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Acaricidal Activity of Crude Ethanolic Extract of *Sphaeranthus indicus*, its Fractions and Subfractions against *Rhipicephalus* (*Boophilus*) *annulatus* (Acari: Ixodidae)

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A B S T R A C T

Plants constitute a rich source of bioactive compounds and its acaricidal effect is increasingly been investigated as a potential solution against acaricide resistance. In the present study, the *in vitro* efficacy of crude ethanolic extract of leaves of *Sphaeranthus indicus*, its fractions (hexane, chloroform, butanol and water) and subfractions of the active acaricidal fraction were evaluated against *Rhipicephalus (Boophilus) annulatus* using the adult immersion technique (AIT). The percentage of adult mortality, inhibition of fecundity and hatching rate were assessed. The crude ethanolic extract of leaves of *Sphaeranthus indicus* revealed hundred per cent adult mortality and inhibition of fecundity at a concentration of 250 mg/mL. Among the four fractions of this extract tested, hexane fraction showed concentration dependent delayed adult tick mortality. At 10 per cent concentration of hexane fraction, 87.41 per cent mortality and 77.78 per cent inhibition of fecundity were observed. Among the 14 subfractions of the active hexane fraction, the subfraction 4 (at 2000 ppm) produced 45.83 per cent mortality and 41.06 per cent inhibition of fecundity. It may be concluded that the hexane fraction of the leaves of ethanolic extract of *S. indicus* and its subfraction 4 revealed significant acaricidal effects.

Introduction

Ticks and tick borne diseases are major constraints for livestock farming in many developing countries. Their impact as disease vectors for human diseases is second to that of mosquitoes and their effect on livestock, wildlife and domestic animals is immeasurably greater (Jongejan and Uilenberg, 2004).

Prospects for control of ticks based on chemical acaricides are limited owing to the development of acaricide resistant ticks as well as public health concerns due to residues in meat and milk. Intensive use of chemical acaricides interferes with enzootic stability, rendering animals susceptible to the diseases (Shkap *et al.,* 2007).

Currently, 904 valid tick species are listed throughout the world (Burger *et al.,* 2014) while 106 species reported from India (Geevarghese *et al.,* 1997). *Rhipicephalus (Boophilus) microplus, R. sanguineus* and *Hyalomma anatolicum* were reported from many states of India (Ghosh and Nagar, 2014). *R. (B.) annulatus* is reported as the commonest species in southern India (Jagannath *et al.,* 1979; Koshy *et al.,* 1982; Rajamohanan, 1982). Some of the future strategies laid down for the sustainable tick control involves development of vaccines, newer generation chemical acaricides, herbal acaricides and transgenic animals (Ghosh *et al.,* 2007). Plants constitute a rich source of bioactive compounds such as phenolics, terpenoids, coumarins and alkaloids (Ahn *et al.,* 1998) which may possess insecticidal, growth inhibiting, anti-molting and repellent activities (Ghosh *et al.,* 2007). The acaricidal effect of plant products is increasingly been investigated as a potential solution against acaricide resistance (Adenubi *et al.,* 2018). Previously, few plants with acaricidal activity were reported from our laboratory based on their ability to kill the ticks, inhibit their fecundity as well as blocking hatching of the laid ova (Ravindran *et al.,* 2011, 2012, 2015; Juliet *et al.,* 2012; Sunil *et al.,* 2013; Divya *et al.,* 2014; Krishna *et al.,* 2014; Ajeesh *et al.,* 2016; Nair *et al.,* 2017)

Sphaeranthus indicus (commonly known as Gorakhmundi in Hindi, Adakyamaniyan in Malayalam) belongs to the family Asteraceae, is an annual spreading herb, which grows approximately 15-30 cm in height. The plant is distributed throughout the plains and wet lands in India, Sri Lanka and Australia (Gogate, 2000).

Used traditionally for the treatment of jaundice, leprosy, fever, pectoralgia, cough, gastropathy, hernia, harmorrhoids, helminthiasis, dyspepsia, skin diseases and as a nerve tonic, the plant is known to possess varied medicinal properties and is reportedly used in Ayurvedic preparations for treating epileptic convulsions, mental illness and hemicranias (Ambavade *et al.,* 2006; Jha *et al.,* 2010). The external application of a paste of this herb is claimed beneficial in treating pruritus, edema, arthritis, filariasis, gout and cervical adenopathy (Paranjape, 2001).

