RESEARCH ARTICLE

Phytochemical Analysis of Ethanolic extract of leaf of Azadirachta indica and *in vitro* Acaricidal Effect against *Rhipicephalus microplus*

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Abstract

The continuous use of chemical ectoparasiticides leads to development of acaricide resistance in ticks. The present study was focused on evaluation of *in vitro* acaricidal efficacy of ethanolic extract of *Azadirachta indica* leaf against *Rhipicephalus microplus* as well as phyto analysis of *A. indica* leaf extract. The phytochemicals in ethanolic extract of *A. indica* leaf was assessed by Gas chromatography-Mass spectroscopy (GC-MS) and Fourier Transform Infra-red Spectroscopy (FTIR). The larvicidal and acaricidal effect of ethanolic extract was investigated by Adult Immersion Test (AIT) and Larval Packet Test (LPT). The FTIR analysis showed presence of carboxylic acids, alkynes, amides, aromatic esters and alkyl hallides as the functional group and GC-MS revealed presence of components; Hexadecanoic acid, octadeca-9,12,15-trienoic acid, 2-(2,5-dimethoxy-4-methylphenyl) ethanamine, 4-ethyl-1,2-dimethoxybenzene.The adult tick mortality, reproductive index, Inhibition of oviposition and larval mortality was observed 86.687%,: 0.038,: 92.59% and 78.67%, respectively at highest concentration of extract (100 mg/mL) and LC_{50} value was determined to be 29.21 mg/mL. There was dose-dependant mortality of adult, larva ticks and reproductive parameters. These results showed that ethanolic extract of *A. indica* leaves is a good source of bioactive phytochemicals with antitick activities.

Keywords: Adult Immersion Test, *Azadirachta indica,* GC-MS, Larval packet test (LPT), *Rhipicephalus microplus.*

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Introduction

Rhipicephalus microplus plays an important role in transmission of bovine Babesiosis and Anaplasmosis apart from production losses and damage to hides (Zamora *et al.,* 2020). The common means of treatment of this one host tick is by chemical acaricides like pyrethroids, macrocyclic lactones and formamidines. The intensive use of these chemicals have led to development of acaricide resistance (George *et al.*, 2004). Natural plant products can be a source of potential acaricides having advantage of being less expensive and minimal environment toxicity.

Azadirachta indica (Meliaceae) commonly known as neem is mostly grown in tropical and sub-tropical regions. It is used in Unani, Chinese, and Ayurvedic medicines throughout the world, particularly in the Indian subcontinent against various diseases (Alzohairy, 2016). Neem has many application as antidermatic, antiviral, antiallergenic, antiscabic, antifeedant, antifungal, anti-inflammatory, larvicidal, nematicidal and insecticidal properties (Pankaj *et al.,* 2011).The bioactive compounds of the neem tree like gallocatechin, epicatechin, epigallocatechin, trilinolein, azadirachtin A and octadecanoic acid-tetrahydrofuran-3,4-diyl ester have significant acaricidal action against *R. microplus* larvae (Cen-Pacheco *et al.,* 2019).

The advancements in ethnobiology will generate interest in the utilisation of medicinal plants to enhance cattle health against cattle ticks and validate the effort to find a novel anti tick agent for pest control. Very scanty literature is available on the acaricidal effect and phytochemical composition of *A. indica* on *R. microplus* in India, therefore, the present study was designed to assess the phytochemical composition

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and *in vitro* acaricidal efficacy of *A. indica* leaf extract on *R. microplus*

Materials and Methods

Collection of Plant Material

A. indica leaf were collected from their natural habitation in and around Bhubaneswar, Odisha and were identified in the Department of Botany, College of Basic Science and Humanities, Bhubaneswar. The adulterants were separated, washed and shade dried for 3–4 weeks. The materials were then finely pulverized by blender, the resulting powder was sieved through a mesh and kept in a clean and dry container. Hot extraction was done in Soxhlet apparatus maintained at 50°C using 40 grams of the plant material and 400 mL of ethanol as solvent.

