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# Isolation of clerodin from *Clerodendron infortunatum* Linn. and its anthelminthic activity

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**Abstract**

The present study shows the anthelmintic activity of Clerodin, extracted from *Clerodendrum infortunatum* leaves against the Pheretima posthuma (P. posthuma).We tried to inhibit the growth of P. posthuma. Extracted Clerodin of *C. infortunatum* leaves were investigated for its anthelmintic activity against the P. posthuma. Salkowski test indicated the presence of clerodin in the hexane extract. Piperazine citrate was used as standard. The Clerodin showed Paralysis time and Death time 4.84 minutes and 9.37 minutes respectively at the concentration of 30 mg/ml, but Piperazine citrate showed paralysis time and death time 7.87 minutes and 13.32 minutes respectively at the same concentration. Overall experimental result revealed that Clerodin has higher anthelmintic activity than natural inhibitor of P. posthuma, Piperazine citrate.

**Keywords:** anthelmintic activity, *Clerodendrum infortunatum*, pheretima posthuma, clerodin, diterpenes

## 1. Introduction

Traditional plants and their medicinal properties are considered as best agents for the eradication of many types of human related diseases across the world. Most of the synthetic medicines give side effects such as vomiting, diarrhoea, abdominal pain and headache (Devi *et al*., 2009) [10]. But, the plant-derived medicines give less side effects in comparison to synthetic medicines. Hence, there is need of the hours to develop plant based medicines/preservatives (Kumar *et al*., 2017) [14]. *Clerodendrum infortunatum* Linn. is a terrestrial shrub, which has blackish stem and hairy leaves with a disagreeable odour (Kirtikar *et al*., 2001) [15] It is an important medicinal plant and commonly known as *Bhant.* This plant is found commonly in India (Ashish *et al.,* 2010) [2]. It is reported that leaves of this plant contain many useful secondary metabolites and enzymes (Sai *et al*., 2002) [21]. Traditionally, the plant leaves extract is administered orally during fever and bowel problem across the North-East India. Fresh juice of this plant leaves is used in the treatment of malaria (Chopra and Chopra, 1992) [8]. The fresh leaf-juice of this plant is also administered into the rectum to cure the infection of ascarides worm (Bhaskar *et al*., 2014) [6]. *C. infortunatum* leaves and flowers parts are used to get rid the problems of scorpion sting, stomach pain and diabetes (Baid *et al*., 2013) [3]. The root paste of the plant is applied on the swelling part of the body as bandage (Barbhuiya *et al.,* 2009) [5]. Other diseases such as diarrhoea, skin disorders, wound infections, post-natal complications, vermifug and external tumours, etc. can be cured by using *C. infortunatum* (Kirtikar and Basu, 1991) [15]. The leaves also contain clerodin, which is widely used as tonic and anthelmintic agent in human life (Haines, 1925).

Helminth infections are considered as most common infections in human life across the world (Mahesh *et al*., 2015) [19]. Helminth was derived from the Greek word “helminthes” which means worms. Anthelmintics are drugs that are used to treat the infections of parasitic worms (Nilani *et al.,* 2012) [2]. They are of huge importance to cure human and animal tropical diseases. The parasites, which are susceptible to the anthelmintics include fluke worms, tape worms, pinworm etc. These worms infect the gastrointestinal tract, liver and other organs of the body (Lee *et al*., 2014) [18]. Some time gastrointestinal worms become resistance to anthelmintic drugs, and give a big challenge to treat the infections (Sreejith *et al.,* 2013) [22]. Parasitic worms also infect livestock and crops food that lead to decrease in nutrient deficiency and yielding capacity. The World Health Organization reported that approximately 2 billion people are facing the problem of parasitic worms infections. Parasitic worm such *Enterobius vermicularis* (pinworm) is known as most successful intestinal nematode, and approximately 400 million people are infected with it worldwide (Kucik *et al*., 2004) [16]. The high parasitic burden causes loss of appetite, restlessness, irritability and insomnia (Cook, 1994) [7]. Many parasites also cause eosinophilia, anaemia, pneumonia and malnutrition (Bundy *et al*., 1994) [4]. Some parasites are considered as good candidate for analysing the link between the “hygiene hypothesis” and disease (Gale, 2002) [11].

