Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2021; 10(5): 303-308
Received: 13-07-2021
Accepted: 15-08-2021

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Anthelmintic activity of leaf extract *Calotropis procera*

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DOI: https://doi.org/10.22271/phyto.2021.v10.i5d.14239

Abstract
Calotropis procera (Asclepiadaceae), giant milkweed, known for its various pharmacological importance for centuries to cure various diseases. It exerts many pharmacological effects such as anti-microbial, anti-pyretic, anti-cancer. *Calotropis procera* leaves extract was found to possess anthelmintic activity against Posthuma using mebendazole as the reference standard. The dose-dependent activity was observed in different concentrations of benzene extract of leaves. The benzene extracts of leaves at concentration 10,20,50mg/ml showed paralysis time as 4.52,3.24,1.15 min and death time as 8.27,5.26,2.66min respectively. At highest concentration produces paralysis and death in a short time which is comparable with mebendazole. Normally saline solution-treated earthworms have not shown any change in physical activity. All the data were expressed as mean ISEM. Data were subjected to one-way ANOVA. The statistical analysis was conducted with graph pad instant software (version 3, USA). The values of $p<0.05$ were considered statistically significant.

Keywords: *Calotropis procera*, anthelmintic activity, benzene extract of leaves, earthworms, mebendazole

1. Introduction
From ancient times plants have been used to cure diseases of man and also animals. This system of therapy is commonly referred to as Eastern, Folk, Unani (or) indigenous medicine [1]. There are lots of plants that have been reported for their medicinal importance, for example, *Caesalpinia crista* (Leguminaceae), *Moringa oleifera* (Moringaceae; Sohanja), *Tachelospermum jasminoides* (Apocyanaceae; Zard chambeli) have been quite commonly used [2]. The medicinal properties ascribed to plants include anthelmintic, analgesic, anti-fertility, anti- microbial, anti-diarrhea, anti-inflammatory, etc. [3-5]
Herbal medicine is one of the oldest forms of health care that is known to be mankind that the use of an herbal drug for human health care is probably as ancient as mankind. One of the important plants that are used in herbal medicine is *Calotropis procera* Linn (Asclepiadaceae). It is commonly known as French cotton which is used as medicine fairly throughout the greater part of India and is referred to as topical plants growing of about 1050 meters. Mainly it refers to the warm climate. Hence it is mostly distributed in Rajasthan. It healthily grows in dry and open habitat that is frequently found along the road side, dry land of the rural and urban region where the soil is extremely drained and where xerophytic conditions are available. The flower of this plant mainly consists of 5 small triangular dirty sepals, 5 thick ovate petals (1cm×1cm), and 5 stamens. Fruits consist of 5 small triangular dirty fruits, up to 15cm long by 10cm wide. Roots occur in the entire condition. The bark is separated of whitish latex excludes from cuts (or) wounds. The leaf of this plant is simple, opposite, sub-sessile, slightly thick, 10-15cm long. Almost all parts of *Calotropis procera* like leaves, roots, bark, fruits, flowers, and latex are occupied for medical purposes and can be used medicinally in various forms such as powder, juice, dry, and extract form [6-9].
Calotropis procera contained many biologically active chemical groups that encompass cardenolides, steroids, tannins, glycosides, terpenoids, sugars, flavonoids, alkaloids, saponins. It brings to play many pharmacological effects such as anti-microbial, anthelmintic, anti-inflammatory-analgesic, antipyretic, anti-cancer activities. Conventional it is used to treat cholera, extracting guinea worms and indigestion, etc.

2. Experimental work

2.1 Materials and Methods

2.1.1 Collection of powder

The leaves of the plant *Calotropis procera* was collected from the area in Nellore (Dist). AP.

2.1.2 Preparation of powder

After the leaves were collected, they were washed with fresh water to remove the soil and adhered matters. Sufficient leaves were dried under the shade dried at room temperature then they were powdered using a grinding mixture to obtain a coarse powder and then passed through a 40 mesh sieve.

2.1.3 Preparation of extract

The extraction method may wary which the scale and purpose of the operation and with the raw material. For many research, purpose chromatography gives both speedy and accurate results.

Solvent extraction by continuous hot Soxhlet extractor

A Soxhlet is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet. Soxhlet extraction is only required where the desired compound has limited solubility in a solvent, and the impurity is insoluble in that solvent.

2.2 Procedure

About 10gm of powdered drug of *Calotropis procera* leaves were placed inside a thimble made from thick paper, which loaded into the main chamber of the Soxhlet extractor maintained at a temperature range from 78.3 °C.