Fourier Transform Infrared Spectroscopy Analysis (FTIR)

The ethanolic extract sample of *A. indica* was subjected to FTIR analysis (Spectrum Two, ATR Sample base plate Diamond, Perkin Elmer). The FTIR was performed with resolution in the spectral area of 4000–450 cm^{-1} to detect the possible functional groups (Pakkirisamy *et al*., 2017).

Preparation of GC-MS Sample

5 mg of ethanolic extract of *A. indica* leaf was 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, Kyoto of 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 -i and an ion source temperature of 250°C. The ethanolic extract of *A. indica* leaf showed various peak areas. The peaks were identified by NIST database and retention index. The retention time signifies the particular compound and the area under the curve signifies the amount or purity of the separated compound. The metabolite was identified by comparing retention time and mass spectra. This data is included in the GC chromatogram, indicating the presence of numerous bioactive compounds in the ethanolic extract of *A. indica* leaf (Avinash *et al*., 2017).

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 near cattle sheds. The ticks were identified under stereozoom microscope (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 (10 ticks each in three replicates). 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 2 weeks, hatched to larvae in about 3 weeks under similar conditions of incubation, which were then used in Larval packet test.

Adult Immersion Test

The *in vitro* tests like larval packet test (LPT) and adult immersion test (AIT) are most common procedures for evaluating the acaricidal activities of different herbal-based formulations.

The test was conducted based on FAO (1984) guidelines, where ticks were divided into 4 groups of 30 ticks (10 ticks in each replicate in each group). Required quantity of extracts was weighed and dissolved in distilled water for making four different dilutions (12.50, 25, 50, and 100 mg/mL). The ticks were immersed in working solutions of 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 at room temperature for 24 hours and transferred to desiccators and kept in BOD incubator (28 ± 2°C temperature and 80 ± 5% relative humidity) for oviposition. The adult tick mortality and weight of the eggs were compared to the control group. The ticks were allowed for oviposition up to 14–15 days, the eggs were incubated and %hatching were estimated visually. The corrected percent mortality, the reproductive index and the inhibition of oviposition were calculated following standard respective formulae in practice.

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 onto the paper rectangles. The solutions were dried by keeping the packets inside the incubator at 37 °C for 30 minutes. Approximately 100 larva in each group were inserted in each rectangular folded packet and then placed inside dessicators in BOD incubator (28°C, 85% RH). After 24 hours, packets were removed and larva were counted for test of mortality.

Statistical Analysis and Log Probit Analysis

The comparison of different groups using one-way ANOVA was conducted using SPSS Software version 20. Dose–response data were analyzed by using log probit method and lethal Dose (LD_{50}) was determined by applying regression equation analysis.

Results and Discussion

Phytochemical Constituents of *A. indica* Leaf Extract.

The bioactive compounds responsible for this acaricidal activities have been studied through FTIR and GC-MS.

The spectrum obtained with FTIR spectroscopy (Fig. 1, Table 1), the peak value in the region of infra-red radiation

showed presence of carboxylic acids, alkynes, amides, aromatic esters and alkyl hallides as the functional group of the active components of *A. indica* leaf extracts which corroborates with the reports of Nair *et al*. (2013). The carboxylic acids present in *A. indica* leaf extract may be oleic acid, palmitic acid, stearic acid, linoleic acid and arachidic acid (Kaushik and Vir, 2000) which is responsible for insecticidal and growth inhibition activity (Farag *et al.,* 2011).

On GC-MS analysis, Hexadecanoic acid, octadeca-9,12,15-trienoic acid, 2-(2,5-dimethoxy-4-methylphenyl) ethanamine, 4-ethyl-1,2-dimethoxybenzene were the prominent compounds recovered based on peak area (Fig.2, Table 2). Hexadecanoic acid has been reported to possess considerable acaricidal activity against *R. microplus* (Park *et al*., 2016; Arceo-Medina *et al*., 2016).

