Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2015; 3(6): 188-192
Received: 07-01-2015
Accepted: 30-02-2015

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Paralysis and death of *Pheretima posthuma* and fungal growth inhibition by leaf extracts of *Clerodendrum infortunatum* L.

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Abstract
In the modern world, the interest in the traditional cures is growing rapidly because of more side effects and raising prices in synthetic drugs. In this outlook herbal medicines play a great role to substitute the synthetic drugs with uncomplicated accessibility through the world. The aim of this study was to evaluate and authenticate the anthelmintic and antifungal efficacy of C. *infortunatum* leaf extracts in adult earthworms (*Pheretima posthuma*) and different fungal strains. The effectiveness of the plant extracts was assessed by monitoring the paralysis and death time of experimental earthworms, as well as the zone of inhibition in different fungal strains. The earth worms were treated with hexane, chloroform, ethyl acetate and ethanol extracts at a concentration of 25, 50 and 100 mg/ml of DMSO. Among these extracts ethanol and ethyl acetate have shown less time of paralysis (16 and 18.67 min) and death (46 and 48 min) at a concentration of 100 mg/mL compared to standard Piperazine citrate. The efficiency of hexane and chloroform extracts was recorded capable of paralysis and death of earthworms comparatively low to ethanol and ethyl acetate extracts. The same extracts were also subjected to antifungal screening against different fungal strains of *Aspergillus niger, Aspergillus fumigates, Aspergillus flavorus* and *Candida albicans* using agar well diffusion method at a concentration of 50, 75, 100 mg/mL and the zone of inhibition in millimeter (mm) has been recorded compared to reference standard Amphotericin (1 mg/mL). Among these extracts most have been proved effective against *Aspergillus fumigates.* The chloroform exhibited highest activity against *Aspergillus fumigates* (10.83 mm) at a concentration of 75mg/mL while as ethanol extract (100mg/mL) has shown intermediate inhibition (3.17 mm) to *Aspergillus flavorus.* None of the extracts have proven to be effective against *Aspergillus niger* and *Candida albicans*. The experimental proof of the current study proposes that leaves of C. *infortunatum* hold specific phytoconstituents which promote their use against the anthelminthes and fungal infections.

Keywords: *Clerodendrum infortunatum, Pheretima posthuma, Aspergillus niger, Aspergillus fumigates, Aspergillus flavorus* and *Candida albicans*

1. Introduction
The presences of the phytochemicals in plants hold-in medicinal properties whose traditional remedial applications can be recognized in modernistic medication. The worlds 80 percent population (4 billion people) exercise, herbal medicines for elementary health care which is estimated by the World Health Organization (WHO) [1]. The plant population is still seized with many species of plants holding active constituents of medicinal value that have not been evaluated for their pharmacological value. Modern isolation and pharmacological screening techniques have emphasized the way to discover new drugs to modern medicine [2].
Different species of *Clerodendrum* genus have been traditionally used over centuries and their antioxidant and hepatoprotective potential have already been proved. The plant parts are also used in Indian folk medicine in the treatment of bronchitis, asthma, fever, burning sensation and epilepsy [3]. The plant was found to contain triterpenes, steroids and flavonoids. The antioxidant, antimicrobial, anti-malaria, anthelmintic and analgesic activities of the plant have further created an upsurge in investigations on the plant [4].
Helminth derived from the Greek word “helminthes” meaning “worm”. Helminth is a broad categorical term referring to various types of parasitic worms that reside in the body [5]. Helminthic infections of the gastrointestinal tract of human beings and animals have been recognized to have adverse effects on health standards with a consequent lowering of resistance to other diseases. The cost of harboring these parasites in terms of human misery and economic loss is incalculable [6]. Although there are many effective drugs available for the treatment of intestinal helminthiasis, the fact remains that they remain out of reach to a majority

of people in the world, especially in African and Asian countries [7]. On the other hand, there are a great many herbal remedies that are effective against common intestinal worms and are easily available and affordable to common people in developing countries [8]. Antihelminthics are those agents that expel parasitic worms (helminthes) from the body, by either stunning or killing them. More than half of the population of the world suffers from various types of infection and majority of cattle's suffers from worm infections [9].

Microorganisms are often a cause of existing diseases, regarding a solemn public health issue in a major part of the population as revealed by either personal or authorized health care systems. The economic crisis, high cost of industrialized medicines, inefficient public access to medical and pharmaceutical care, in addition to the side effects caused by synthetic drugs are some of the factors contributing to the central role of medicinal plants in healthcare [10]. Plants are always surrounded by an enormous number of potential enemies such as bacteria, viruses, fungi, insect etc. [11] Plants produce a great deal of secondary metabolites, many of them with antifungal activity. Well-known examples of these compounds include flavonoids, phenols and phenolic glycosides and saponins, [12, 13, 14, 15].