Piperazine citrate was used as reference standard drug in this experiment. Piperazine citrate causes hyperpolarization of muscle that leads to opening of $Cl^-$ channels, and causes flaccid paralysis in the cells. The present investigation was designed to explore the anthelmintic activity of the hexane extract of the leaves of the *C. infortunatum* against the *Pheretima posthuma.* The *P. posthuma* was used in this experiment due to its anatomical and physiological resemblance with the intestinal parasites of human body, and known as model organism for screening of anthelmintic drug.

## 2. Materials and Method

### 2.1 Plant collection and identification

The plants of *Clerodendrum infortunatum* were collected from the different regions of Bihar (Bhagalpur, Gaya and Patna). Identification of plants (a high resolution photograph is shown in Fig. 1(A) was done at taxonomical section, Department of Botany and Biotechnology, College of Commerce, Arts and Science, Patna - 20.

### 2.2 Preparation of extract

The leaves were cleaned with running tap water and followed by distilled water. Thereafter, leaves were dried at room temperature for 72 hours. The dried leaves were crushed in an electric grinder to convert into power. 2.5gm of leaves powder were weighed on electric balance, and transferred into a conical flask. 3 litres of hexane was added into the flask, and left for 72 hours. In next step, the content of the flask was filtered through Whatman filter paper no.1. Afterwards, the filtrate was concentrated by rotary evaporator. The concentrated hexane extract was partitioned with hexane and methanol and, concentrated in a vacuum at 44–45°C. The residue was further extracted with methanol and partitioned with ethyl acetate.

### 2.3 Isolation of clerodin

Methanol fraction of hexane was dissolved into 50 ml of methanol, and kept into an oven for crystallization. Crystal was separated by using microfilter assembly and weighed, and stored into freeze for further use.

### 2.4 TLC method for the detection of clerodin

10mg of residue of methanol was subjected to silica gel column, and eluted with hexane containing increasing amount of ethyl acetate. Fractions were monitored by TLC contaning silica gel plate. Spots were visualized under the UV light by spraying 20% of $H_2so_4$ solution on the TLC plates. Hexane and ethyl acetate were used as mobile phase in different ratio (v/v) such as hexane (100%), hexane and ethyl acetate (95:5), hexane and ethyl acetate (90:10), hexane and ethyl acetate (85:15), hexane and ethyl acetate (80:20), hexane and ethyl acetate (75:25), hexane and ethyl acetate (70:30). Sixth fraction was showed spot on TLC plate, so this fraction was sent to CBMR for NMR. The structures of these compounds were investigated by comparison of their 1H and 13C NMR.

### 2.5 Test for clerodin

**Salkowski test**: 2 ml of the hexane extract of *Clerodendrum infortunatum* was mixed with 2ml chloroform. Thereafter, 3 ml concentrated H2SO4 was added carefully and shaken well. Appearance of reddish brown colour indicated the presence of clerodin.

### 2.6 Anthelmintic activity of extracts of *Clerodendrum infortunatum*

The anthelmintic activity of the *Clerodendrum infortunatum* was investigated by following the method of Ajayieoba *et al*. (2001) with some modifications. *Pheretima posthuma* worms were isolated from moist soil and washed with normal saline. The parasitic worms of 3 -5 cm in length and 0.1-0.3 cm in width were used for experiment. Various concentrations (10-30 mg/ml) of the extracted clerodin were used to analyze the anthelmintic properties of the worm. Paralysis time and Death time of the worms were recorded for each concentration of the clerodin. Piperazine citrate was used as standard reference. The time, when no movement was absorbed in the worms was considered as time of paralysis. The paralysed warms showed movement when shaken vigorously. The timing of losing of motility of the warms was considered as time of death.