The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The chamber containing the solid material slowly filled with warm solvent within which some of the desired compounds were dissolved. when the Soxhlet chamber was full, then the solvent runs down to the distillation flask through a siphon tube.

The extraction was carried out by using the solvent benzene for about 72 hours. Until it becomes colorless.

Then the extract was concentrated under reduced pressure and dried vacuum condition to get a semisolid mass. The dried extract thus obtained was dark greenish was kept in a desiccator to get a semisolid mass. The dried extract thus obtained was dark greenish was kept in a desiccator and was used for the further experiment as well as used for identification of their chemical groups present.

2.3 Preliminary phytochemical screening

2.3.1 Alkaloids

The preliminary phytochemical investigations were carried out for the qualitative detection of phytoconstituents. Qualitative tests were conducted for all the extracts to identify the various phytoconstituents. The various tests and reagents used are given below and the observations are recorded in a table.

a. Dragendroff's test

1ml of extract and add 1ml of Dragendroff's reagent (potassium bismuth iodide solution). An orange-red precipitate indicates the presence of alkaloids.

b. Mayer's test

1ml of extract and add 1ml of Mayer's reagent (potassium mercuric iodide solution), whitish or cream-colored precipitate indicates the presence of alkaloids.

c. Hager's test

1ml of extract and add 3ml of Hager's reagent (saturated aqueous solution of picric acid). Yellow-colored precipitate indicates the presence of alkaloids.

d. Wager's test

1ml of extract and add 2ml of Wager's reagent (iodine in potassium iodide). Reddish-brown colored precipitate indicates the presence of alkaloids.

2.3.2 Carbohydrates and glycosides

a. Molisch's test

2ml of the prepared filtrate was mixed with 20ml of alcoholic solution of α naphthol 10% in addition to 2ml of sulphuric acid, a bluish violet zone is formed, this indicates the presence of carbohydrates and or glycosides.

b. Fehling's test

In a test tube, 5ml of the filtrate was treated with the 5ml Fehling's solutions(A&B) and heated. The appearance of a red precipitate indicates the presence.

c. Benedict's test

To 1ml of the filtrate, 5ml of Benedict's reagent was added. The mixture was heated; the appearance of red precipitate indicated the presence.

2.3.3 Flavones and flavonoids

1ml of 10%ethanolic extract of the studied plant was mixed with 0.5ml of hydrochloric acid (10%) and magnesium metal. A developed reddish color indicates the presence of flavonoids.5ml of 1% hydrochloric acid extract was shaken with sodium hydroxide; a yellow color appeared indicating the presence of flavonoids.

2.3.4 Saponins

1gm of the plant under investigation was boiled with 10ml water for few minutes and filtered. The filtrate was vigorously shaken. The persistent froth(1cm height) was observed for 1 hour, which indicates the presence of saponins.

2.3.5 Steroids

For testing the presence of unsaturated sterols triterpenes, 1gm of the air-dried powder of the studied plant was extracted with a few ml of ethanol then filtrated and the filter was evaporated till dryness. The residue was dissolved in 10ml chloroform, filtered and the titrate was divided into two equal portions for proceeding with the following tests.

a. Libermann-burchard test

To the first portion of chloroform filtrate 1ml of acetic acid anhydride was added, followed by 2ml of sulphuric acid down the wall of the test tube. The appearance of reddish-violet color at the junction of two layers and a bluish-green color in the acetic acid layer indicates the presence. To the second portion of chloroform filtrate, an equal volume of sulphuric acid was added. The appearance of red color indicates the presence.

b. Tannins

About 2gm of the air-dried powder of the plant was extracted with ethanol (50%) and tested for the presence of tannins using the following tests.

One drop of ferric chloride was added to 2ml of the extract, the appearance of bluish or greenish-black coloration

indicates the presence of pyrogallol or catechol tannins, respectively. 5ml of alcoholic extracts of the studied plants were mixed with 2ml vanillin hydrochloride acid solution if a precipitate was formed. This indicates the presence of Gallic acid.

c. Phenol and phenolic compounds

The small quantity of alcohol and aqueous extract in water is tested for the presence of phenolic compounds with dilute ferric chloride solution (5%), 1% solute of gelatin contains 10% sodium chloride, 10% lead acetate, and aqueous bromine solution.