Pharmacological activities such as immunomodulatory (Bafna and Mishra, 2006), antimicrobial (Singh *et al.,* 1988; Duraipandiyan *et al.,* 2009), anxiolytic (Ambavade *et al.,* 2006), wound healing (Sadaf *et al.,* 2006; Jha *et al.,* 2009), anti-oxidant (Shriwaikar *et al.,* 2006; Prabhu *et al.,* 2009), hepatoprotective (Tiwari and Khosa, 2010) and anti-inflammatory activities (Nanda *et al.,* 2010) were reported for this plant. The acaricidal activity of the plant was previously not reported. Hence, the present study aims at studying the acaricidal activity of crude ethanolic extract of *S. indicus*, its different fractions and subfractions against adult female *R. (B.) annulatus* ticks.

Materials and Methods

Ticks

Fully engorged adult female ticks apparently of the same size were collected randomly from the infested calves. These animals were not treated with any acaricides for 60 days prior to the collection of ticks. The ticks were washed in tap water and mopped dry using an absorbent paper.

Plant materials

The plant was collected from Wayanad district of Kerala in May 2011. It was identified by a botanist and voucher specimen was deposited at Calicut University Herbarium (Accession number, CALI: 6635), Calicut, Kerala.

Preparation of ethanolic crude extract

One kilogram of dried leaves of the plant was pulverized. A portion of the powdered material (120g) was used for ethanolic extraction in a soxhlet apparatus attached with a solvent recovery unit (Rotavac, Buchi, Switzerland). The crude extract produced was then dried at room temperature, weighed and dissolved in methanol to prepare different concentrations of extract (50mg/mL, 100mg/mL and 250mg/mL) for testing the acaricidal activity.

Preparation of extract, fractions and subfractions

The crude ethanolic extract was fractionated using solvents of ascending polarity such as hexane, chloroform, n-butanol and water. The crude extract (200 g) was transferred to a separating funnel and extracted with hexane to obtain the hexane soluble fraction. Then, the hexane insoluble fraction was extracted with chloroform to yield chloroform soluble fraction. Further, the chloroform insoluble fraction was extracted with n-butanol and subsequently with water to yield n-butanol soluble and aqueous fractions. Solvents were removed using Rotavac unit and dried at room temperature and used for testing the acaricidal activity and phytochemical screening.

Thin layer chromatography

Thin layer chromatography was performed to find out the exact solvent system for column chromatography. Composition of each active fraction was assessed by one way ascending thin layer chromatography on silica gel pre-coated aluminium paper plates with thickness of 0.25mm (Merck F245). The sample (1mg) was applied to a plate, about 1 cm from the base. The plate was then dipped into a chamber containing fixed combination of solvent system and placed in a sealed container. Different subfractions in the sample mixture moved at different rates due to differences in solubility in the solvent and due to differences in their attraction to the stationary phase. Mobile phase of different compositions were tried and the solvent system with maximum efficient separation of subcomponent was selected as the solvent system for column chromatographic separation. Plates were visualized under 254 nm and 365 nm UV light (Ultraviolet Radiation Obligatory eye protection: Vilber Lourmat serial No V01 5636).

Column Chromatography

Silica gel (100 – 200 mesh, 200g, Merck), activated at 80˚C for 1 hour and 5g of respective active fraction was packed in 3x60 cm glass column and eluted with suitable solvent system, chloroform: hexane (80:20) for the active hexane fraction and hexane: chloroform (80:20) for subfraction identified in TLC for separation. Packing of the column was done in low polarity solvents such as hexane and the flow rate was adjusted to 1-3

drops per minute. Subfractions eluted from the active fraction were collected in separate glass tubes. Each subfraction was concentrated by removing the solvents in rotary vacuum evaporator and used for testing acaricidal properties.

