concentration.

Table 4: Muscle Relaxant activity extract from *Calotropis gigantea*

Drug	Volume (ml)	Dose (µg)	Height(mm)	Responses
Acetylcholine	0.1	1	3	Increased
Acetylcholine	0.4	4	5	Increased
Acetylcholine	0.8	8	9	Increased
Acetylcholine	1.2	12	11	Increased
T1 (1:100)	0.4	4	-	-
T1 + ach	0.1	1	6	Increased
T1 + ach	0.4	4	9	Increased
T1 + ach	0.8	8	12	Increased
T2 (1:500)	0.4	4	-	Increased
T2 + ach	0.1	1	24	-
T2 + ach	0.2	2	13	Increased
T3(1:1000) + ach	0.3	3	15	Increased
T3 + ach	0.4	4	16	Increased
T3 + ach	0.8	8	18	Increased
T3 + ach	1.6	16	24	Increased
				Increased

AE: aqueous extract, **PC**: positive control, **NC**: negative control. Values are expressed as mean ± standard deviation of the three replicates. Zone of inhibition not include the diameter of the well.

CONCLUSION

The results obtained in the MES test in rats that, the standard drug as well as the different extracts of stem barks of *Calotropis gigantea* protected against MES induced seizures. Extract of *Calotropis gigantea* had a slower onset of action and lesser degree of Muscle relaxant activity.The Antioxidant , Antibacterial, Muscle Relaxant Activities of Calotropis gigantean from Eethanolic, & Aqueous extract was studied and compare to each other.

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