Dual-target anti-tick activity of Tagetes erecta – based topical cream through acetylcholinesterase inhibition and salivary protein targeting

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Abstract

Ticks are major vectors of pathogens, imposing significant health and economic burdens in humans and animals. Conventional synthetic acaricides face increasing limitations due to resistance and environmental concerns, highlighting the need for eco-friendly alternatives. We developed a topical cream based on ethanolic extracts of *Tagetes erecta* flowers, a plant traditionally valued for insect-repellent and medicinal properties. The cream showed substantial tick-repellent efficacy under laboratory conditions, indicating its potential as a natural acaricidal agent. Mechanistic studies revealed over 60% inhibition of acetylcholinesterase (AChE) at 1000 µg/mL, with an IC_{50} of 267.6 µg/mL, comparable to known inhibitors. In silico molecular docking and dynamics simulations targeted AChE (RmAChE1) and the tick salivary protein BSAP1 (PDB ID: 7NE8). Key phytoconstituents of *T. erecta*, Yangambin, displayed favorable binding affinities to both proteins (Yangambin: − 6.539 kcal/mol with RmAChE1; − 4.055 kcal/mol with BSAP1). Molecular dynamics over 100 ns confirmed complex stability, with RMSD values < 3 Å for Yangambin–BSAP1 and stabilization of Yangambin–RmAChE1 after 40 ns (~ 2 Å). Additional compounds, such as 1h,3h-furo[3,4-c] furan, Stigmata, Pyrrolidineacetic acid, and Nonylamine N,N-di(allyl), also formed stable complexes with RmAChE1, supporting sustained inhibitory activity. This integrative study demonstrates that *T. erecta*-based formulations exert dual activity through AChE inhibition and salivary protein interaction. The findings highlight *T. erecta* -derived topical creams as promising, safe, and multifaceted candidates for next-generation, plant-based tick control agents.

Introduction

Ticks are hematophagous ectoparasites responsible for transmitting a wide spectrum of pathogens that cause significant viral, bacterial, protozoal, and parasitic diseases in humans and animals (Kamyingkird et al. 2024; Sahina et al. 2024[a]). Globally, ticks transmit more pathogen species than any other group of blood-feeding arthropods, adversely affecting humans, livestock, and companion animals (Rathinam et al. 2022; Sahina et al. 2024[b]). The public health impact of tick-borne diseases (TBDs) became particularly evident in Europe and North America following the discovery of Borrelia burgdorferi as the causative agent of Lyme disease in the 1980s. Lyme disease continues to be a major concern, with reported cases rising from an average of 28,000 annually to 62,551 cases in 2022 (Pfäffle et al. 2013). In India, Kyasanur Forest Disease (KFD), another severe TBD, has resulted in thousands of infections between 1957 and 2017 (Chakraborty et al. 2019; Balasubramanian et al. 2021), including outbreaks with fatalities in Kerala and Karnataka (Sadanandane et al. 2017; Munivenkatappa et al. 2024).

Current tick control strategies primarily depend on synthetic acaricides, which offer rapid and effective population suppression (Madder et al. 2011). However, widespread and prolonged use has led to the development of acaricide resistance in many tick species, including mutations reducing acetylcholinesterase (AChE) sensitivity in *Rhipicephalus microplus* (Temeyer et al. 2013; Salman et al. 2020). Moreover, the environmental toxicity and adverse effects of acaricides on non-target species, including humans, have driven the growing demand for safer, eco-friendly alternatives (Rosado-Aguilar et al. 2017).