Table 1: Phytochemical screening of leaf extracts of *Calotropis procera*

Serial No.	Test	Results
1	Carbohydrates	+
2	Alkaloids	+
3	Phenols	+
4	Tannins	+
5	Saponins	-
6	Glycosides	+
7	Flavonoids	+
8	Terpenes	-
9	Steroids	+

Fig 1: leaf extracts of *Calotropis procera* for Phytochemical screening

2.4 Worm collection

Indian adult earthworm [*Pheritima posthuma* (*P. posthuma*) was used to study the anthelmintic activity of plant extracts. The adult earthworm was collected from Govt Vermicompost, Kodavaluru Village, Nellore (Dist), Andhra Pradesh, India. Worms with the length of 5-6 cm and a width of 0.2-0.3cm were utilized for the whole experiment. The earthworms obtained resembled intestinal roundworm parasites of human beings both automatically and physiologically and hence were considered for anthelmintic activity.

Fig 2: warm collection

2.5 Drugs and chemicals

Mebendazole, Saline water, benzene, and DMSO were the drugs and chemicals in the experiment.

Fig 3: Mebendazole

2.5.1 Preparation of test drug and reference drug

Extract for Invitro study were prepared as having concentrations 10mg/ml, 20mg/ml, 50mg/ml. Samples of benzene extracts were prepared by dissolving 100mg, 200mg, and 500mg. Crude extract of each in 1ml dimethylsulfoxide and made the volumes up to 10ml. Samples achieved where 10mg/ml, 20mg/ml and 50mg/ml respectively. Normal saline solution was used as control and mebendazole was used as the standard drug for the study.

3. Anthelmintic activity

Anthelmintic study of the extract was carried out at concentrations 10, 20, 50mg/ml against Indian earthworms (*P. posthuma*) by affirming the method of Hussain et.al., Five groups of Indian earthworms, each containing five earthworms approximately of equal size were used for the study. Three groups of earthworms were tested with extract of different concentrations (10mg/ml,20mg/ml,50mg/ml) and one group were treated with 10mg/ml with reference standard as mebendazole, and one group was used as control which is treated with normal saline. The anthelmintic on earthworms were observed and the time required for paralysis and death was recorded.

4. Statistical analysis

All the data were expressed as the mean±SEM. Data were subjected to one-way ANOVA followed by the Dunnett test. The statistical analysis was conducted with graph pad instant software (version 3, USA). The values of $p<0.05$ were considered statistically significant.

5. Results and Discussion

Preliminary phytochemical investigations of the extract revealed the presence of carbohydrates, alkaloids, phenols, glycoside, flavonoids, tannins, steroids. The effect of different concentrations of benzene extracts of *Calotropis procera* and mebendazole on *Pheretima posthuman* is depicted in Table 2. The dose-dependent onset of paralysis and mortality were observed in earthworms treated with the extract which was compared with mebendazole as a reference drug. The benzene extract at concentrations 10, 20, 50mg/ml showed paralysis time as 4.52, 3.24, 1.15 mon and death time as 8.27, 5.26, 2.66 min respectively. The highest concentration is produced paralysis and death in a short time which is comparable with mebendazole. The mebendazole treated group at concentration 10mg/ml showed paralysis time 14.23 min and dearth time of 17.26min. The normal saline solution-treated earthworms have not shown any change in physical activity

and remained active with whole-body movements. The paralyzed earthworms have not shown any movements and remain scattered in the media whereas earthworms that did not show paralysis remained mobilized and aggregates many times. Invitro effects of different concentrations of benzene extract *Calotropis procera* leaves, normal saline (control), and mebendazole (standard) on survival in Indian earthworms.

Table 2: Dose-dependent onset of paralysis and mortality of earthworms

S. No	Group	Drug (Mg/Ml)	Concentration	Paralysis (Time Taken In Min)	Death (Time Taken In Min)
1	Control	Saline	-	-	-
2	Standard	Mebendazole	10mg/ml	14.23±0.03	17.26±0.05
3	Test	Extract	10mg/ml	4.52±0.06	8.27±0.05
			20mg/ml		
			30mg/ml		

Fig 4: Normal saline-treated with worms remained active. Paralysis or mortality was not observed

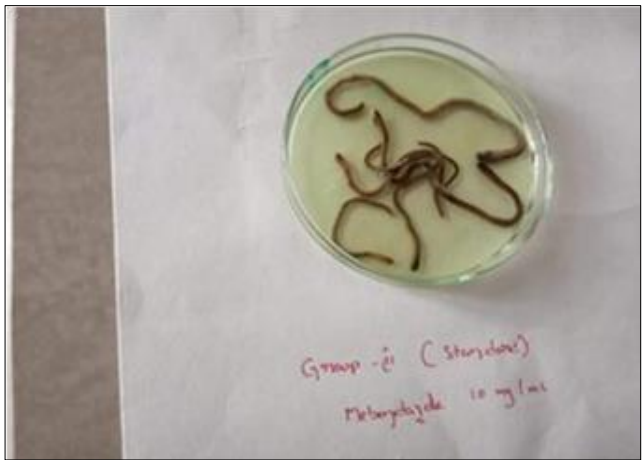

Fig 5: Effect of mebendazole on paralysis & mortality time for 10mg/ml

(a) (b) (c)

Fig 6: Effect of benzene extract of *Calotropis procera* leaves on paralysis & mortality time for 10mg/ml (a), 20mg/ml (b), 50mg/ml (c) concentrations.