### 2.7 NMR Spectroscopy

All experiments were performed on a Bruker Avance (III) 800 MHz NMR spectrometer operating at $^1$H frequency of 800 MHz and $^{13}$C frequency of 200 MHz. The spectrometer was equipped with a 5 mm inverse detection cold probe and an actively shielded z-gradient. All spectra were measured at temperature of 298 K using CDCl3 as solvent. Chemical shifts are given on the  scale and were referenced to residual $CHCl_3$ at 7.29 ppm for proton and to the solvent at 77.00 ppm for carbon. The pulse programs used for recording The conventional one dimensional (1D) $^1$H (at 800 MHz frequency) and $^{13}$C (at 200 MHz frequency) NMR spectra and two dimensional (2D) homonuclear correlation spectra, COSY, and inverse proton detected 2D heteronuclear correlation spectra, HSQC and HMBC in phase-sensitive mode, were recorded using the standard Bruker pulse program library. The various acquisition parameters and the names of the Bruker pulse programs are summarized in Supplementary material Table S1. The HSQC experiment was optimized for a one-bond heteronuclear coupling constant of 145 Hz, whereas the HMBC experiment was optimized for long-range coupling constants of 8 Hz.

## 3. Results and Discussion

Presence of clerodin in the hexane extract of *Clerodendrum infortunatum* was detected by the TLC method (Fig. 1B). The TLC plates showed the bands of clerodin when hexane and ethyl acetate were used as mobile phase in the ratio of 75:25 (v/v),(fig1). Later, the presence of clerodin in the hexane extract was confirmed by Salkowski test.

**Fig A** **Fig B**

**Fig 1:** (A) Photograph of Indian herb –botanically named *Clerodendrum infortunatum* and (B) TLC plate band of purified compound clerodin.

### 3.1 Solution purity and chemical structure of clerodin confirmed using $^{1}H$ and $^{13}C$ NMR

In order to ascertain the structure of Clerodin (Fig. 2), the $^{1}H$ and $^{13}C$ NMR spectra of the isolated and purified compound were recorded in 100% $CDCl_3$ solvent and compared with the previously reported $^{1}H$ and $^{13}C$ spectra [Li *et al*, 2016] in terms of chemical shifts, coupling constant and overall spectral patterns. Importantly, the $^{1}H$ and $^{13}C$ NMR spectral features were completely matched with the ones previously reported for the Clerodin [Lee *et al*, 2014] [18]. The resulted NMR assignments of all the carbon chemical shifts are shown in Figure 2**.** The resonance assignment shown here has been confirmed through the composite use of $^{1}H$-$^{1}H$ correlation spectrum (2D COSY) and $^{1}H$-$^{13}C$ correlation spectra (HSQC and 2D HMBC). The details are described in Supplementary material (See Appendix SI including Figure S1). Overall, the presence of single set of peaks corresponding to Clerodin resonances clearly reflected that the compound has been isolated in a highly purified form.

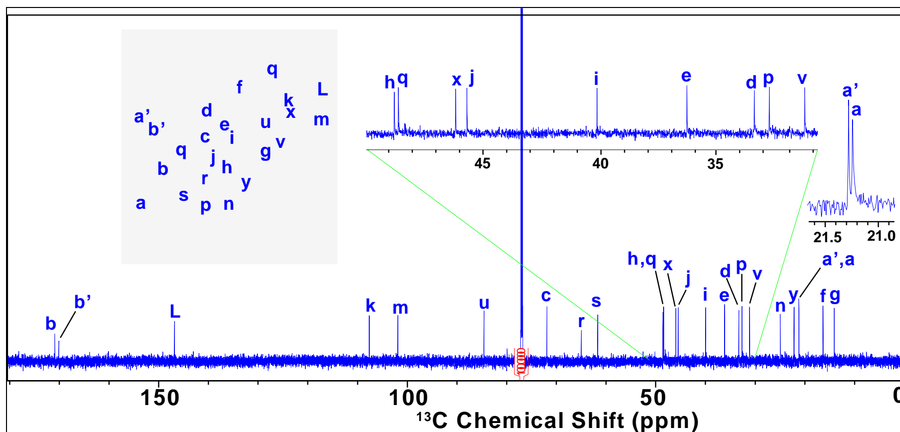


**Fig 2:** The 1D $^{13}C$ NMR spectrum of purified Clerodin compound recorded at 800 MHz NMR spectrometer.

**Table 1: Anthelmintic activity of Clerodin extracted from *C. infortunatum***

| Treat with Compound | Concentration mg/ml | Pheretima Posthuma | |
|---|---|---|---|
| | | P | D |
| Isolated Clerodin | 10 | 42.32±0.13 | 61.24±0.19 |
| | 15 | 31.23±0.12 | 49.43±0.21 |
| | 20 | 19.43±0.23 | 28.67±0.31 |
| | 25 | 9.45±0.16 | 16.42±0.13 |
| | 30 | 4.84±0.15 | 9.37±0.18 |
| Piperazine citrate | 10 | 55.34±0.21 | 72.43±0.12 |
| | 15 | 36.21±0.32 | 59.32±1.02 |
| | 20 | 21.32±0.19 | 38.45±0.21 |
| | 25 | 15.41±0.12 | 23.67±0.18 |
| | 30 | 7.87±1.03 | 13.32±0.23 |