Keeping this in view, the present study has been considered to screen anthelmintic and antifungal activity of different extracts of leaves from *Clerodendrum infortunatum* L. which may facilitate these extracts for future study.

2. Materials and Methods

2.1. Collection and Processing of Plant Material

The fresh leaves of *C. infortunatum* were collected from the road side of Bhadra River Channels, Shimoga, Karnataka, India. The leaves (after cutting into small pieces) were shade dried for several days. The plant material was then oven dried for 24 hours at considerably low temperature (40 °C) for moisture free and better grinding. The dried samples were then ground in coarse powder using high capacity grinding machine. The coarse powder was stored in air-tight container with marking for identification and kept in cool, dark and dry place for future use.

2.2. Preparation of crude extract

The air-dried and finely ground (500 g) leaves of the plant were extracted in a Soxhlet apparatus which is on top of a collecting flask beneath a reflux condenser successively with hexane, chloroform, ethyl acetate and ethanol from low polarity to high polarity. A suitable solvent was added to the flask and the setup was heated under reflux. The steam of the solvent which, when contacts with the material will dissolve metabolites and brings back metabolites to the flask. The extracts were filtered, pooled and concentrated to dryness under reduced pressure in a rotary evaporator (Buchi, Flawil, Switzerland) to yield dried hexane, chloroform, ethyl acetate and ethanol extract of leaves. The extracts so obtained from each of solvents were labeled and yield was calculated in terms of grams/weight of the powdered material.

2.3. Pilot solubility tests of plant extracts

Solubility tests were carried out for the analysis of solubility of crude extracts in different solvents like, hexane, chloroform, ethyl acetate, acetone, DMSO, ethanol, methanol, water, 1N NaOH, and 1N HCl.

2.4. Test organism for Anthelmintic activity

Adult earthworms (*Pheretima posthuma*) were selected to evaluate anthelmintic activity *in vitro* due to its anatomical and physiological resemblance with the intestinal round worm parasites of human beings [16, 17]. Earthworms were collected from Agricultural University, Shimoga. All the worms were washed with normal saline to remove all fecal matters. The earthworms 5-7 cm in length and 0.3-0.4 cm in width weighing 0.8-3.0 g were used for experimental protocols.

2.5. Evaluation of Anthelmintic activity

The anthelmintic activity was performed according to the method of Ghosh *et al*, [18]. Test samples of each extract were prepared at the concentration of 25, 50, 100 mg/ml of DMSO. 10 mg/ml of standard Piperazine citrate was used as the reference while normal saline served as the control [19]. Earthworms were divided into six groups, each containing three worms, separately released into 20 ml of desired formulation of normal saline in petri dishes. Group I earthworms were released in 20 ml normal saline in a clean Petri plate as a negative control. Group II earthworms were released in 20 ml of normal saline containing standard drug Piperazine citrate (10 mg/ml). Group III, IV, V and VI earthworms were released in 20 ml of normal saline with 25, 50 and 100 mg/ml of hexane, chloroform, ethyl acetate and ethanol extracts respectively and each concentration has been taken in triplicates. Finally, observations were made for determining the time taken for paralysis and death of the worm. Time for paralysis was noted when no movement of any sort could be observed except when the worms were shaken vigorously. Death was concluded when the worms lose their motility followed with fading away of their body color [20].

2.6. Microorganisms used for antifungal activity

Four clinical strains *Aspergillus niger*, *Aspergillus fumigates*, *Aspergillus flavorus* and *Candida albicans* were used for assessing the antifungal activity and standard Amphotericin (1 mg/ml) was used as positive control.

2.7. Screening of antifungal activity

Antifungal activity was determined by the disc diffusion method. Muller Hilton and saboured Dextrose Broth were used as a medium for fungal strains respectively. The fungal cultures were incubated at 27±2 °C for 48 h [21]. The assessment of antifungal activity was based on the measurement of diameter of inhibition zone formed by dissolving the plant material extract in DMSO. The leaf extracts of *C. infortunatum* were prepared at a concentration of 50, 75 and 100 mg/ml in triplicates and one control was used for each extract. All extracts were inoculated with fungal organisms to be tested and then incubated at 27±2 °C (for 48 h). Turbidity produced was measured thereafter against a blank.

3. Results and Discussions

In the present study the anthelmintic and antifungal tests were conducted using standard protocols. The methods were chosen for analyzing the biological properties of the plant extracts considering their beneficial sides. It is well established that different plant extracts contain a wide spectrum of bioactive compounds and these compounds are responsible for different biological activities [22].