High performance thin layer chromatography (HPTLC)

Chromatographic separation using HPTLC analysis (Camag, Switzerland) was performed on Merck TLC plates precoated with silica gel 60 F254 (20cm ×10cm with 200µm layer thickness) from E. Merck, Germany. Sample solution (0.5µL and 3µL) was applied onto the plates as a band with 8mm width using Camag 100 microlitre sample syringe (Hamilton, Switzerland) with a Camag Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (20 x 10cm) with the mobile phase chloroform: hexane (80:20) for hexane fraction and hexane: chloroform (80:20) for active subfraction. Scanning was performed using Camag TLC scanner 3 at 254nm, 366nm and 550nm through fluorescence mode and operated by win CATS software (version 1.4.1, Camag). Plates are visualized under UV 254nm, UV 366nm and in visible light after derivatizing with Anisaldehyde – sulfuric acid (ANS) reagent.

Phytochemical analysis

The active fraction was subjected to phytochemical tests for plant secondary metabolites like tannins, saponins, steroid, alkaloids and glycosides in accordance with Harbone (1991).

Adult immersion test (AIT)

Adult immersion test (AIT) was performed based on the method previously described Drummond *et al.,* (1973). Deltamethrin at a concentration of 0.03 mg/mL was used as positive control and methanol as negative control. A total of 24 ticks were used for each dilution with four replicates of six ticks. Ticks were weighed prior to the experiment and were immersed for 2 minutes in the respective dilution (10mL) in a 50mL beaker with gentle agitations. Ticks were recovered from the solution, dried using tissue paper towels and placed in separate plastic specimen tubes (25 x 50mm). The tubes were incubated at 28 ± 2˚C and 80% relative humidity in a BOD incubator. These ticks were observed for oviposition and mortality for 15 days. Mortality of the adult tick and weight of the eggs laid by the treated ticks were recorded in comparison with the control. The eggs laid by the ticks in each replicates were collected, weighed and observed at the same condition of incubation for the next 30 days for visual estimation of hatching. Ticks under different treatments were compared with that of the controls. Adult tick mortality, percent inhibition of fecundity and hatching of laid eggs by treated ticks were evaluated FAO (2004).

Adult tick mortality

The number of dead ticks in the specimen tubes was observed within 15 days after incubation. The percentage of adult tick mortality was then determined.

Inhibition of fecundity and hatching

The eggs laid by ticks in each tube were collected, weighed and observed at the same conditions of incubation for the next 30 days for visual estimation of hatching. Ticks under different treatments were compared with that of controls.

The percentage inhibition of fecundity was calculated as follows:

Index of egg laying (IE) = weight of eggs laid (mg) / weight of females (mg)

Percentage inhibition of fecundity (IF) = [IE (control group) -IE (treated group)] x 100 / IE (control group).

Statistical analysis

Statistical analysis of data was performed using standard procedures of Snedecor and Cochran (1994). Data were expressed as the mean ± SEM. Groups were compared using one-way ANOVA for repeated measurements using SPSS software. Duncan's test was used for post-hoc analysis. A value of $P<0.05$ was considered significant.

Results and Discussion

The efficacy of crude ethanolic extract of leaves of *S. indicus* against female *R. (B.) annulatus* is presented in table 1. Mortality of the adult ticks increased with increasing concentrations of the extract. A statistically significant effect on adult mortality could be observed at higher concentrations of 100 mg/mL and 250 mg/mL compared to vehicle control (methanol) and deltamethrin. The inhibition of fecundity ranged from 36.47 (at 50 mg/mL) to 100 per cent (at 250 mg/mL) on the treated ticks.

The per cent extractive values of hexane, chloroform, n-butanol and aqueous fractions of *S. indicus* are 16, 7, 2 and 5.75 respectively. Significant acaricidal activity against *R. (B.) annulatus* was observed only with hexane fraction of the crude ethanolic extract of leaves of *S. indicus* in comparison to other fractions. The per cent adult tick mortality caused by hexane fraction varied from 49.99 to 87.49 per cent when tested at concentrations ranging from 25 to 100 mg/mL (Table 2). Mortality was mainly seen after 7 days of treatment. The inhibition of fecundity varied from 36.21 to 77.78 per cent. The per cent mortality and inhibition of fecundity were concentration dependent. The hexane fraction did not exert any effect on the hatching of eggs laid by the treated ticks. The n-butanol fraction though exhibited some dose dependent effect on tick mortality and inhibition of fecundity; it was not significant compared to the hexane fraction. The chloroform and aqueous fractions did not exhibit any effect on adult tick mortality or inhibition of fecundity.