The various functional groups found in FTIR analysis correlates with the compounds identified in the GC-MS analysis. These fatty acids and their esters from the leaf of *A. indica* are mainly responsible for the acaricidal and growth inhibitory activity against *R. microplus* similar to insecticidal and growth inhibition activity of fatty acids and their esters of *Melia azedarach* against *Spodoptera littoralis* (Farag *et al.,* 2011).

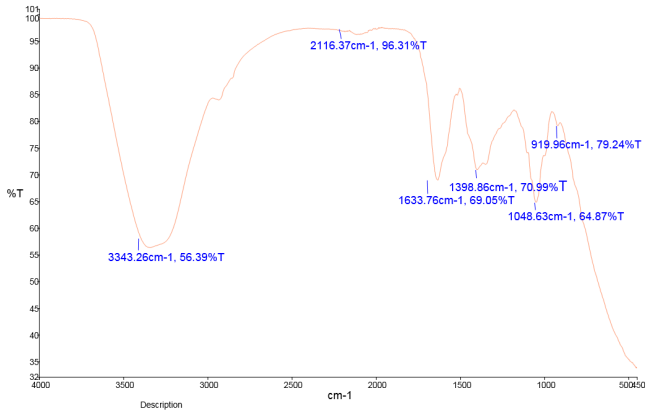

Fig 1: FTIR analysis of *A. indica* leaf ethanolic extract

Table 1: FTIR peak values of ethanolic extract of *A. indica* leaf

Peak value	*Functional group*	
3343.26	Carboxylic acid	0-H stretch
2116.37	alkynes	C ≡ C stretch
1633.7	amides	C=O stretch
1398.86	Aromatic esters	O=C-O-C
1048.63	Alkyl hallide	C-I stretch
919.96	alkenes	C-H stretch

Efficacy of *A. indica* Leaf Extract on Adult and Larva *R. microplus*

Based on the beneficial effects of phytochemicals present in *A. indica,* this study estimates its potential as a bioinsecticide against *R. microplus*. The effect of extract of *A. indica* leaf at different concentration on adult and larvae are presented in Table 3. The effect of the *A. indica* leaf extract is found dose dependent on all the parameters under study. Mortality % and IO % increased significantly ($p < 0.05$) with increase in concentration of extract. Similarly, RI reduced significantly with increase in the concentration.

A maximum reproduction index at lowest concentration and minimum at highest concentration was registered during

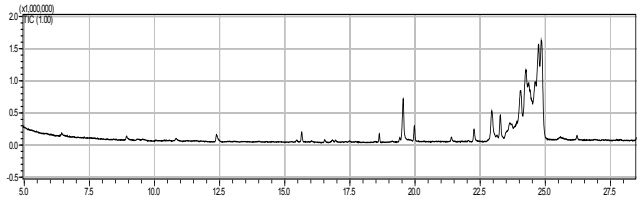

Fig 2: GC- MS spectral analysis of ethanolic extract of *A. indica* leaf

Table 2: Phytoconstituents of significance obtained on GCMS-MS analysis of *A. indica* leaf

Serial no.	*R. Time*	*Area%*	*Name*
1	6.432	0.41	2-(2-butoxyethoxy)ethanol
2	8.933	0.49	2,2,4,4,6,6,8,8,10,10,12,12,14,14-tetradecamethyl-1,3,5,7,9,11,13-heptaoxa-2,4,6,8,10,12,14-heptasilacyclotetradecane
3	9.334	0.46	3-phenylprop-2-enoic acid
4	10.832	0.60	5,6,7,7*a*-tetrahydro-4*H*-1-benzofuran-2-one
5	16.531	0.58	propan-2-yl tetradecanoate
6	19.543	11.17	Hexadecanoic acid
7	19.973	2.49	ethyl hexadecanoate
8	22.262	2.29	(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol
9	22.946	10.98	octadeca-9,12,15-trienoic acid
10	23.146	1.72	methyl octadec-8-ynoate
11	23.273	6.83	octadeca-9,12,15-trienoic acid ester
12	23.721	3.95	15-methylhexadecanoic acid
13	24.602	20.06	2-(2,5-dimethoxy-4-methylphenyl)ethanamine
14	24.743	33.77	4-ethyl-1,2-dimethoxybenzene

