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Ovicidal and larval repellent efficacy of *Tagetes erecta* Linn. on *Rhipicephalus sanguineus* (Latreille, 1806) (Acari: Ixodidae)

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ABSTRACT: A study was undertaken on the ovicidal and larval repellent activity of *Tagetes erecta* leaf and flower extracts on *Rhipicephalus sanguineus* (Latreille, 1806), an important tick species in the world from an economic and medical point of view. Ethanol and methanol extracted plant products tested against the eggs and larvae of *R. sanguineus* indicated that the ethanol extract of flower had maximum ovicidal activity (86.1%), followed by the ethanol extract of leaf (75%) at 25 mg ml^{-1} concentration. In all analyses, the homogeneity of variance was significant. The probit analysis clearly indicated that the ethanol extract of the flower has a higher ability to kill the eggs. In the case of larval repellency tested, both extracts of leaf showed the highest repellency (83%) at 2.5 mg ml^{-1}. Significant tick repellency (> 90%) was found in both methanol and ethanol extracts of flower at 2.5 mg ml^{-1}. GC-MS analysis of extracts revealed the presence of bioactive insecticidal compounds such as yangambin, cyclohexane and neophytadine.

KEYWORDS: Tick, ovicidal, larvicidal, repellency, marigold, bioactive compounds

INTRODUCTION

Ticks (Acari: Ixodidae) are the obligate ectoparasites of animals and are responsible for the transmission of numerous infectious agents such as pathogens to vertebrates, including viruses, bacteria, protozoa, and helminths (De la Fuente *et al*., 2008). In recent studies, tick and tick-borne diseases being much concentrated because of their increasing incidence and significant harm to livestock and human health (Balasubramanian *et al*., 2019). *Rhipicephalus sanguineus* (Latreille, 1806), brown dog tick is the most important tick species in the world as a vector of various disease-causing pathogens like *Coxiella burnetti, Rickettsia conorii* and *R. rickettsii* for animals as well as for human beings (Sonenshine and Roe, 2014). In India, the causative pathogens of Indian tick typhus (ITT), a type of rickettsial spotted fever (similar to Rocky Mountain spotted fever), and Babesiosis are transmitted by *R. sanguineus* (Srikant Ghosh and Gaurav Nagar, 2014; Brites-Neto *et al*., 2015). Studies with an emphasis on tick controls are limited to India. Chemical

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acaricides like amitraz, synthetic pyrethroids used to control resulted in the development of resistance to the ticks (Eiden *et al*., 2015; Rodriguez-vivas *et al*., 2017). Plant-derived natural acaricides are the suitable alternative for chemical acaricides with minimum toxicity, high rate biodegradation and less resistance development (Quadros *et al*., 2020). Although many plant species have been traditionally used to control ticks (Adenubi *et al.,* 2016), the efficacy of extracts of many of the plant species have not been investigated and validated. The plant *Tagetes erecta Linn.* belonging to the *Asteraceae* family, commonly known as marigold, is commonly cultivated in India and has acaricidal properties (Wanzala *et al*., 2014; Fabrick *et al*., 2020). Hence a study was undertaken on the ovicidal and larval repellent activity of ethanol as well as methanol extracts of *T. erecta* leaf and flower on *R. sanguineus*.

MATERIALS AND METHODS

From the blood-fed adult ticks collected from cattle and naturally infected dogs, *R. sanguineus* species ticks were selected after the identification by morphological identification key (Hans *et al*., 2001). Ticks were placed in a plastic container (7 X 5 cm) capped with a piece of cotton cloth tubes were placed in an incubator (28 ±1°C and RH ≥ 80%). Freshly laid eggs and subsequent larva were used for the experiments.

Marigold plant at the full bloom stage was confirmed as *T. erecta* from the faculty of Department of Biological science, Gandhigram University, Dindigul, Tamil Nadu. The leaves and flowers of the *T. erecta* plants were washed and dried at room temperature (25–30°C) and powdered using an electric grinder. The powdered flower and leaf are stored in a sealed bottle at room temperature. About 75–100 g of dried plant powder was weighed and kept in a thimble chamber of the soxhlet apparatus. Ethanol and methanol extraction of each sample was carried out in 75" 85 cycles at a temperature ranging from 40–55°C, and the extract was concentrated by evaporation at 40-55°C and then dried and kept at 4°C for the bioassays.