The hatching of the eggs laid by the ticks treated with n-butanol, chloroform and water fractions was apparently normal compared to control.

The acaricidal hexane fraction was further analyzed to ascertain the most effective subfraction. Suitable solvent system comprising chloroform: hexane (80:20) was identified based on TLC and HPTLC finger print profiles (Fig. 1). Fourteen subfractions were separated from hexane fraction by column chromatography with chloroform: hexane (80:20) as mobile phase. The acaricidal hexane fraction of *S. indicus* gave positive result for alkaloids, tannins and flavonoids.

The results of adult immersion test of fourteen subfractions of the active hexane fraction of crude ethanloic extract of leaves of *S. indicus* against *R. (B.) annulatus* are presented in table 3. Among fourteen subfractions, sub-fractions 2, 3, 4, 6 and 10 exhibited toxic effect on ticks. The subfraction 4 recorded the highest mortality (at 2000ppm concentration) of treated ticks.

The per cent mortality and inhibition of fecundity for active subfraction 4 were 45.83 and 41.06 per cent respectively. None of the subfractions significantly affected egg hatchability.

Fig.1 HPTLC finger print profile of hexane fraction of ethanolic extract of leaves of *S. indicus* at wavelength 366 nm eluted with Chloroform: Hexane (80:20)

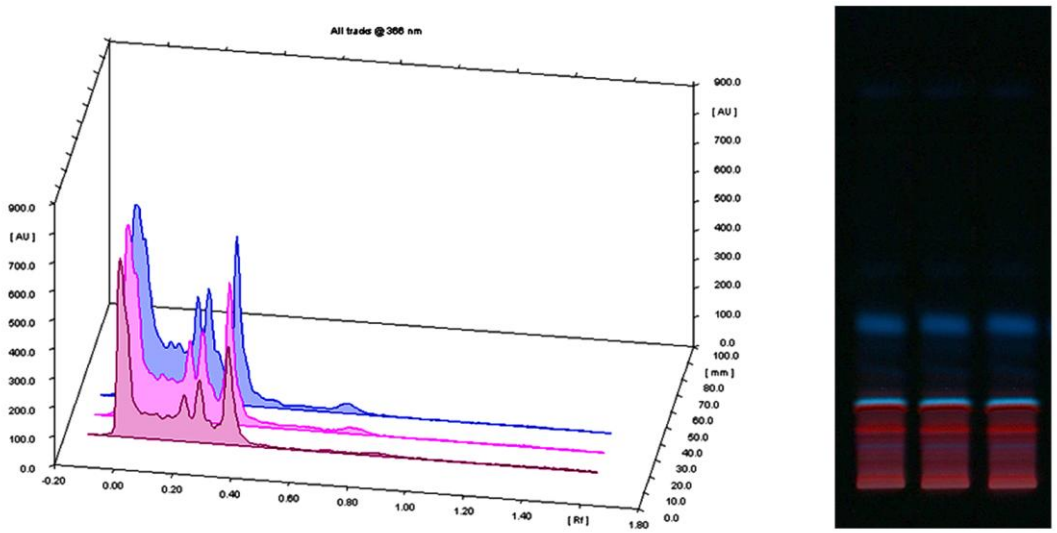

Table.1 Acaricidal effects of crude ethanolic extract of leaves of *S. indicus* against *R. (B.) annulatus*