Table 3: Effect of different concentrations of ehanolic extract of *A.indica* on adult and larva of *Rhipicephalus microplus.*

Conc of extracts (mg/ml)	*Adult Mortality (%) (Mean ± SE)*	*RI (Mean ± SE)*	*IO% (Mean ± SE)*	*Larval Mortality(%) (Mean ± SE)*
Control	00.00	0.507 ± 0.070	00.00	0
12.5	20 ± 5.774^{d}	0.073 ± 0.004^{d}	85.683 ± 0.786^{b}	38.667 ± 1.763^{d}
25	43.333 ± 3.333^{c}	0.065 ± 0.003^{c}	87.231 ± 0.661^{b}	52.667 ± 1.453^{c}
50	70 ± 5.774^{b}	0.049 ± 0.007^{b}	90.284 ± 1.462^{a}	63.333 ± 1.764^{b}
100	86.667 ± 3.333^{a}	0.038 ± 0.009^{a}	92.583 ± 1.811^{a}	75.667 ± 1.453^{a}

*Means bearing different superscript in the same column differ significantly

the study. The larval mortality caused by the *A. indica* was tested at different concentrations increased significantly with increase in concentration. The LPT showed that there was dose dependent mortality of larvae as compared to the control group where no mortality was observed during the study period. Various reports on efficacy of extracts in different solvents for neem leaf varied from 30-70% (Micheletti *et al*., 2009; Shyma *et al.,* 2014; Mahran *et al.,* 2020) while seed extract showed an adult mortality of 80% (Srivastava *et al.,* 2008). Studies from South Africa depicted reduced tick prevalence on bovine population (Webb and David, 2002) on weekly spraying of neem seed extracts. Neem seed oil tested against the larvae of *Boophilus decoloratus* ticks showed cent percent mortality (Choudhury, 2009). The aqueous extracts of neem leaf showed highest acaricidal activity as compared to aqueous/chloroform/hexane extracts (Avinash *et al*., 2017)

The pronounced effect of neem leaf on reproduction of engorged females *R. microplus* and subsequent egg laying agrees with the previous reports (Borges *et al*., 2011; Giglioti *et al*., 2011; Aimee *et al*., 2017). The LD_{50} values of ethanolic extracts of leaf of *A. indica* were found to be 29.21mg/ml as calculated from the regression equation.

Conclusion

The phytochemical analysis reveals the presence of terpenes and fatty acids which have potential acaricidal and growth inhibitory activity against *R. microplus*. The bioactive compounds of leaf have the potential to be used as an effective alternative in the control of *R. microplus*. Secondary metabolites of neem offer sustainable pest control. The extract of leaf of *A. indica* possess *in vitro* anti tick activity against *R. microplus*.

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ANNOUNCEMENT: SVSBT-NS-2022

IX Annual Convention and National Seminar of SVSBT

The ***IX Annual Convention*** and ***National Seminar*** of The Society for Veterinary Science & Biotechnology ***(SVSBT) on "Recent Biotechnological Advances in Health and Management to Augment Productivity of Livestock and Poultry"*** will be **organized at Ramayanpatti, Tirunelveli - 627 358, Tamil Nadu, during September 22-24, 2022** (Thursday, Friday & Saturday) by Veterinary College & Research Institute, Tirunelveli - 627 358, TANUVAS, (TN). The detailed Brochure cum Invitation showing Theme Areas/ Sessions, Registration Fee, Bank Details for online payment and deadlines, etc. has been floated on the Whats Apps and e-mails. Accordingly, the organizing committee of ***SVSBT NS-2022 invites abstracts*** of original and quality research work on theme areas of seminar limited to 250 words by e-mail on svsbttnns2022@gmail.com or mopandian69@gmail.com latest by 30th August, 2022 for inclusion in the Souvenir cum Compendium to be published on the occasion.

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