The phytochemicals in the extracts were identified as per Harborne (1998) and Kokate (2001). The analysis of the flower and leaf extracts was performed using a gas chromatograph coupled to a mass spectrometer (GC–MS) equipped with an auto-injector and a fused-silica capillary column. Helium was used as the carrier gas at a flow rate of 1.2 ml per minute. Injector and detector temperatures were set at 250°C and 280°C, respectively. Column temperature was set to 60°C for 5 minutes, then gradually increased to 160°C at 4°C for one minute and finally increased to 270°C at 15°C for one minute.

A stock solution of plant extracts was prepared in dimethyl sulpho oxide (DMSO). From the stock solution, concentrations of 5, 10, 15, 20, and 25 mg ml^{-1} meant for ovicidal repellency bioassay and concentrations of 0.5, 1, 1.5, 2 and 2.5 mg ml^{-1} for larval repellency bioassay were prepared.

Ovicidal assay: Twenty numbers of eggs were placed in glass vials (5cm x 2cm) with filter paper at the bottom and topically sprayed with 5 ml of different extracts with concentrations of 5, 10, 15, 20 and 25 mg ml^{-1}. Control eggs were treated with one per cent DMSO only. Three replicates were maintained for each treatment, and the experiment was conducted in an incubator (28 ± 1°C and RH ≥ 80%) and regularly observed until hatching began. The hatched larvae were separated every day from the unhatched eggs and observed for two more weeks before they were declared unhatched and dead. The ovicidal activity (%) was assessed by the following formula:

$$\frac{\text{Number of unhatched eggs X 100}}{\text{Total number of eggs introduced}}$$

Repellency Bioassay: The experiment was carried out in a test model with a funnel based on the combination of ambushing and hunting behavior of ticks. Test and control Whatman No.1filter papers (2.5 × 2.5 cm) were treated with 5 ml of different concentrations (0.5, 1, 1.5, 2 and 2.5 mg ml^{-1}) of sample solutions and air-dried for one hour. The control filter paper was impregnated with one per cent DMSO. The treated and control paper was placed in the middle of the tail tube of the funnel (5 × 0.5 cm). Twenty larval ticks were introduced on a base plate (7 × 1.5 cm). Ticks that were climbed on the upper part of the filter paper were considered not repelled, and those on the bottom of the filter paper, naked part of the apparatus, and on the base plate were considered repelled. Each experiment was repeated three times. The percentage repellency was calculated as:

$$100 - \frac{\{\text{Mean no. of ticks in test} \quad X\ 100\}}{\{\text{Mean no. of ticks in control}\}}$$

Statistical analysis: Probit analysis (EPA 2006) was used to analyse the ovicidal and larval repellency percentage with the calculation of confidence interval (CI) of the mean number of ticks repelled as well as the egg mortality by the treatment. Each replication was considered independently. Statistical significance on dose response with each concentration was determined by ANOVA. All significant levels are set at $P<0.05$. SPSS windows version IBM 20 was used for data analysis.

RESULTS AND DISCUSSION

The leaves and flowers of *T. erecta* in ethanol (EtOH) and methanol (MeOH) extracts were analysed for their phytochemical contents. Phytochemicals such as alkaloids, flavonoids, saponins, tannin, cardiac glycosides, and terpenoids were found. Tannins were found strong positive in both the extracts of leaves and flowers, followed by alkaloids and flavonoids. Cardiac glycosides, terpenoids and saponins were found in EtOH flower extracts (Table 1).

The bioactive compounds present in the leaves and flowers extracts, identification and characterization were based on their retention time in an HP-5MS column (Table 2, 3). Based on abundance, the three major compounds present in the MeOH extract of leaf were 1-butanol, 3-methyl-formate (39.10%), benzofuran (8.10%) and octyl-beta-D-glucopyranoside (7.15%). The EtOH leaf extract contained yangambin (69.44%) followed by alpha-tocopherol (14.65%) and neophytadine (4.73%) as major compounds. Diethyl phthalate (27.98%), Alpha D-Glucopyranose (19.63%) and 4H-Pyran (5.79%) as three major compounds in the MeOH extract of flower. Major compounds found in EtOH extracts of the flower are the yangambin (30.81%), 3H-Forofuran (29.78%), and Beta D-glucopyranose (10.98%).