Sl. No	Concentration mg/ml	Mean ticks weight per replicate ± SEM (g)	Mean % adult mortality within 15 days ± SEM	Mean eggs mass per replicate ± SEM (g)	Index of fecundity ± SEM	Percentage Inhibition of Fecundity (%)	Hatching % (Visual)
1.	Methanol	1.0182±0.0382 ^{bc}	0±0^{a}	0.4381±0.0300^{d}	0.4299±0.0240^{c}	0	100
2.	50	1.0824 ±0.0373^{c}	20.8275 ±4.1675^{b}	0.2969±0.0366^{c}	0.2731±0.0282^{bc}	36.47	100
3.	100	0.5525 ±0.0135^{a}	41.665±4.812^{c}	0.1242±0.0733^{b}	0.2269±0.0132^{b}	47.22	100
4.	250	0.9699 ±0.0369^{b}	100 ± 0^{d}	0 ± 0^{a}	0 ± 0 ^{a}	100	0
5	Deltamethrin 0.03	0.9653±0.0361^{b}	16.662 ±6.803^{b}	0.2081 ±0.0276^{bc}	0.2140±0.0236^{b}	57.3	10

n = 4, Values are Mean ± SEM, means bearing different superscripts a, b, c, d (P<0.05), indicate significant difference when compared with the control and recommended concentration of deltamethrin.

Table.2 Acaricidal effects of hexane fraction extracted from the ethanolic extract of leaves of *S. indicus* against *R. (B.) annulates*

Sl. No	Concentration mg/ml	Mean ticks weight per replicate ± SEM (g)	Mean % adult mortality within 15 days ± SEM	Mean eggs mass per replicate ± SEM (g)	Index of fecundity ± SEM	Percentage Inhibition of Fecundity (%)	Hatching % (Visual)
1.	Methanol	0.8885±0.0308^{ab}	0±0^{a}	0.3622±0.0290^{c}	0.4104±0.0390^{c}	0	100
2.	25	0.8264 ±0.0247^{a}	49.997 ±6.8035^{b}	0.2152 ±0.0219^{b}	0.2618±0.0306^{b}	36.21	100
3.	50	0.8034 ±0.0270^{a}	58.330±10.757^{b}	0.1776±0.0386^{b}	0.2212±0.05051^{b}	46.10	100
4.	100	0.7910 ±0.0595^{a}	87.4975± 4.1675^{c}	0.0069 ±0.0096^{a}	0.0912±0.0165^{a}	77.78	100
5	Deltamethrin 0.03	0.9653±0.0361^{b}	16.662 ±6.803^{a}	0.2081 ±0.0276^{b}	0.2140±0.0236^{b}	57.3	10

n = 4, Values are Mean ± SEM, means bearing different superscripts a, b, c, d *(*P<0.05), indicate significant difference when compared with the control and recommended concentration of deltamethrin.

Table.3 Acaricidal effects of sub-fractions obtained from the active hexane fraction of *S. indicus* against *R. (B.) annulatus* at 2000ppm

Sl. No	Concentration µg/ml	Mean ticks weight per replicate ± SEM (g)	Mean % adult mortality within 15 days ± SEM	Mean eggs mass per replicate ± SEM (g)	Index of fecundity ± SEM	Percentage Inhibition of Fecundity (%)	Hatching % (Visual)
1.	Methanol	0.6624±0.0240^{ab}	0±0^{a}	0.4400±0.0310 ^{e}	0.6712±0.0659^{d}	0	100
2.	Subfraction 1	0.6163±0.0280^{a}	0±0^{a}	0.3101±0.0062^{bc}	0.5063±0.0243^{c}	24.57	100
3.	Subfraction2	0.6546±0.0219^{ab}	12.497± 7.977^{a}	0.337±0.0108^{c}	0.5124±0.0300^{c}	23.66	100
4.	Subfraction3	0.7671±0.0330^{c}	4.1650±0.0416^{a}	0.4091±0.0307^{de}	0.5311±0.0203^{c}	20.87	100
5.	Subfraction4	0.6748±0.0348^{ab}	45.830±14.235^{b}	0.2643±0.0100^{b}	0.3956±0.0279^{b}	41.06	100
6.	Subfraction5	0.6748±0.0348^{ab}	0 ± 0^{a}	0.3355±0.0110^{c}	0.4978±0.0270^{c}	25.83	100
7	Subfraction6	0.7005±0.0116^{abc}	12.497± 7.9775^{a}	0.3551±0.0251^{cd}	0.5063±0.0306^{c}	24.57	100
8	Subfraction7	0.6270±0.0348^{ab}	0±0^{a}	0.3156±0.0210^{bc}	0.5104±0.0508^{c}	23.96	100
9	Subfraction8	0.6567±0.0216^{ab}	0±0^{a}	0.3414±0.0070^{c}	0.5209±0.0140^{c}	22.39	100
10	Subfraction9	0.6892±0.0286^{abc}	0±0^{a}	0.3517±0.0195^{cd}	0.5111±0.0266^{c}	23.85	100
11	Subfraction10	0.6552±0.0261^{ab}	4.1650±4.1650^{a}	0.3328±0.0105^{c}	0.5107±0.0288^{c}	23.91	100
12	Subfraction11	0.6734±0.0090^{ab}	0±0^{a}	0.3431±0.0158^{c}	0.5101±0.0265^{c}	24.00	100
13	Subfraction12	0.6734±0.0090^{ab}	0±0^{a}	0.3370±0.0220^{c}	0.5008±0.0329^{c}	25.39	100
14	Subfraction13	0.6878±0.0067^{abc}	0±0^{a}	0.3521±0.0150^{cd}	0.5120±0.0213^{c}	23.72	100
15	Subfraction14	0.7112±0.0168^{bc}	0±0^{a}	0.3605±0.0232^{cd}	0.5077±0.349^{c}	24.36	100
16	Deltamethrin(30)	0.9653±0.0361^{d}	16.6625±6.803^{a}	0.2081±0.0276^{a}	0.2140±0.0236^{a}	57.3	10

