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In vitro Acaricidal Activity and Phytochemical Screening of *Calotropis gigantea* Flowers against *Riphicephalus microplus*

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ABSTRACT

Background: Tick infestation particularly, of *Riphicephalus microplus* is a costly affair for farmers particularly in times of emerging resistance. Biological control using plant extracts will serve as environment friendly and cost-effective method. The present study was taken up to investigate the phytoconstituents and acaricidal activities of ethanolic extract of *Calotropis gigantea* flowers.

Methods: The acaricidal efficacy of ethanolic extracts of *Calotropis gigantea* flowers against engorged adult females of *R. microplus* were accessed by measuring mortality, inhibition of oviposition, hatching rate and larval mortality through Adult Immersion and Larval Packet Test. Gas chromatography-Mass spectrometry (GC-MS) analysis was done to reveal various compounds found in the flowers of *Calotropis gigantea* to justify their acaricidal activity.

Result: As per the results of the *in vitro* tests (adult immersion test and larval packet test), there was adult mortality of 56.67%, RI of 0.17, IO of 66% and larval mortality of 60.33% at concentration of 100 mg/ml. LC_{50} values were found to be 83.18 mg/ml. The GC-MS analysis revealed the presence of two major phytocompounds: dibutyl phthalate and n-Hexadecanoic acid in the extracts suggesting potential acaricidal properties. Results of the present study revealed the ethanolic extracts of *Calotropis gigantea* flower have potential anti-tick activity against both larval and adult stage of *Rhipicephalus microplus*.

Key words: Adult immersion test, *Calotropis gigantea*, GC/MS analysis, Larval packet test, *Riphicephalus microplus*.

INTRODUCTION

Around 80% of the global cattle population is exposed to infestation by ticks with an estimated impact of 7.3 US $/ head/ year (FAO, 2004). Ticks cause tick paralysis,tick worry, irritation, unrest, direct injury to hides due to tick bites, loss of blood due to the feeding of ticks and weight loss due to massive infestation of ticks (Drummond, 1983). Ticks act as carrier of numerous bacterial, viral and protozoan diseases *via* virtue of its ability to transmit pathogen from one host to another during blood feeding (FAO, 1998). The Asian blue tick, *Rhipicephalus microplus* is a one host tick commonly found in Asia including India and has been recorded on cattle, other livestock population and some wild animals (Haque *et al*., 2011; Patel *et al*., 2019). It is responsible for transmission of *Babesia bigemina, B. bovis, Anaplasma maginale, Coxiella burnetii* and *Borrelia theileri.* (Peter *et al*., 2005). As tropical and sub-tropical climate is suitable for ticks to complete their life cycle, they are more prevalent in these areas, especially in the study area (Odisha). Odisha lying just south of the Tropic of Cancer, has a tropical climate and the weather is also greatly influenced by the sea.

The average south-west monsoon rainfall in the state during July-September is 150 cm and these conditions also favour the dissemination of ticks and related diseases.

Presently, use of chemical acaricides *viz*., synthetic pyrethroids, organophosphates, formamidines and macrocyclic lactones is the most common means to eradicate ticks on the animal body as well as environment. The costly treatment of livestock results in costlier end product. The overdependence on chemicals has led to spawning of residues polluting soil and water bodies as well as finding their way into milk and meat (Gromboni *et al*., 2007). As the life cycle of parasitic arthropods is short and they possess high fecundity, there is also development of acaricide resistance which deems the chemical treatment programme undesirable.

In contemporary times, there is a necessity to look for alternative affordable control measures against tick infestation, especially for the small-scale farmers with meagre income, who constitute the majority of animal rearers

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in the developing countries, including India. Biological control being environmentally friendly can be taken alternatively or along with acaricide drugs. The herbal remedies include use of plants, plant extracts and their essential oils, are been investigated for acaricide efficacy against economically important tick species like *Rhipicephalus microplus* by researchers around the world (Borges *et al.,* 2011; Juliet *et al*., 2012). The plant extracts are accessible due to local availability and have history of some insecticidal or medicinal properties and traditionally used by the people (Sharma *et al*., 2012; Shyma *et al*., 2021). As it is nontoxic to humans, these can be a potential alternative to the current chemicals available for control of tick infestations.

The purple-white flowers of *Calotropis gigantea* is used as a very famous traditional folk medicine by many cultures owing to its diverse phytochemicals. To investigate this, the ethanolic extract of flowers of *Calotropis gigantea* were examined for their chemical composition and acaricidal effect on *Rhipicephalus microplus*.

