Academic Sciences Asian Journal of Pharmaceutical and Clinical Research
Vol 6, Issue 1, 2013 ISSN - 0974-2441

Research Article

IN-VITRO ANTHELMINTIC & CYTOTOXIC POTENTIAL OF DIFFERENT EXTRACTS OF *CALOTROPIS PROCERA* LEAVES

YOGESH MURTI*, ABHAY P SINGH AND DEVENDER PATHAK

Rajiv Academy for Pharmacy, N.H. # 2, Delhi-Mathura Bye-Pass, P.O. Chattikara, Mathura-281001.Email: ymurti@gmail.com

Received: 29 March 2012, Revised and Accepted: 07 November 2012

ABSTRACT

Different extracts of *Calotropis procera* leaves were evaluated for *in-vitro* anthelmintic activity against Indian earthworms *Pheritima posthuma,* and for *in-vitro* cytotoxic activity against the Hep-2 (human larynx epithelial carcinoma) cell line. Dose dependent activity was observed in different extracts of plant leaves. It was observed that 70% hydroethanolic extract of *Calotropis procera* leaves exhibited better *in-vitro* anthelmintic activity as compared to *n*-butanol and chloroform extract of *Calotropis procera* leaves. The results of *in-vitro* cytotoxic activity suggested that the *n*-butanol extract had most pronounced cytotoxicity against the Hep-2. Further investigations are required to obtain the clinically important lead molecules for the drug development.

Keywords: *Calotropis procera* leaves; Traditional medicines; Anthelmintic activity; Cytotoxic activity; SRB Assay; Human larynx epithelial carcinoma.

INTRODUCTION

Calotropis procera (Ait.) R.Br. (giant milkweed) belong to the family Asclepiadaceae, locally known as "*aak*" is being used as herbal medicine by people living in desert areas[1] and also found sculptured on Shiva temple symbolizing mythological cum medicinal value enjoyed by the plant in ancient India[2]. In the traditional Indian Medicinal system, this plant has been used for a variety of disease conditions including asthma, cold, cough, piles, ulcers, diarrhoea, heart diseases, leprosy, rheumatism and diseases of skin, spleen, liver and abdomen[3-5]. The leaf of *Calotropis procera* is sub-sessile, 6-15 cm by 4.5-8 cm, broadly ovate, ovate-oblong, elliptic or obovate acute, pubescent, when young and glabrous on both sides on maturity. The lamina which is dorsiventral with mesophyll differentiated into a palisade and spongy tissue[6]. Extracts from this plant have been found to possess various pharmacological activities[7,8]. The flowers as well as latex of the plant have been claimed to be useful as an anthelmintic[9,10] and latex as well as roots of the plant have also been reported to possess cytotoxic activity[11-15]. The present study was undertaken to find the unexplored anthelmintic and cytotoxic potential of the different extracts of *Calotropis procera* leaves.

MATERIAL AND METHODS

Plant material

Fresh leaves were collected from the *Calotropis procera* plant growing in medicinal garden of Rajiv Academy for Pharmacy, Mathura, U.P., India. The plant specimens were authenticated and voucher specimen is NISCAIR/RHMD/Consult/-2008-09/1144/176 for future reference. The leaves were cleaned by washing with running water and shade dried and then milled to coarse powder by mechanical grinder.

Preparation of the leaves extracts

The leaf powder (2.0 kg) was extracted by maceration for seven days with 70% ethanol (3 x 2.5 liters) at room temperature and the combined ethanolic extract was evaporated under reduced pressure to yield hydroethanolic fraction as brownish green viscous residue (60 g). The mark was further extracted sequentially by maceration with *n*-butanol (3 x 2.5 liters) and chloroform (3 x 2.5 liters). The organic layers were evaporated under reduced pressure to yield *n*-butanol fraction (32 g) and chloroform fraction (19 g). The prepared extracts were kept under refrigeration for screening of anthelmintic activity.

In-vitro anthelmintic assay

The *in-vitro* anthelmintic activity was evaluated on adult Indian earthworm *Phaeritima posthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings[16-19]. The earthworms were collected and washed with normal saline. The earthworms used were of 5 to 6 cm in length and 0.2-0.3 cm in widths for experiment protocol[20, 21].