Ovicidal Activity: Among the four extracts tested, the EtOH extract of flower recorded the highest ovicidal activity (86.1%), followed by the EtOH extract of leaf (75%) at 25mg ml^{-1}. The MeOH extracts of both leaf and flower showed 65 and 69.3 per cent respectively, ovicidal activity at 25 mg ml^{-1} (Table 4). The homogeneity of variance was significant at all the analyses and the ANOVA was significant (P value <0.05). The R^2 indicate that EtOH extracts of flowers had maximum

Table1. Phytochemical compounds in methanol and ethanol extracts of *T. erecta* leaves and flower

Phytochemicals	Test	Leaf		Flower	
		MeOH	EtOH	MeOH	EtOH
Alkaloid	Wagners	—	+++	—	+++
Flavonoid	Lead acetate	—	+++	—	+++
Saponin	Froth	+	—	+++	+++
Tannin	Ferric chloride	+++	+++	+++	+++
Cardiacglycoside	Keller-Killianis	—	—	—	++
Terpenoid	Salkowski	—	—	—	++

+ mild positive ++ Average — Negative, +++ strong positive

Table 2. Biochemical compounds in methanol and ethanol extracts of *T. erecta* leaves by GC-MS analysis

Methanol Extract			Ethanol Extract		
Compound	R T*	%	Compound	R T*	%
Cyclohexanamine,N-3-butenyl-N-methyl-	4.158	1.54	1-Butanol,3-methyl-formate	4.496	1.36
1-Butanol,3-methyl-formate	4.604	39.10	2-cyclohexen-1-one,3-methyl-6-	8.400	1.99
Aceticacid, pentylester	5.299	2.10	Neophytadiene	28.774	4.73
4h-pyran-4-one,2,3-dihydro-3, 5-dihydroxy	5.545	5.02	phytolisomer	35.909	2.61
benzofuran,2,3-dihydro-	7.244	8.10	hexadecanoicacid	43.255	1.01
benzaldehyde,2-hydroxy-6methyl	14.458	2.01	beta.-tocopherol	44.852	0.51
octyl-.beta.-d-glucopyranoside	20.866	7.15	methyl(z)-5,11,14,17-eicosatetraenoate	46.262	1.16
nonylamine,n,n-di(allyl)-	22.575	6.55	28-norolean-17-en-3one	46.523	1.59
Hexylamine,N,N-di(allyl)-	25.151	7.06	yangambin	47.643	69.44
Muco-Inositol	31.750	4.74	alpha.-Tocopherol-beta-D-mannoside	48.843	14.65

* Retention time

Table 3. Biochemical compounds in methanol and ethanol extracts of *T. erecta* flowers by GC-MS analysis

Methanol Extract			Ethanol Extract		
Compound	R T*	%	Compound	R T*	%
Thymine	4.175	4.18	1-Butanol,3-methyl-formate	4.160	0.85
2-Butanone,4-hydroxy-3-methyl-	5.25	4.38	2-Butanone,4-hydroxy-3-methyl-	4.512	1.93
4H-Pyran-4-one,2,3-dihydro-3, 5-dihydroxy-	5.551	5.79	4H-Pyran-4-one,2,3-dihydro-3, 5-dihydroxy-	5.539	1.20
Ketone,methyl2-methyl-1, 3-oxothiolan	7.838	3.27	2-Pyrrolidineaceticacid	7.027	0.54
beta.-Alanine,N-acryloyl-, isobutylester	8.85	2.52	Phenol, 2,6-dimethoxy-	11.225	4.14
Phenol, 2,6-dimethoxy-	11.225	7.46	beta.-D-Glucopyranose,4-O-. beta.-D-galact	19.853	0.69
Diethyl Phthalate	19.833	27.9	Nonylamine,N,N-di(allyl)-	25.149	0.51
Hexylamine,N,N-di(allyl)-	25.147	2.99	n-Hexadecanoicacid	32.339	0.28
n-Hexadecanoicacid	31.462	0.68	Stigmasta-5,20(22)-Dien-3-OL	43.998	3.32
.alpha.-D-Glucopyranose,4-O-. beta.-D-galac	35.951	19.6	Yangambin	45.925	30.81
(2,3,5,6-Tetrafluorophenyl)methyl 3-(2,2-dic	38.20	2.65	1h,3h-furo[3,4-c]furan,tetrahyd	47.264	29.78
.gamma.-Sitosterol	47.729	2.82	.gamma.-Sitosterol	47.86	4.74