Plant-derived bioactive compounds have emerged as promising candidates in this context (Sahina and Esakkiammal 2022). *Tagetes erecta* (Family: Asteraceae) (marigold), an aromatic annual herb of the Asteraceae family, is recognized for its traditional medicinal properties, including antimalarial and febrifuge effects (Srivastava et al. 2023). Research has demonstrated its insect-repellent properties against mosquitoes and acaricidal potential against ticks such as *Rhipicephalus sanguineus* (Politi et al. 2012; Sahina et al. 2021). Related species, including *Tagetes minuta* and *Tithonia diversifolia*, have been used traditionally for controlling livestock ticks in various regions (Wellington et al. 2017). Certain secondary metabolites present in these plants are known to interact with key enzymes like acetylcholinesterase, disrupting nervous system function and contributing to acaricidal effects. Acetylcholinesterase (AChE1) (E.C. 3.1.1.7.) is a synaptic enzyme and a major target of both organophosphates (OPs) and carbamates. It is essential for the transmission of nerve impulses in both vertebrates and arthropods, catalysing the hydrolysis of acetylcholine into acetate and choline. Both carbamates and OPs inhibit AChE1 activity by binding to the esteratic and anionic sites of the enzyme, thereby suppressing its function through a similar mode of action (Malak et al. 2023), In addition to targeting enzymes critical for tick survival, disrupting tick feeding mechanisms by inhibiting salivary proteins that modulate host responses is an effective control strategy. One such protein is $BaSO_4$-adsorbing protein 1 (BSAP1) from *Ornithodoros savignyi*, which inhibits the extrinsic blood coagulation pathway, thereby facilitating prolonged blood feeding and pathogen transmission (Ehebauer et al. 2002).

This study aims to develop a natural tick-repellent topical cream formulation using *T. erecta* flower extract and assess its repellency against ticks. Furthermore, molecular docking investigations were performed to elucidate the interaction mechanisms of key

alkaloids from the extract with acetylcholinesterase 1 (AChE1) from *R. microplus* (RmAChE1). Complementary docking and molecular dynamics simulations were also conducted to explore the binding stability of lead compounds with BSAP1 (PDB ID: 7NE8) from *O. savignyi*. Together, these approaches seek to provide experimental and molecular insights towards the development of safer, plant-based tick control agents targeting both neural and salivary protein pathways in ticks.

Methods

Plant Material Collection and Ethanolic Extraction

Fresh flowers of *T. erecta* were collected from plants cultivated in a home garden under natural environmental conditions in Kerala, India (Allappy district). The plant material was grown without the application of chemical pesticides or fertilizers. The flowers were thoroughly washed three times with tap water to remove debris and impurities and then shade-dried at room temperature under laboratory conditions until completely desiccated. The dried material was pulverized into a fine powder and stored in airtight containers until extraction. 100 g of the powdered sample was subjected to Soxhlet extraction using ethanol at 55°C for 85 cycles. The resulting extract was concentrated under reduced pressure using a rotary evaporator at 40°C and stored at 4°C in air tight container for subsequent analyses.

Phytochemical Profiling

Phytochemical profiling of the *T. erecta* extract was performed using gas chromatography–mass spectrometry (GC–MS) equipped with an auto-injector and a fused-silica capillary column, with helium as the carrier gas at 1.2 mL/min. Injector and detector temperatures were set at 250°C and 280°C, respectively, and the oven temperature was programmed from 60°C (held 5 min) to 160°C at 4°C/min (held 1 min), then to 270°C. Compound identification was achieved by comparison with reference mass spectral libraries.

Isolation and Confirmation of Yangambin

The lignoid compound yangambin was isolated from *T. erecta* flower extract by suspended in 10% acetic acid, filtered, and the aqueous fraction was extracted with dichloromethane. The organic phase was dried under reduced pressure to obtain the total lignoid fraction, which was subsequently purified by column chromatography on silica gel 60 (0.063–0.200 mm) using a gradient of hexane, chloroform, and ethanol. The ethanol fraction yielded pure yangambin. Its structure was confirmed using ^1H/^13C NMR spectroscopy. For NMR analysis, the isolated compound was dissolved in deuterated dimethyl sulfoxide (DMSO-d6) and analyzed on a Bruker Avance NEO 400 MHz spectrometer. Spectral data were compared with literature values to validate identity and purity.