Different extracts prepared from *Calotropis procera* leaves were examined systematically for their *in-vitro* anthelmintic activity against *Phaeritima posthuma.* The *in-vitro* anthelmintic assay procedures were carried out as per method of Mathew et al.[22] and Dash et al.[23] with slight modifications[24-27]. Five groups of equal size Indian earthworm consisting of six earthworms in each groups were released into 50 mL of desired formulation. Each group was treated with one of the following: Vehicle (0.9% w/v NaCl), piperazine citrate (15 mg/mL), and different extracts of (50 mg/mL, 25 mg/mL, 12.5 mg/mL) in normal saline. Observations were made for the paralysis time and subsequently for death time of the worms. The mean paralysis and/or death time for each group was recorded. The time taken by the worms to become motionless, considered as paralysis time, was recorded and the lethal time was also recorded by observing the time taken to become motionless on application of external stimuli by pricking with pin. Piperazine citrate (15 mg/mL) was taken as reference drug. (Table 1,2 & 3)

Table 1: *In-vitro* anthelmintic activity of 70% hydroethanolic extract of *Calotropis procera* leaves

Treatment	Concentration (mg/ml)	Paralysis Time (min.)	Death Time (min.)
Vehicle	___	___	___
Piperazine citrate	15.0	13.32±0.360	31.10±0.230
70% Hydroethanolic Extract	50.0	5.52±0.143	12.25±0.232
70% Hydroethanolic Extract	25.0	13.53±0.704	27.50±0.207
70% Hydroethanolic Extract	12.50	18.58±0.64	29.05±0.628

Table 2: *In-vitro* anthelmintic activity of *n*-butanol extract of *Calotropis procera* leaves

Treatment	Concentration (mg/ml)	Paralysis Time (min.)	Death Time (min.)
Vehicle	___	___	___
Piperazine citrate	15.0	13.32±0.360	31.10±0.230
***n*-Butanol extract**	50.0	6.32±0.26	14.25±0.12
***n*-Butanol extract**	25.0	16.03±0.20	29.52±0.61
***n*-Butanol extract**	12.5	21.03±0.22	48.26±0.16

Table 3: *In-vitro* anthelmintic activity of chloroform extract of *Calotropis procera* leaves

Treatment	Concentration (mg/ml)	Paralysis Time (min.)	Death Time (min.)
Vehicle	___	___	____
Piperazine citrate	15.0	13.32±0.360	31.10±0.230
Chloroform extract	50.0	7.21±0.497	13.0±0.369
Chloroform extract	25.0	15.00±0.290	28.20±1.90
Chloroform extract	12.5	26.53±0.350	57.25±0.25

Results expressed as ±SEM of six worms in each group.

In-vitro cytotoxic assay

Cell line and cell culture

The Human larynx epithelial carcinoma cell line (Hep-2) was obtained from National centre for cell sciences, Pune. Hep-2 cells were grown in Earl's Minimal essential medium supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum, penicillin (100 µg/mL), streptomycin (100 µg/mL) and amphoterecin B (5 µg/mL) and the cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were sub-culture twice a week.

SRB assay

The *in-vitro* cytotoxicity of different extracts of *Calotropis procera* leaves was determined against Hep-2 using sulforhodamine B (SRB) dye[28]. The monolayer cell culture was trypsinized and the cell count adjusted to 1.0 x 10^5 cell/mL using medium (MEM) containing 10% new born calf serum. To each well of the 96 well microtitre plate, 0.1mL of diluted cell suspension (approximately 10,000 cells) was added and kept for 24 hours and incubated at 37°C for 3 days in 5% CO_2 atmosphere for cell monolayer formation. After 24 hours, when a partial monolayer was formed at the bottom of the well, the supernatant was flicked off, washed the monolayer once and 100µL of different isolated extracts (70% hydroethanolic, chloroform and *n*-butanol) were added to the culture (cell) in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere and microscopic examination was carried out and observations recorded every 24 hrs. After 72 hrs, 25µL of 50% trichloro acetic acid was added to the wells gently such that it forms a thin layer over the drug dilution to form an overall concentration of 10%. The plates were incubated at 4°C for one hour. After that, plates were flicked and washed five times with tap water to remove traces of medium, drug and serum, and were then air-dried. The air-dried plates were stained with 100 ml of SRB and kept for 30 minutes at room temperature. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100 µL of 10 mM tris base was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using micro plate reader at a wavelength of 540 nm. Suitable blanks and positive controls were also determined. Values of CTC_{50} obtained are summarized in table 4, which is the concentration at which 50% of the cells are dead after 72 hrs of drug exposure.

Table 4: *In-vitro* cytotoxic activity results of different extracts of *Calotropis procera* leaves against the human larynx epithelial carcinoma (Hep-2) cell line

S. No.	Sample	CTC_{50} (µg/ mL)
1.	70% Hydroethanolic extract	170
2.	Chloroform extract	110
3.	*n*-Butanol extract	3.7

Statistical analysis

The values are expressed as mean ± SE (n=6) and the statistical analysis was performed by Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

In the present work, three different extracts, 70% hydroethanolic, chloroform and *n*-butanol from leaves of *Calotropis procera* were used to evaluate *in-vitro* anthelmintic activity against Indian earthworms *Pheritima posthuma,* and *in-vitro* cytotoxic activity against the Hep-2 (human larynx epithelial carcinoma) cell line.