3.1 Solubility test

After the analysis of solubility of extracts, it has been detected that the extracts are partially soluble in water and ethanol, but all of the extracts have been found completely soluble in DMSO.

3.2 Anthelmintic activity

On exposure to the various concentrations of the hexane, chloroform, ethyl acetate and ethanol extracts, the *Pheretima posthuma* showed an onset of paralytic state; the time required for the parasites to attain paralysis has been shown in Fig. 1, while Fig. 2 specifies the death of earthworms. Treatment with 25, 50 and 100 mg/ml concentration of the ethanol extract induced loss of motility and consequent paralysis in 28, 24 and 16 min followed by death time of 59, 53, and 46 min respectively. In comparison, the 10 mg/ml dose of the reference drug caused paralysis to set in after 24 min of post-incubation followed by the time of death in 51 min. The observations indicate significant difference between *ethanol extract* treated group and the control group. Ethyl acetate extract has also shown significant results in terms of paralysis and death. Worms treated with 25, 50 and 100 mg/ml of ethyl acetate extract attained paralysis in 34.33, 30.67 and 18.67 min followed by the time of death in 64, 55.67 and 48 min respectively. Hexane and chloroform extracts have also shown promising activities revealed in Fig. 1 and Fig. 2 which are very close in range to a standard.

Earthworms have the ability to move by ciliary movement. The outer layer of the earthworm is a mucilaginous layer and composed of complex polysaccharides. This layer being slimy enables the earthworm to move freely. Any damage to the mucopolysaccharide membrane will expose the outer layer and this restricts its movement and can cause paralysis. This action may lead to the death of the worm by causing damage to the mucopolysaccharide layer. The predominant effect of Piperazine citrate on worm is to cause a flaccid paralysis which results in expulsion of the worm by peristalsis. Piperazine citrate by increasing chloride ion conductance of worm muscle membrane produces hyper polarization and reduced excitability that leads to muscle relaxation and flaccid paralysis [23]. Therefore, the leaf extracts of *C. infortunatum* have proved their potent anthelmintic activity and the study can be advanced with the isolation of pure compounds from the crude extracts.

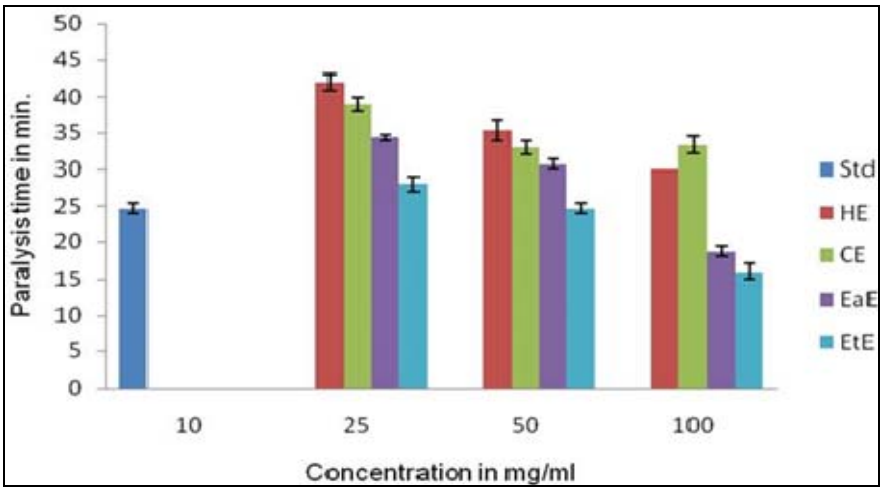

Fig 1: Paralysis time of earthworms

HE= Hexane Extract; CE=Chloroform Extract; EaE= Ethyl acetate extract; EtE= Ethanol Extract.
Values are the mean ± S.E.M. of three earthworms.