Where P= Time taken for Paralysis (min), D= Time taken for Death of worms (min)

### 3.2 Anthelmintic activity

The extracted clerodin of *Clerodendrum infortunatum* showed anthelmintic properties against the *Pheretima posthuma.* In the clerodin treated sample, Paralysis time and death time were observed 4.84 minutes and 9.37 minutes respectively at the concentration of 30 mg/ml, but Piperazine citrate treated sample showed paralysis time and death time 7.87 minutes and 13.32 minutes respectively(table1) at the same concentration of the extract. This result indicates that hexane extract of *C. infortunatum* possesses more potential than Piperazine citrate to kill the *P. posthuma,* while Piperazine citrate is known as natural inhibitor of *P. posthuma.*

## 4. Conclusion

The above findings clearly indicate that extracted clerodin from *Clerodendron infortunatum* has anthelmintic activity against the worm *Pheretima posthuma.* The clerodin also showed high anthelmintic activity than natural inhibitor, Piperazine citrate. Hence, this extract can be used as plant based anthelmintic drug to inhibit the infection of helminths.

## 5. Supplementary Material

**Table 2:** The acquisitions and processing parameters used for NMR experiments performed to confirm the resonance assignment of Clerodin.

| Parameters | 1D $^{1}H$ NMR (zg) | 1D $^{13}C$ NMR (zgpg30) | 2D $^{1}H$-13C HSQC (hsqcetgp) | 2D $^{1}H$-$^{13}C$ HMBC (hmbcgpndqf) | 2D $^{1}H$-$^{1}H$ COSY (cosygpqf) |
|---|---|---|---|---|---|
| RF pulse Offsets (ppm) | 5.27 | 80.0 | 5.27/80 ($^{1}H/^{13}C$) | 4.59/110 ($^{1}H/^{13}C$) | 4.59 |
| Spectral widths (ppm) | 20 | 239 | 16/220 ($^{1}H/^{13}C$) | 15.2/240 ($^{1}H/^{13}C$) | 10.0 |
| Size of FID (direct/indirect) | 64 k | 64 k | 4 k/200 | 16 k/400 | 3966/512 |
| Zero-filling before FT | 64 k | 64 k | 4 k/512 | 4 k/512 | 4k/1k |
| Recycle delay | 1.0 sec | 1.0 sec | 1.5 sec | 1.4 sec | 1.5 sec |
| Number of scans per FID | 16 | 3072 | 16 | 8 | 2 |

**Table 3:** Chemical Shifts of Clerodin $^{1}H$ and $^{13}C$ resonances

| Peak | Annotation | 13C Shift (ppm) | Amplitude [abs] | 1H Shift (ppm) |
|---|---|---|---|---|
| 1 | B | 170.9827 | 35380.47 | Carbonyl |
| 2 | b' | 170.1936 | 29734.02 | Carbonyl |
| 3 | L | 146.8906 | 56364.8 | 6.489 |
| 4 | k | 107.7121 | 66425.42 | 6.0379 |
| 5 | m | 101.9554 | 45608.55 | 4.8372 |
| 6 | u | 84.6327 | 56171.23 | 4.0515 |
| 7 | c | 71.9575 | 57636.59 | 4.7126 |
| 8 | r | 65.0631 | 51770.11 | tertiary |
| 9 | s | 61.7434 | 51258.91 | 4.9236,4.3984 |
| 10 | h | 48.6416 | 71234.21 | 1.6761 |
| 11 | q | 48.469 | 59839.03 | 3.011,2.2409 |
| 12 | x | 46.0407 | 59144.73 | 3.59 |
| 13 | j | 45.5604 | 63875.81 | tertiary |
| 14 | i | 40.0475 | 61505.21 | tertiary |
| 15 | e | 36.2331 | 64315.88 | 1.4808 |
| 16 | d | 33.3797 | 59565.58 | 1.48, 1.67 |
| 17 | p | 32.7443 | 59739.55 | 1.049, 2.13 |
| 18 | v | 31.2387 | 54306.35 | 1.7596, 1.674 |
| 19 | n | 25.0319 | 55241.27 | 1.91,1.421 |
| 20 | y | 22.2643 | 42874.59 | 1.6039 |
| 21 | a' | 21.2798 | 50972.41 | 2.1291 |
| 22 | a | 21.2411 | 45917.55 | 1.9734 |
| 23 | f | 16.4403 | 38278.88 | 0.8635 |
| 24 | g | 14.1753 | 28096.76 | 0.9966 |