* Retention time

Table 4. Effect of methanol and ethanol extracts of *T. erecta* on *R. sanguineus* eggs (% mortality ± SE)

Conc. ($mg\ ml^{-1}$)	Leaf		Flower	
	MeOH	EtOH	MeOH	EtOH
5	12.50±0.12	18.33±0.18	21.50±0.13	24.50±0.14
10	13.33±0.12	20.33±0.14	32.10±0.12	39.20±0.13
15	21.66±0.14	25.00±0.18	39.60±0.12	58.20±0.12
20	58.33±0.13	46.60±0.13	62.70±0.18	75.50±0.12
25	65.00±0.13	75.00±0.12	69.30±0.18	86.10±0.10

Table 5. Statistical analysis of ovicidal activity of methanol and ethanol extracts of *T. erecta* against *R. sanguineus* eggs

Extract	LC_{50}	LC_{90}	R^2	df	P value*	Upper Cl	Lower Cl	Regression
Leaf–MeOH	20.41	72.44	0.79	4	0.04	4.38	0.18	Y=2.00-2.28x
Leaf–EtOH	19.49	89.12	0.67	4	0.05	4.37	-0.50	Y=2.50-1.93x
Flower–MeOH	15.84	77.62	0.86	4	0.02	3.17	0.50	Y=2.79-1.84x
Flower-EtOH	10.71	34.67	0.94	4	0.005	3.58	1.38	Y=2.44-2.48x

*P value is significant at 0.05 level; MeOH – Methanol; EtOH – Ethanol; LC50 - Lethal concentration at 50%; LC90 - Lethal concentration at 90%; R^2 - Proportion of the variance; df – degree of freedom; Upper Cl – Upper confidance interval at 95%; Lower Cl – Lower confidence interval at 95%.

Table 6. *R. sanguineus* larval repellency (%) in different concentrations of methanol and ethanol extracts of *T.erecta*

Conc. ($mg\ ml^{-1}$)	Leaf		Flower	
	MeOH	EtOH	MeOH	EtOH
0.50	12.50	25.00	21.40	41.60
1.00	33.30	67.50	75.00	78.50
1.50	41.20	75.00	87.50	92.80
2.00	63.30	87.50	91.80	99.00
2.50	83.00	86.10	96.80	99.00
Lower CI	1.32	1.88	2.64	2.77
Upper CI	4.16	3.68	4.75	4.93

ovicidal activity of (R^2=0.94). MeOH extract of leaf required higher concentration (20.41 $mg\ ml^{-1}$) for 50 per cent egg mortality, whereas EtOH extract of flower required only 10.71 $mg\ ml^{-1}$ for 50 per cent and 34.6 $mg\ ml^{-1}$ for 90 per cent egg mortality (Table 5). The probit analysis clearly indicates that the EtOH flower extract has maximum potential to kill the eggs of *R. sanguineus.* The toxicity values of treated extracts of *T. erecta* based on LC_{50} values could be arranged in descending order as follows: EtOH flower extract > MeOH flower extract > EtOH leaf extract > MeOH leaf extract. The control eggs treated with the one per cent DMSO recorded with zero mortality.

Larval Repellency: All tested larval ticks showed repellency against all extracts tested except control. The larval repellency observed in MeOH extract of leaf ranged from 12.5 per cent in the lowest 0.5 mg ml^{-1} to 83 per cent in the highest 2.5 mg ml^{-1} concentration. EtOH extract of leaf larval repellency ranged from 25 in 0.5 mg ml^{-1} to 83 per cent 2.5 mg ml^{-1} concentration (Table 6). The repelled larval ticks were found in the naked regions of the test apparatus or resting on the platform. There was a significant (R^2= 0.97, P-value = 0.001) dose – tick repellency response relationship. Tick repellency (> 90%) was found in both MeOH and EtOH flower extracts at 2 mg ml^{-1} concentration producing an RC_{50} of 0.77 and 0.58 per cent respectively (Fig. 1).