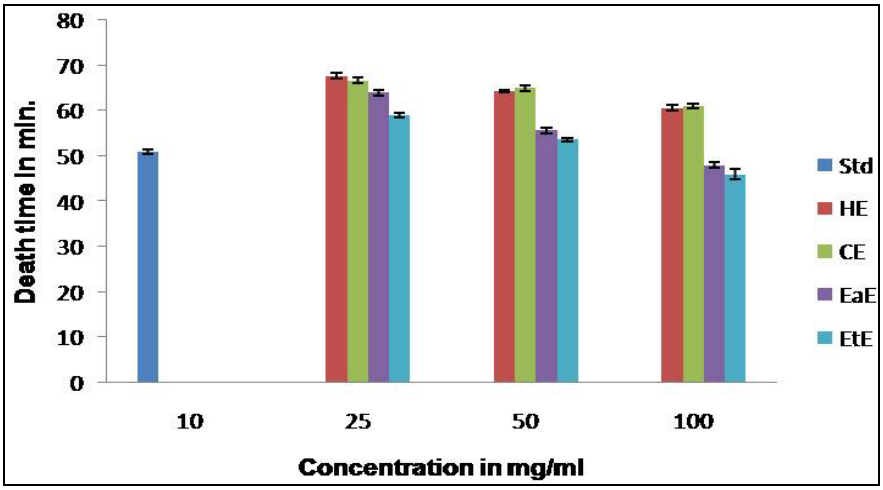

Fig 2: Death time of earthworms

HE= Hexane Extract; CE=Chloroform Extract; EaE= Ethyl acetate extract; EtE= Ethanol Extract.
Values are the mean ± S.E.M. of three earthworms.

Antifungal activity

All the leaf crude extracts of *Clerodendrum infortunatum* were subjected for screening against four fungal strains and the zone of inhibition is detailed in table 1. It is clear from the inhibition zones that most of the leaf extracts of *C. infortunatum* are effective against *Aspergillus fumigates* and ethanol extract has shown intermediate inhibition on *Aspergillus flavorus*. The highest value of inhibition zone is 10.83 mm in diameter in case of chloroform extract (75 mg/mL) against *Aspergillus fumigates* while as hexane and ethyl acetate have also proven to be most effective against *Aspergillus fumigates* (9.76 ± 0.44 and 10.33 mm) at a concentration of 75 mg/mL. The lowest value of inhibition zone was 3.17 mm in diameter by ethanol extract (100 mg/mL) on *Aspergillus flavorus*. None of the extracts have shown significant activity against *Aspergillus niger* and *Candida albicans*.

Table 1: Antifungal activity of leaf extracts of *Clerodendrum infortunatum*.

SI. No.	Samples	Fungal Strains Inhibition zone (mm)			
		Aspergillus niger	***Aspergillus fumigates***	***Aspergillus flavorus***	***Candida albicans***
1.	**Standard** (1mg/mL)	8.50 ± 0.50	7.50 ± 0.50	6.00 ± 1.00	4.03±0.07
2.	**HE** 50 mg/mL 75 mg/mL 100 mg/mL	 NA NA NA	 7.50 ± 0.29 9.76 ± 0.44 8.50 ± 0.29	 NA NA NA	 NA NA NA
3.	**CE** 50 mg/mL 75 mg/mL 100 mg/mL	 NA NA NA	 9.33 ± 0.33 10.83 ± 0.44 6.00 ±0.58	 NA NA NA	 NA NA NA
4.	**EaE** 50 mg/mL 75 mg/mL 100 mg/mL	 NA NA NA	 9.33 ± 0.17 10.33 ± 0.33 9.50 ±0.29	 NA NA NA	 NA NA NA
5.	**EtE** 50 mg/mL 75 mg/mL 100 mg/mL	 NA NA NA	 NA NA NA	 3.50 ± 0.29 3.50 ± 0.50 3.17 ± 0.44	 NA NA NA

HE= Hexane Extract; CE=Chloroform Extract; EaE= Ethyl acetate extract; EtE= Ethanol Extract, NA = Not appeared, Values are the mean ± S.E.M.

Some extracts of *Clerodendrum infortunatum* proved very promising and showed significant inhibition of tested fungal strains. The broad spectra of activity of this plant may help to discover new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases.

Plant *C. infortunatum* can produce antifungal compounds to protect themselves from biotic attack that could be essential for fungi infection resistance [24]. The understanding of the inhibitory mechanism would provide better directions toward the development of efficient production and application of technologies associated with bio fungicide plant materials.

4. Conclusion

The importance of any ethnomedicinal survey lies in the potential discovery of plants, which may through proper scientific investigations may yield novel compounds to treat both old and emerging diseases. From current investigation, it has been concluded that the leaf extracts of plant *C. infortunatum* are having substantial anthelmintic activity, the constituent present in the herb might be responsible for this activity. The current findings also clearly indicate promising antifungal properties of *C. infortunatum* against different fungal strains. Thus, *C. infortunatum* appears to be an effective material for the development of antimicrobial drugs and ecofriendly biopesticides. This provides some evidence of the leaves being used in treating various skin diseases. However, it is necessary to find out whether this plant is not dangerous for use for the reason it is being employed by people.

5. Acknowledgements

The authors wish to thank UGC, New Delhi for supporting financially. Thanks are also due to the Registrar, Kuvempu University, for his keen interest and for providing facilities for this work. The authors are grateful to the Principal, Govt. Medical College, Shimoga, Karnataka for the generous gift of fungus cultures.

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