MATERIALS AND METHODS

Collection of plant material

The fresh flowers of *Calotropis gigantea* were collected from their natural habitat in and around Bhubaneswar and research work was carried out in Department of Veterinary Parasitology, Odisha University of Agriculture and Technology, Bhubaneswar during 2018-2020. They were identified in the Department of Botany, College of Basic Science and Humanities, Bhubaneswar. The adulterants were separated, washed and dried in shade for two-three weeks. Dried flowers were finely pulverized by steel grinder, the resulting powder was sieved through a mesh (2 mm size) and kept in separate clean and dry container. Hot extraction was done in Soxhlet apparatus maintained at 70°C using 50 grams of the plant material and 400 ml of ethanol as solvent for 7 hours and the solvent was recovered in rotary evaporator (Gopalasatheeskumar, 2018).

Collection of target organism

Engorged *R. microplus* female ticks were handpicked or collected by using blunt end forceps with gentle pressure, from the body of cattle and in the vicinity of cattle sheds. The ticks were identified under stereozoom microscope as per taxonomical details (Walker *et al*., 2003) and transferred into specimen bottles, washed and dried.120 ticks were used for adult immersion test, divided into four groups (four treatments) each comprising of 30 ticks with 10 ticks each in three replicates (Drummond, 1983). 30 ticks were used as control where no treatment was applied. About 20 ticks were kept separately (28±1°C, 85±5% RH) covered by filter paper for oviposition. Eggs were laid upto 14-15 days and were allowed to hatch to larvae in 15-20 days under similar conditions of incubation. The larvae were utilised in larval packet test.

Adult immersion test

The test was conducted based on FAO (1984) guidelines, where ticks were divided into 4 groups (10 ticks in each group). Required quantity of extracts were weighed and dissolved in distilled water for making four different dilutions (12.50 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml). The ticks were immersed in working solutionof the plant extracts for 5 min for each dilution. For each concentration, three replications were maintained. The control group was immersed in distilled water. The treated ticks were kept in room temperature for 24 hrs, then transferred to desiccators and kept in BOD incubator (28±2°C temperature, 80±5% relative humidity) for oviposition. The adult tick mortality, weight of the eggs was compared to the control group. The ticks were allowed for oviposition up till 14-15 days, the eggs were incubated and % hatching were estimated visually.

Corrected percent of tick mortality was calculated as per Bagherwal *et al*. (1995).

$$\text{Corrected per cent mortality} = \frac{(\%\ \text{Mortality of test group} - \%\ \text{Mortality of control group})}{(100 - \%\ \text{Mortality of control group})} \times 100$$

The reproductive index/index of egg laying and percentage inhibition of oviposition/fecundity was calculated using formulae (Goncalves *et al*., 2007).

$$\text{Reproductive index (RI)} = \frac{\text{Weight of eggs laid in gm}}{\text{Weight of female tick in gm}}$$

$$\text{Inhibition of oviposition (IO\%)} = \frac{\text{RI of control group}}{\text{RI of treated group}} \times 100$$

Larval packet test

The larval packet test (LPT) was conducted according to FAO (1984) guidelines with minor modifications. Rectangle packets of 3.75×8.5 cm was made using Whatman filter papers. 0.6 ml of the working solutions of plant extracts was impregnated into the paper rectangles. The solutions were dried by keeping the packets inside the incubator at 37°C for 30 minutes. Approximately 100 nos. of larva were inserted in each rectangular folded packet and then placed inside dessicators in BOD incubator (28°C, 85% RH). After 24 hours interval, packets were removed and larva counted for test of mortality.

Interpretation of data, statistical analysis and log probit analysis

The data generated were analyzed by statistical analysis and groups were compared using one-way ANOVA for repeated measurements using SPSS Software version 20. A value of $p<0.05$ was considered as significant. Dose-response data were analysed by using probit method. The lethal concentration (LC_{50}) was determined by applying regression equation analysis to the probit transformed data of mortality.

Preparation of GC-MS sample

5 mg extract of fractions dissolved in 1 ml of GC-MS grade ethanol and 1 µl of it was used for injection in the gas chromatography-mass spectrometer of model GCMS-QP 2010, Shimadzu, Kyotoof Japan. Electron impact (EI) ionization was used at 70 eV. Helium was used as the carrier gas, with a flow rate of 1.0 ml min^{-1} and an ion source temperature of 250°C. The ethanolic fraction of flowers of *Calotropis gigantea* of eastern region of Odisha showed peak areas. The peaks were identified by NIST database and retention index. Identification of metabolite was accomplished by comparing retention time and mass spectra.

