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Acaricidal activity of *Juglans regia* hull extracts against unfed larvae of *Rhipicephalus microplus* ticks

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Abstract
The walnut (*Juglans regia*) hull extracts in three different solvents (acetone, chloroform and ethanol) were tested against unfed larvae of *Rhipicephalus microplus* ticks using larval immersion test. Mortality rates of larval ticks were in dose-dependent manner. The 100% mortality of larvae was achieved at 10% concentration for acetone and chloroform extracts whereas ethanolic extract showed 100% mortality of tick larvae from 7.5% concentration onwards. The LC_{50} values of 1.52, 1.22 and 1.43% were determined for chloroform, acetone and ethanolic extracts, respectively. However, the lowest LC_{95} value was calculated for ethanolic extract indicating its better acaricidal activity. Significant larvicidal activity of *J. regia* hull extracts against tick larvae qualifies them as green pesticides which could be combined with other tools for integrated pest management.

Keywords: Acaricidal activity, *Juglans regia*, larval immersion test, *Rhipicephalus microplus*

Introduction
Rhipicephalus microplus ticks are distributed throughout the tropical and subtropical regions of the world and are responsible for huge economic losses to dairy farmers by their direct (tick-worry, udder injury, decrease in milk production and damage to hides) and indirect (transmit babesiosis and anaplasmosis) effects [1]. Generally chemical acaricides are used to control these ticks. Ticks have become resistant to these acaricides due to their frequent widespread use, which ultimately rendered the chemical-based tick control programs as ineffective. The detrimental effects of chemical acaricides have led to the development of alternative and eco-friendly methods of tick control, and one such method involves use of natural products comprising of extracts and essential oils of plants which have efficient activity against both acaricide-susceptible [2, 3, 4] and acaricide-resistant [5] tick populations. Despite the advancing progress in health sciences, medicinal plants are still thought to be a vital source of different drugs in several countries around the globe. Plants with medicinal value can be considered as alternatives to the commercially available chemical drugs due to the presence of secondary metabolites [6].

The *Juglans regia* belongs to the family of Juglandaceae, is a big deciduous tree, which chiefly grows in Iran, Baluchistan, Himalayan regions of India, Armenia and several other regions with temperate climatic conditions [7]. Walnut is considered to be a rich source of fatty acids, polyphenols, tocopherols, essential amino acids and minerals [8]. The bark of the walnut tree contains various chemical substances comprising of flavonoids, phenolic compounds, alkaloids and steroids [9]. Besides the well-known bioactivities of the walnut kernel that include antioxidant, antibacterial, and anti-inflammatory [10], several studies reported that walnut leaves [11] and green husk [12] could also induce the same great health benefits. However, there is lack of information regarding acaricidal activity of walnut hull extracts against ixodid ticks. Thus, the aim of present study was to evaluate the acaricidal properties of walnut hull (outer covering of green fruit) extracts against unfed larvae of *R. microplus*.

Materials and Methods
Extract preparation
Fresh green walnut hull was collected from Kashmir region in polythene bags and brought to the laboratory. It was identified by Taxonomist, Centre for Biodiversity and Taxonomy,

University of Kashmir (voucher specimen number KASH-2920, dated: 03/05/2020). They were cleaned manually and air-dried in shade (temperature not exceeding 40°C) for 2-3 weeks. Various solvents *viz.* acetone, chloroform and ethyl alcohol were used for the preparation of different extracts of *J. regia* as per method given by Harborne [12]. All extractions were done by maintaining a plate temperature of (70-80°C) in Soxhlet apparatus. The final drying was done in a rotatory evaporator (5-6 rpm, 55°C). The dried extracts were transferred to glass containers and stored at – 20ºC for further use.

Collection of ticks

Fully engorged female ticks were collected from cattle sheds located at Chak-Siyan, Jammu (India). These ticks were brought to the laboratory in plastic vials covered with muslin cloths to allow exchange of air. After thorough washing and drying with paper towels, ticks were identified as per Walker [14]. After placing in individual vials, ticks were incubated at 28±1°C and relative humidity of 85±5% to obtain eggs. After two weeks, ticks were discarded and the eggs were kept in similar conditions of incubation to obtain larvae. About 2-3 weeks old unfed larvae were used to perform Larval Immersion Test (LIT).