In-vitro anthelmintic assay

The perusal of the anthelmintic activity data reveals that 70% hydroethanolic extract at the concentration of 12.5 mg/mL showed paralysis and death in 18.58 and 29.05 min. respectively. Similarly *n*-butanol and chloroform extract at the concentration of 12.5 mg/mL showed both paralysis and death in 21.03 and 48.26 min. & 26.53 and 51.25 min. respectively. The effect increased with concentration.

In-vitro cytotoxic assay

The *in-vitro* cytotoxic study results showed that the CTC_{50} value for the three extracts viz., 70% hydroethanolic extract, chloroform extract and *n*-butanol extract was 170, 110 and 3.7 µg/ ml, respectively against the Hep-2 (human larynx epithelial carcinoma) cell line. Thus, the *n*-butanol extract had most pronounced *in-vitro* cytotoxicity against the Hep-2.

DISCUSSION

It was observed that 70% hydroethanolic extract shown better activity as compared to *n*-butanol and chloroform extract of *Calotropis procera* leaves and reference control piperazine citrate. Different extracts caused paralysis followed by death of the worms at all tested dose levels. The potency of the extract was found inversely proportional to the time taken for paralysis or death of worms. The activity confirms the dose dependent nature of extract.

The *n*-butanol extract of *Calotropis procera* leaves exhibited a strong cytotoxicity for Hep-2 cell lines. Chemical constituents reported from the extracts of leaves are alkaloids, flavonoids, tannins, steroids, saponins and glycosides[29]. Three cytotoxic chemical constitutes reported are calotropin, uscharin and calotoxin[30]. These biologically active compounds may be responsible for the *in-vitro* cytotoxic activity of *n*-butanol extract against the Hep-2 cell lines. Further isolation and identification of the active compounds as lead in the crude extracts is recommended for the drug development.

CONCLUSION

In conclusion the 70% hydroethanolic extract of *Calotropis procera* leaves had significant anthelmintic activity. The *in-vitro* cytotoxic study results showed that the *n*-butanol extract had most pronounced cytotoxicity against the HEp-2 cell line. The results

provide a support for the use of *Calotropis procera* leaves in traditional medicine and suggest its future advance investigation. So, further identification of chemical constituents responsible for these activities as well as pharmacological and toxicological studies and are needed to pinpoint the findings.

ACKNOWLEDGEMENTS

Authors acknowledge the support of JSS College of Pharmacy, Tamil Nadu for providing *in-vitro* cytotoxic assay data. Authors are also grateful to C.P.C.R.I., Kasaragod, Kerala, for providing the earthworms for testing anthelmintic activity.