RESULTS AND DISCUSSION

According to several studies, *Rhipicephalus microplus* is growing increasingly resistant to numerous commercially available acaricides on the market (de Oliveira *et al*., 2015). In certain circumstances, it has been seen to be resistant to the use of commercially available multi-acaricides (Bhaskar *et al*., 2021). Among all other techniques being considered, use of plant-based acaricides, known as phyto-acaricides might be a useful option for managing the problem (Kumar *et al*., 2016).

Calotropis, commonly termed as “giant milkweed”or “Sweta Arka” is a shrub belonging to the family Asclepiadaceae, geographically located in Africa, Asia and South America. The Soxhlet extraction of flowers of *Calotropis gigantea* using ethanol yielded 19.80 g of extract with 39.6% yield. The extracts of *Calotropis gigantea* exhibited significant maximum and minimum mortality of 56.67% and 3.33% exhibited at 100 and 12.5 mg/ml respectively. Reproductive index showed inverse relationship with the concentration of plant extract. The extract showed antitick activity by significantly inhibiting the oviposition (IO%) in a dose dependant manner (Table 1). A maximum percentage of larval mortality was exhibited at 100 mg/ml (60.33%) and minimum at 12.5 mg/ml (14.33%) which differed significantly (Table 2). LC_{50} values were found to be 83.18 mg/ml for ethanolic extracts of flowers of *C. gigantea* as derived from the regression analysis. The acaricidal activity of nanoparticles (titanium dioxide) synthesized from of *Calotropis gigantea* flowers against the larvae of *Rhipicephalus microplus* yielded promising results (Sampath *et al*., 2013). However, (Shyma *et al.,* 2014) have reported slightly higher incides with regards to adult mortality and inhibition of oviposition from leaves of *Calotropis procera* while the reproductive indices and larval motility were quite similar to our results. The whole-plant extracts from *C. procera* (40 mg/ml) showed a larval mortality of 96.0% ±0.57. (Khan *et al*., 2019) while *in vitro* hot water extract at 24% concentration exhibited 43% mortality with LC_{50} value of 21.1 mg/ml (Nitya *et al*., 2013). The acaricide effect of flowers of combined aqueous herbal extracts of *Azadirachta indica* leaves and *Calotropis procera* flowers showed lethal effects on egg laying, hatching (22.35%) and total larval mortality at 50 mg/ml (Zaman *et al*., 2012). Al-Rajhy *et al*., (2003) tested cardenolide extract from *Calotropis procera* and reported better efficacy than Azadirachtin on the basis of LC_{50} values against larvae and adult stages of *Hyalomma dromedarii*.

The ethanolic extract of *Calotropis gigantea* flowers was subjected to GCMS-MS analysis for determination of the presence of total numbers of peaks and their retention time, peak area along their peak report. The spectral analysis (Fig 1, Table 3) showed a sharp peak with retention time of

Table 1: Effect of different concentrations of ethanolic extract of *Calotropis gigantea* on adult *Rhipicephalus microplus*.

Conc of extracts (mg/ml)	Live tick weight (gm) (Mean±SE)	Mortality (%) (Mean±SE)	Weight of eggs laid (gm) (Mean±SE)	RI (Mean±SE)	IO% (Mean±SE)
Control	0.746±0.016	0	0.380±0.061	0.507±0.070	0
100	0.73±0.010	56.667±3.333^{a}	0.125±0.011	0.171±0.017^{a}	66.144±3.347^{a}
50	0.762±0.006	30±5.774^{b}	0.149±0.016^{a}	0.195±0.020^{b}	61.435±3.890^{b}
25	0.735±0.005	13.333±3.333^{c}	0.155±0.006^{a}	0.211±0.007^{b}	58.384±1.359^{c}
12.5	0.776±0.005	3.333±3.333^{d}	0.198±0.006	0.255±0.008	49.618±1.643^{d}

*Means bearing different superscript in the same column differ significantly.

Table 2: Effect of different concentrations of ethanolic extract of *Calotropis gigantea* on larval motility of *Rhipicephalus microplus*.

Concentration of extract	No of treated larva (Mean±SE)	Live larva (Mean±SE)	Dead larva (Mean±SE)	% of larval mortality (Mean±SE)
Control	100	100	0	0
100	100	43±5.033	60.333±1.764	60.333±1.764^{a}
50	100	48.667±1.202	51.333±1.202	51.333±1.202^{b}
25	100	60.333±1.202	39.667±1.202	39.667±1.202^{c}
12.5	100	85.667±2.028	14.333±2.028	14.333±2.028^{d}

*Means bearing different superscript in the same column differ significantly.

Table 3: Retention time of active compound and peak area of GCMS spectral analysis of ethanolic extract of *Calotropis gigantea* flowers.