Preparation of test concentrations

The extracts obtained from *J. regia* hull were dissolved in 1% ethanol-Triton X-100 (1% Eth-TX). Different working concentrations (0.625, 1.25, 2.5, 5.0, 7.5 and 10.0%) were prepared from the stock solution in 1% Eth-TX to conduct LIT.

Larval immersion test

The LIT described by Shaw [15] was used for the evaluation of acaricidal activity of *J. regia* hull extracts against two to three weeks old unfed larvae of *R. microplus* ticks. Briefly, approximately 100 larvae were taken into a 1.75-mL micro-centrifuge tube, containing 0.5 mL volume of each concentration of extract. The tube was shaken for few seconds for larval immersion and kept as such for 10 minutes. A filter paper (7.0 cm by 7.0 cm, Whatman No.1) was folded in half diagonally and sealed on one side with adhesive tapes. Then the tube lid was opened and the larvae were transferred to filter paper packet and the open end of packet was sealed with adhesive tape. These packets were incubated in BOD at a temperature of 28±1°C and relative humidity of 85±5% for 24 hours and subsequent percent mortality (number of dead larvae x100/total larvae) of larvae was determined. For each concentration of extract, the test was repeated twelve times. The 1% Eth-TX was used as negative control whereas deltamethrin (0.02%, Sigma-Aldrich) was used as positive control.

Statistical analyses

Data obtained were analysed by probit method [16] using Graph Pad Prism 4 software. Probit transformation of percentage mortality and natural logarithm transformation of concentration was carried out to determine the lethal concentrations at 50% (LC_{50}) and 95% (LC_{95}) and their respective 95% confidence limits (CL).

Results and Discussion

One-host *R. microplus* ticks are found round the year in the study area due to favourable climatic conditions for their development and propagation [17]. The commonly available chemical acaricides failed to control these ticks in field conditions as observed in our earlier studies [18, 19]. This has led to search for botanical acaricides to control ticks in field conditions and various studies have been conducted to evaluate plant extracts and essential oils against economically important ixodid ticks with promising results [20].

Various extracts of green walnut hull were assessed against larvae of *R. microplus* by estimating the larval mortality (%). The values of mortality (%), mortality slopes and goodness of fit (R^2) are presented in Table 1. The mortality rated were concentration dependent for all the extracts and 100% mortality was observed at the concentration of 10% for chloroform and acetone extracts while ethanolic extract showed 100% mortality at 7.5% concentration onwards. There was no mortality of tick larvae in negative control group which were exposed to diluent only whereas tick larvae in positive controls showed 100% mortality at the concentration of 0.02% of deltamethrin. Earlier, Wang *et al.* [21] have evaluated leaf extracts of *J. regia* against mites *Tetranychus cinnabarinus* and *T. viennensis* and they found both contact and systemic toxicity to these mites. It has been proven that the green walnut husk contains a common plant-borne fatty acid ester, methyl palmitate which possesses strong acaricidal activity [22]. Methyl palmitate induced contact toxicity has also been reported in mite, *T. viennensis* [23]. Further, the stem bark extracts (benzene, methanol and ethanol extracts) of *J. regia* exhibited significant anthelmintic activity on adult Indian earthworm, *Pheretima posthuma* as compared to that of standard drug piperazine citrate [24]. Henceforth, the chemical constituents, pharmacological and therapeutic characteristics of *J. regia* assemble it as a promising medicinal plant with wide range of pharmacological activities, along with its effectiveness and safety [25].

Table 1: The effect of various *J. regia* hull extracts on unfed larvae of *R. microplus*

Extract	Conc. (%)	Mortality (%) (mean±SE)	Slope±SE (95% CI)	R^2	LC_{50} (%) (95% CI)	LC_{95} (%) (95% CI)
Chloroform	0.625	30.9±0.9^{a}				
	1.25	42.3±0.6^{b}	2.32±0.75	0.71	1.52	7.69
	2.5	55.2±0.9^{c}	(0.24-4.41)		(1.46-1.58)	(7.09-8.34)
	5.0	68.8±0.7^{c}				
	7.5	85.1±2.1^{d}				
	10.0	100.0±0.0^{e}				
Acetone	0.625	40.8±0.7^{ab}				
	1.25	50.9±0.7^{abc}	2.27±0.71	0.72	1.22	6.48
	2.5	59.1±0.9^{bc}	(0.32-4.21)		(1.17-1.27)	(5.96-7.04)
	5.0	70.8±0.7^{c}				
	7.5	93.9±1.4^{d}				