The leaves and flowers of *T. erecta* have been used in India and other South East Asian countries traditional medicine to treat various pain and inflammatory conditions (Singh *et al*., 2020; Rahman *et al*., 2020). The current study establishes the ovicidal and anti-larval properties of the EtOH and MeOH leaf and flower extract of *T. erecta* against *R. sanguineus*. The acaricidal activity of the *T. erecta* extracts in our study is consistent with results from other studies. However, direct comparisons are difficult to make as no other study has evaluated the relationship between the extract concentrations and the percentage of ticks killed. Even though the exact acaricidal mechanisms are yet to be established, it is possible that *T. erecta* may act through the inhibition of the release and/or action of repellent mediators (e.g., Alkaloids, flavonoids, saponins, and tannins) since it inhibited egg hatching and larval repellency (Ravikumar, 2010; Vijay *et al*., 2013). The egg mortality increased as extract concentration increased, and more than 50% mortality was induced by all the plant extracts at 20mg ml^{-1} concentration. Politi *et al.*(2012) reported that the 70 percent of EtOH extract of aerial parts of *T. patula* reduced egg laying by 21.5 per cent and eliminated 99.78 percent of the larvae of *R. sanguineus*. However, in our study, the EtOH extracts of flower produce 39.2 per cent mortality in 10mg ml^{-1} concentration. Furthermore, different *Tagetes* spp., extracts have also been shown to significantly kill various kinds of insect pests such as stored product beetles, mosquitoes and armyworms (Nikkon *et al*., 2011; Nchu *et al*., 2012; Politi *et al*., 2012).

The presence of alcoholic sugar xylitol, Butanol, 3-methyl formate, cyclohexane and neophytadiene in the leaf of the MeOH and EtOH extracts may account, at least in part, for the observed insecticidal and medicinal effects (Puterka *et al.,* 2003; Barakat,

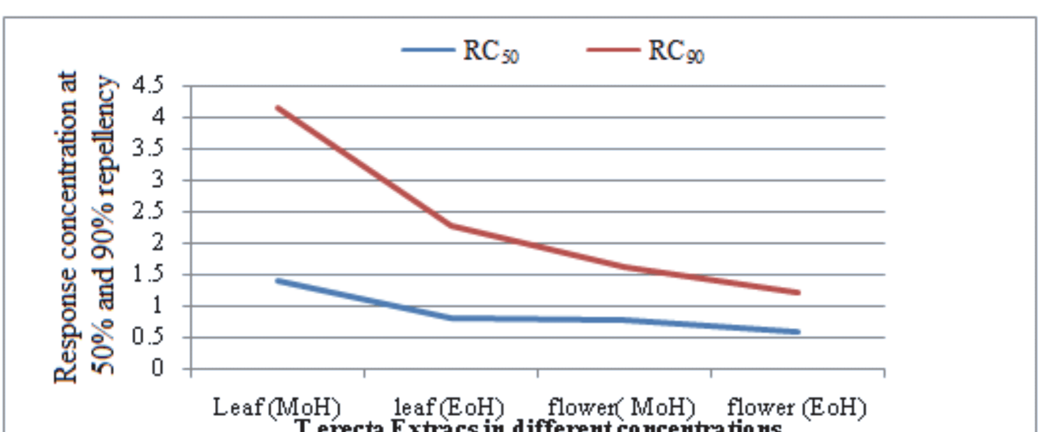

Fig. 1 Relationship between *R. sanguineus* larval repellency (RC_{50} & RC_{90}) and extracts of *T. erecta* in the bioassay

2011; Caceres *et al*., 2015; Edwin and King, 2017; Etify *et al*., 2017). The chemical compound yangambin was found highest percentage in both EtOH extracts of leaf and flower extracts. Several studies have shown that yangambin inhibits postembryonic development, morphological alteration, and oviposition reduction in harmful insect pests (Marise *et al*., 2007). The extract of *T. erecta* leaf and flower showed repellent effects on larval ticks at all concentrations tested with RC_{50} of 0.58% to 1.4%w/v. Elango and Rahuman (2011) and Vijay *et al*. (2013) reported 70 per cent acaricidal activity for *Haemaphysalis bispinosa* and 77 per cent larvicidal activity for *R. microplus* in MeOH extracts of *T.erecta* flowers. The current results indicate that the ethanol extracts of the *T.erecta* flower were more effective in exhibiting the repellent action against the larval ticks tested. The present study clearly establishes the acaricidal properties of the leaf and flower extract of *T. erecta*. EtOH extracts of this plant may be used as a source of anti-tick agents. However, further studies are needed to further elucidate the efficacy of the identified compounds in EtOH extracts of leaf and flower.

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