Extract	Peak	Retention time	Area %	Height %	Chemical compound
Ethanolic fraction	1	5.065	0.55	1.10	Piperidine, 4-methyl-1-nitroso-
	2	5.452	0.94	1.23	2,9-Dioxo-5,5,6,6-decanetetraca
	3	5.653	0.59	1.31	2,2,3,3-Tetraethyloxirane
	4	5.675	1.03	1.22	Oxazolidine, 3-ethyl-
	5	5.975	1.02	1.08	1-Hexene, 4-methyl-
	6	6.585	0.50	1.29	iso-Butyl 2-bromopropionate
	7	7.048	1.11	1.08	1-Hexene, 4-methyl-
	8	7.491	0.79	1.16	Pentane, 2,3-dimethyl-
	9	7.684	0.89	1.24	Heptafluorobutyric acid, 2,2-dim
	10	10.310	0.66	0.65	Carbonic acid, butyl hexyl ester
	11	11.646	0.84	0.96	2-Methylpentyl formate
	12	15.474	3.97	4.53	Tetradecanoic acid
	13	17.509	2.38	3.35	Phthalic acid, 2,7-dimethyloct-7
	14	19.419	32.19	22.52	Dibutyl phthalate
	15	19.533	38.14	40.73	n-Hexadecanoic acid
	16	21.943	1.88	2.81	Cyclopropaneoctanoic acid, 2-[[
	17	22.826	8.66	9.83	9,12-Octadecadienoic acid (Z,Z)
	18	25.813	3.85	3.91	Naphtho[2',3':4,5]imidazo[1,2-a

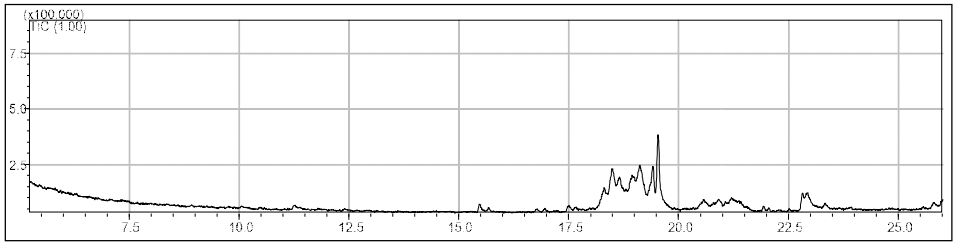


Fig 1: GC- MSspectral analysis of ethanolic extract of *Calotropis gigantea* flowers.

19.419 min. representing dibutyl phthalate. The peak area was highest at 38.14, height 40.73 and retention time 19.533 min. which corresponded to the compound n-Hexadecanoic acid.

The two compounds dibutyl phthalate and N-hexadecanoic acids discovered in our GC-MS study are also previously identified to be possessing antiparasitic activities. For instance, Dibutyl phthalate (di-n-butyl phthalate, DBP) is a known phthalate ester and has been described as an effective compound against demodicidosis (Yuan *et al*., 2001). Hexadecanoic acids have been identified as the active compounds responsible for arthropocidal activities in plant-based material (Magano *et al*., 2008). The cytotoxic activity of N-hexadecanoic acid occurred due to its interaction with DNA topoisomerase-I (Ravi and Krishnan, 2017). There are reports of N-hexadecanoic acid having anti-inflammatory, nematicide, pesticide activities (Praveen *et al*., 2010). The role of N-hexadecanoic acid as a potent mosquito larvicide has been documented (Rahuman *et al*., 2000).

Our findings of ant-tick activities of *Calotropis gigantea* flower can be reasonably attributed to the GC-MS findings of the presence of dibutyl phthalate and N-hexadecanoic acid in the extracts of *Calotropis gigantea*. The reported results might potentially be attributed to the additive and synergistic interactions of the chemicals found in the *Calotropis gigantea* flower.

Although *in vitro* plant extracts studies have shown hopeful results, *in vivo* and toxicity studies are essential before their use. It is very difficult to develop a perfect formulation of botanical acaricide for tick control due to scantily available literature of the active acaricide component. More research is needed to determine all the active components as well as the mechanism of toxicity that induced tick death. Specific toxicity testing is needed to show its harmlessness in non-target animals and people. Micro and nano encapsulating techniques are also being adapted to address this issue.

CONCLUSION

The ethanolic extract of *Calotropis gigantea* flowers reported remarkable acaricidal activity against *Rhipicephalus microplus*, justified through identification of dibutyl phthalate and n-Hexadecanoic acid. Further research through *in vivo* tests can evaluate the plant extract as an alternative to commercially available synthetic acaricides.

Conflict of interest

All authors declare that they have no conflict of interest.

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