Table 3: Effect of benzene extract of *Calotropis procera* leaves on paralysis & mortality time for (a)Standard (b)Test 1 (c) Test 2 (d) Test 3

(a)

Standard		
Paralysis		**Death**
14.11		17.1
14.2		17.23
14.24		17.28
14.29		17.33
14.33		17.4
AVG	14.234	17.268
SEM	0.034713	0.050537

(b)

Test 1(10mg/ml)		
Paralysis		**Death**
4.3		8.1
4.45		8.23
4.53		8.29
4.62		8.33
4.7		8.41
AVG	4.52	8.272
SEM	0.06921	0.052

(c)

Test 2 (20 mg/ml)		
Paralysis		**Death**
3.17		5.21
3.21		5.24
3.25		5.26
3.28		5.28
3.32		5.32
AVG	3.246	5.262
SEM	0.0261992	0.018547

(d)

Test 3 (50 mg/ml)	
Paralysis	**Death**
1.03	2.33
1.1	2.36
1.18	2.41
1.22	3.01
1.24	3.2

6. Discussion

P. Posthuma exposed to different concentrations of benzene extract of *Calotropis procera* leaves revealed a dose-dependent alteration in physical activity, paralysis, and death. The results were subjected to one-way ANOVA followed by the Dunnett test and mean ±SEM were calculated for all concentrations of the extract. The anthelmintic efficacy on earthworms (*Pheretima posthuma*) summarized in table 2 reveals that benzene extract at different concentrations has shown paralysis and earthworms and it was compared with mebendazole as a reference drug. Mebendazole produce metabolic disruption at several different sites, most of which are involved in energy production in the parasite leading to the death of earthworms.

All the concentrations of leaves extracts of *Calotropis procera* showed a change in the movement of earthworms which eventually progressed to death but the significant effect was observed at 50mg/ml concentration the present anthelmintic components of *Calotropis procera* are not known; however, phytochemical screening literature are reported to contain carbohydrates, alkaloids, phenols, glycosides, flavonoids, tannins, steroids, and several phytochemicals have potential to alter the metabolic pathway of earthworms and thereby produce mortality in earthworms. Tannins are one of such potential chemical constituents responsible for the anthelmintic activity which can bind with the free proteins present in the gastro intestinal tract of earthworms and cause death.

Several mechanisms contribute to the anthelmintic effect of tannins like including the formation of protein complexes by increasing supply of digestible proteins, imposing larval starvation by engaging free protein available in tubes, producing energy production by uncoupling oxidative phosphorylation, and interfering with nematodes cuticle that leads to paralysis. Plants rich in tannins as main chemical constituents have potent anthelmintic activity and may therefore estimate as a strong choice to control nematodes. Also, steroids are known to affect membrane permeability and pore formation of parasites thereby leads to mortality of parasites. Alkaloids in parasites reduce nitrate generation thereby decreases ribosomal and mitochondrial protein synthesis and interferes with the synthesis and activities of DNA and RNA, inhibits glucose supply, and cause paralysis of worms by acting on the central nervous system.

The significant effectiveness of benzene extract of *Calotropis procera* leaves as anthelmintic activity might be due to the presence of phytochemicals like tannins that further needs to screen for precise anthelmintic mechanism. Further *in vivo* study is required for evaluation of *Calotropis procera* for its effectiveness and pharmacological rationale as an anthelmintic agent. Although further study is needed still the plant exhibits significant anthelmintic potential which is useful for the plant management of liver disease caused by worms.

7. Conclusion

The anthelmintic activity of benzene extract of *Calotropis procera* leaves was evaluated. From the above results, it was confirmed that benzene extract obtained from the leaves of *Calotropis procera* showed anthelmintic activity at various concentrations respectively (10mg/ml,20mg/ml,50mg/ml). At (50mg/ml) concentration the maximum anthelmintic activity has been achieved while compared to that of the standard solution.

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