REFERENCES

1. *Calotropis* R. Br., in The wealth of India-a dictionary of Indian raw materials and industrial products (Publications and Informations Directorate, CSIR, New Delhi), 3rd Volume, 1992, p.78.
2. Gupta S M, Plants in Indian temple art (BR Publishing Corp., New Delhi) 1996, p.56.
3. Razzak H M A, Unani System of Medicine in India (Central Council for Research in Unani Medicine, New Delhi), 1991, p. 29.
4. Kartikar K R and Basu N, Indian Medicinal Plants (Lolit Mohan Basu, Allahabad), 1935, p. 1606.
5. Verma R, Satsangi G P and Shrivastava J N, Ethno-medicinal profile of different plant parts of *Calotropis procera* (Ait.) R. Br., *Ethnobotanical Leaflets*, 2010, 14, 721-742.
6. Murti Y, Yogi B and Pathak D, Pharmacognostical standardization of leaves of *Calotropis procera* (Ait.) R. Br. (Asclepidaceae), *Int J Ayur Res*, 2010, 1(1), 14.
7. Ahmed K K M, Rana A C and Dixit V K, *Calotropis* species (Ascelpediaceae)- A comprehensive Review, *Phcog Mag*, 2005, 1(2), 48-52.
8. Kumar V L and Arya S, Medicinal uses and pharmacological properties of *Calotropis procera,* In: Govil J N, Editor, Resent Progress in Medicinal Plants, (Houstan, Texas: Studium Press), 2006, 11, p. 373.
9. Al-Qarawi A A, Mahmoud O M, Sobaih H E M and Adam S E, A preliminary study on the anthelmintic activity of *Calotropis procera* latex against *Haemonchus contortus* infection in *Najdi sheep*, *Vet Res Commun*, 2001, 25, 61-70.
10. Iqbal Z, Lateef M, Jabbar A, Muhammad G, Khan M N, Anthelmintic activity of Calotropis procera (Ait.) Ait. F. flowers in sheep, *J Ethnopharmacol*, 2005, 256-261.
11. Bhagat M, Arora J S and Saxena A K, *In vitro* cytotoxicity of extracts and fractions of *Calotropis procera* (Ait.) roots against human cancer cell lines, *Int J Green Pharm*, 2010, 4, 36.
12. Mathur R, Gupta S K, Mathur S R and Velpandian T, Anti-tumor studies with extracts of *Calotropis procera* (Ait.) R. Br. root employing Hep2 cells and their possible mechanism of action, *Indian J Exp Biol,* 2009, 47, 343-348.
13. Oliveira J S, Bezerra D L, Freitas C D T, Filho J D B M, Moraes M O et al, *In vitro* cytotoxicity against different human cancer cell lines of laticifer proteins of *Calotropis procera* (Ait.) R. Br, *Toxicology in Vitro*, 2007, 21, 1563-1573.
14. Choedon T, Mathan G, Arya S, Kumar V L and Kumar V, Anticancer and cytotoxic properties of the latex of *Calotropis procera* in a transgenic mouse model of hepatocellular carcinoma, *World J Gastroenterol,* 2006, 12(16), 2517-2522.
15. Quaquebeke E V, Simon G, Andre A, Dewelle J, Yazidi M J et al, Identification of a novel cardenolide (2”-oxovoruscharin) from *Calotropis procera* and hemisynthesis of novel derivatives displaying potent *in vitro* antitumor activities and high *in vivo* tolerance: Structure activity relationship analyses, *J Med Chem,* 2005, 48, 849-856.
16. Vidyarthi R D, A Text book of Zoology (S. Chand and Co., New Delhi), 14th Edition, 1967, p. 45.
17. Thorn G W, Adams R D, Braunwald E, Isselbacher K J and Petersdorf R G, Harrison’s Principles of Internal Medicine (Mcgraw Hill Co., New York), 1977, p.1088.
18. Vigar Z, Atlas of Medical Parasitology (P.G. Publishing House, Singapore), 2nd Edition, 1984, p. 216.
19. Chatterjee K D, Parasitology, Protozoology and Helminthology (In Guha Ray Sree Saraswati Press Ltd., Calcutta), 6th Edition, 1967, p. 87.
20. Nirmal SA, Malwadkar G and Laware R B, Anthelmintic activity of *Pongamia glabra* Songklanakarin, *J Sci Technol*, 2007, 29(3), 755-757.
21. Aswar M, Aswar U, Watkar B, Vyas M, Wagh A and Gujar K N, Anthelmintic activity of *Ficus bengalensis, Int J Green Pharm,* 2008, 1, 170-172.
22. Mathew A S, Patel K N and Shah B K, Investigation on antifeedant and anthelmintic potential of *Adhatoda vasica* Nees, *Indian J Nat Prod*, 1998, 14(1), 11-16.
23. Dash G K, Mishra B, Panda A, Patro C P and Ganapaty S, Anthelmintic activity of *Evolvulus nummularious*, *Indian J Nat Prod*, 2003, 19(3), 24.
24. Dash G K, Suresh P, Sahu S K, Kar D M, Ganapathy S and Panda S B, Evaluation of *Evolvulus alsinoides* Linn for anthelmintic and antimicrobial activities, *Indian J Nat Remed,* 2002; 2(2),182-185.
25. Rastogi S, Rastogi H and Singh V, Anti-inflammatory activities of methanolic extract of *Plumeria rubra* leaves, *Indian J Nat Prod,* 2009, 25(4), 15-.
26. Ajayeoba E O, Onocha, P A and Olarenwaju OT, *In vitro* anthelmintic properties of *Buchholzia coriaceae* and *Gynandropsis gynandra* extracts, *Pharm Biol*, 2001, 39, 217-220.
27. Deore S L, Khadabadi S S, Kamdi K S, Ingle V P, Kawalkar N G et al, *In vitro* anthelmintic activity of *Cassia tora*, *Int J ChemTech Res,* 2009, 1(2), 177-179.
28. Bhahwal A S, , Kumar A, Gupta P, Sharma M, Sethi V K et al, Cytotoxic and apoptotic activities of novel amino analogues of boswellic acids, Bioorg Med Chem Lett, 2007, 17, 6411-6416.
29. Hassan S W, Bilbis F L, Ladan M J, Umar R A, Dangoggo S M et al, Evaluation of antifungal activity and phytochemical analysis of leaves, roots and stem barks extracts of *Calotropis procera* (Asclepiadaceae), *Pak J Biol Sci*, 2006, 9(14), 2624-2629.
30. Atal C K and Sethi P D, Isolation, properties and kinetic studies of *Calotropis*, *Planta Med*, 1962, 10, 77-85.