n = 4, Values are Mean ± SEM, means bearing different superscripts a, b, c *(*P<0.05), indicate significant difference when compared with the control and recommended concentration of deltamethrin.

In the present study, crude ethanolic extract of *S. indicus* demonstrated a significant concentration dependent effect on adult mortality and inhibition of fecundity. Qualitative phytochemical analysis of the acaricidal hexane fraction of the extract detected the presence of tannins, flavonoids and alkaloids. Subfraction 4 of hexane fraction of crude ethanolic extract of *S. indicus* showed the acaricidal activity.

Previous reports on *S. indicus* revealed that the aerial part of the plant is quite rich in flavonoids, essential oils, glycosides and eudesmanolides along with some uncharacterized sesquiterpenes, phenolic glycosides and sesquiterpene lactones (Galani and Patel, 2009). Besides, C-glycoside, 5-hydroxy-7-methoxy-6-C-glycosylflavone, n-pentacosan, sterols, stigmasterol, β-sitosterol, hentriacontane, β-D-glucoside of hentriacontane, n-triacontanol, sphaeranthine, isoflavone, 5, 4-dimethoxy-3-prenylbiochanin 7-O-β-galactoside (Jadhav *et al.*, 2007; Mishra *et al.*, 2007; Tiwari and Khosa, 2010) were also isolated from this herb. Biologically active compounds such as eudesmanolides (Shekhani *et al.*, 1991), methyl chavicol, α-ionone, δ–cadinene, p methoxycinnamaldehyde, thymoquinol-dimethyl ether, pdephene-2 and T-cadnol (Kaul *et al.*, 2005), ocimene, α-terpinene, methyl chavicol, α- citral, geraniol, α-ionone, β-ionone, δ-cadinene, p-methoxy cinnamaldehyde, sphaeranthine, stigmasterol, β sitosterol, hentriacontane, sesquiterpene lactone, sesquiterpene glycoside, sphaeranthanolides, flavones, isoflavone glycoside (Ambavade *et al.*, 2006) were also isolated from the whole plant of *S. indicus*. Sesquiterpene alkaloids isolated from *S. indicus* exhibited strong deterrent activity against several insects (Liu *et al.*, 1990). Sesquiterpene lactones were also shown to provide resistance to insect feeding (Burnett *et al.*, 1974). Previously different extracts of the plant revealed insecticidal activities against *Callosobruchus chinensis* (Baby, 1994), *Tribolium castaneum* (Tiwari and Saxena, 2003), *Sitotroga cerealella* (Srinivasan and Nadarajan, 2006), *S. indicus* also exhibited larvicidal effect against mosquitoes (Arivoli *et al.*, 2016).

It can be concluded that, the hexane fraction of the ethanolic extract of *S. indicus* and its subfraction 4 have significant acaricidal effects. Further, analysis of the subfraction 4 by GC MS will detect the phytochemical compound responsible for the activity.

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