	10.0	100.0±0.0^{e}				
Ethanolic	0.625	24.5±1.7^{a}				
	1.25	38.4±2.3^{b}	3.27±0.71	0.84	1.43	4.56
	2.5	55.6±3.5^{c}	(1.29-5.24)		(1.39-1.47)	(4.31-4.83)
	5.0	79.2±1.9^{d}				
	7.5	100.0±0.0^{e}				
	10.0	100.0±0.0^{e}				
Eth-TX	1.0	0.0±0.0^{a}				
Deltamethrin	0.02	100.0±0.0				

Eth-TX: Ethanol-Triton X-100 (1%), CI: Confidence interval
Mean followed by same letters do not differ statistically at a significance level of 5%.

The probit mortalities of tick larvae against log concentrations of chloroform, acetone and ethanolic extracts are shown in Fig. 1. The lethal concentrations (LC_{50} and LC_{95}) and their 95% confidence intervals are given in Table 1. The LC_{50} values of 1.52, 1.22 and 1.43% were recorded for chloroform, acetone and ethanolic extracts, respectively. Although the lowest LC_{50} value of 1.22% was recorded for acetone extract, the lowest LC_{95} value of 4.76% was determined for the ethanolic extract indicating better acaricidal activity against the larvae of *R. microplus* tick.

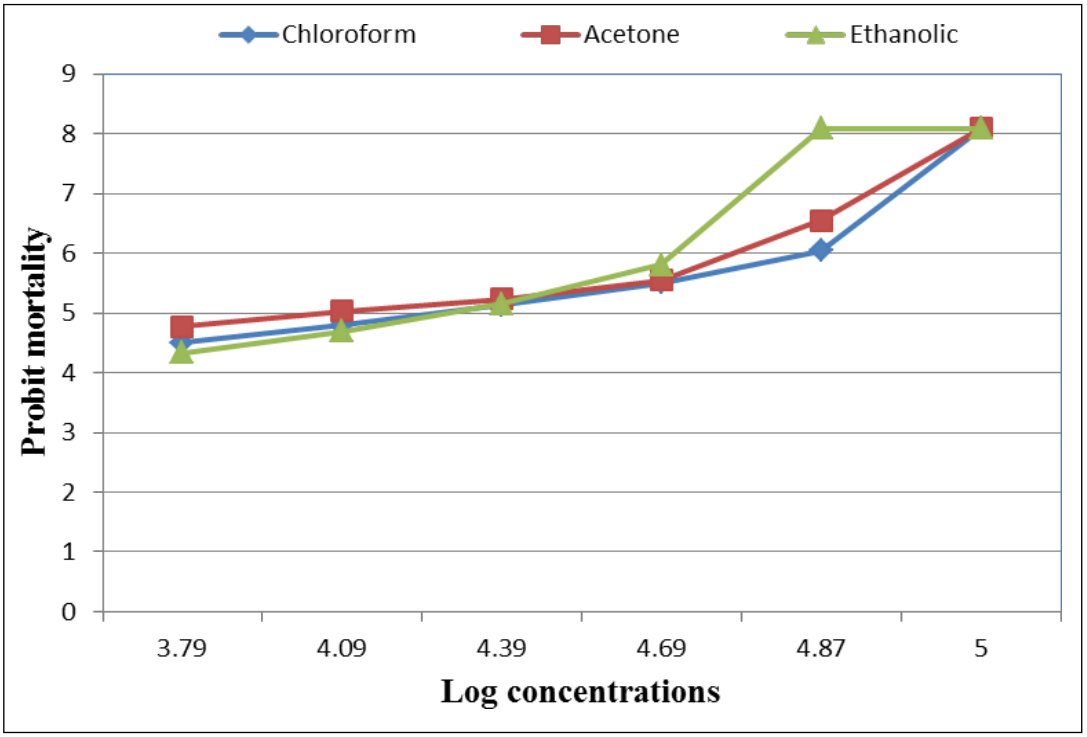

Fig 1: Probit mortality of unfed larvae of *R. microplus* against log concentrations of different hull extracts of *J. regia*

Conclusion

From the results of present study, it can be concluded that the different extracts prepared from the green hull of *J. regia* have acaricidal activity and more studies will be conducted to investigate its *in vivo* efficacy against larval and mature *R. microplus* ticks.

Conflict of interest

There are no conflicts of interest among the authors.

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