## 6. Appendix SI

An overlay of 2D HSQC and 2D HMBC spectra recorded in purified Clerodin compound (and dissolved in 100% $CDCl_3$) is shown in Figure S1 and has been used to further confirm the assignment of $^{1}H$ and $^{13}C$ resonances based on the presence of one-bond C-H and two/three bond C-H correlation peaks, respectively. Due to spectral congestion, the zoomed spectral region of $^{13}C$ HSQC spectrum is also shown in Figure S1B to highlight the standard assignment procedure of $^{1}H$ resonances coupled to $^{13}C$-resonances (through one-bond coupling) based on tracking them through CH correlation peaks. The $^{1}H$ resonance assignment was further validated through the presence of specific sequential $^{1}H$-$^{1}H$ correlation peaks in the 2D COSY spectrum as shown in Figure S1C and its zoomed spectral region in Figure S1D.

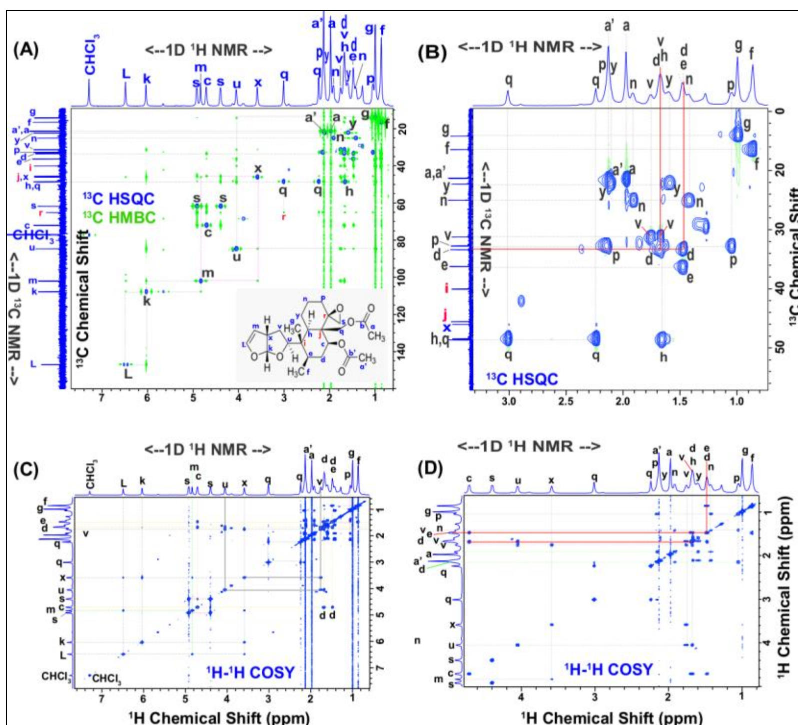


**Fig 2**

Figure S1: (A) The overlaid $^{13}C$ HSQC and HMBC spectra of purified Clerodin compound recorded at 800 MHz NMR spectrometer. The long range C-C correlations further confirmed the resonance assignment. (B) The zoomed spectral region of $^{13}C$ HSQC spectrum showing standard assignment procedure of $^{1}H$ resonances one-bond coupled to $^{13}C$-resonances based on tracking them through CH correlation peaks. (C) and (D), respectively, represent the complete and zoomed region of 2D COSY spectrum used to further confirm the resonance assignment through walking along the sequential $^{1}H$-$^{1}H$ correlations based on direct one-bond coupling interactions.

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