Tick Collection and Maintenance

Ticks were collected from vegetation using the standard dragging method. Following morphological identification (Estrada-Pena A and Jongejan F 1999), the ticks were thoroughly washed with distilled water and dried on filter paper. Nymphal and adult ticks collected by dragging were used for experimental assays. Fully engorged female ticks were incubated for 14–21 days at 27°C and relative humidity ≥ 80% in plastic containers (7 cm × 5 cm), for oviposition (Ali et al. 2024).

Topical cream Formulation

A 150 g topical cream was prepared using the phase inversion temperature (PIT) method (Radhakrishnan and Raj 2020). Ingredients were divided into oil and aqueous phases (Table 1). The oil phase was heated to 75°C and homogenized at 200 rpm using a hotplate stirrer, while the aqueous phase, containing 1% (w/w) *T.erecta* extract, was dissolved in distilled water at 75°C. The two phases were combined at 65°C and emulsified at 600–700 rpm for 45 minutes. The resulting emulsion was gently hand-stirred until ambient temperature to produce a uniform cream.

Table 1
Composition of topical tick repellent cream prototypes incorporating *T. erecta* extract.

Contents	Prototypes															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Oil Phase																
Stearic acid	4	4	4	4	4	6	8	6	8	10	12	15	15	10	10	10
Cetostearyl alcohol	3	3	3	3	3	3	4	3	4	5	6	2	2	3	2	2
Clove oil	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lubricant oil	-	0.7	-	-	-	-	-		-	-	-	-	-	-	-	-
Paraffin oil	-	-	0.7	0.7	1	0.6	1	0.6	1	1	1.2	2	2	2	2	2
Tween 80	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Aqueous Phase																
TEA	2	2	2	2	2	6	8	6	8	10	12	5	5	3	3	6
Glycerol	8	8	8	8	8	8	8	12	16	10	12	10	10	15	10	15
Rose water	-	-	0.6	0.6	0.6	1	1	1	1	1	1	3	2	2	2	2
Methylparaben	-	-	-	0.2	0.1	0.14	0.18	0.14	0.18	0.2	0.7	0.2	0.2	0.02	0.02	0.2
Propyl paraben	-	-	-	0.2	0.02	0.06	0.08	0.06	0.08	0.1	0.1	0.1	0.1	0.01	0.01	0.1
Water	sq	sq	Sq	sq	sq	sq	sq	sq	sq	Sq	sq	sq	sq	sq	sq	sq
Extract	0.5	0.5	0.5	0.5	0.5	1	1	1	2	2	2	1	1	1	1	1

Repellency evaluation

The repellent efficacy of the formulated cream prototypes was evaluated against adult and larval ticks of *Haemaphysalis* spp. using the paper plate bioassay method (Burtis et al. 2023). Rectangular paper plates (18.5 cm × 14.5 cm) were used for all experiments. Four treatments were tested in parallel: (i) *T. erecta*–based formulated cream, (ii) a commercially available tick repellent containing benzyl benzoate (positive control), (iii) lemongrass oil as a natural reference repellent, and (iv) a blank cream without plant extract, serving as the negative control. Approximately 100 mg of each treatment was uniformly applied as a 2-cm-wide continuous strip along all four edges of the plate, leaving the central area untreated to serve as the tick release zone. Each treatment was tested on a separate plate, and all assays were conducted in triplicate. Ten nymphal ticks were released at the centre of each plate, and their movement towards and across the treated zones was observed for up to 6 h under laboratory conditions. Tick crossing behaviour was recorded at hourly intervals. Repellency was expressed as the percentage reduction in the number of ticks crossing the treated area compared to the control and calculated using the following formula:

$$Repellencypercentage\,(\backslash\%) = 100 - \frac{Mean\,no.\,of\,ticks\,on\,test}{Mean\,no.\,ofticks\,on\,control} \times\ 100$$

Optimization and Stability profiling

The formulations were characterized for their physicochemical and sensorial attributes, including appearance, pH, consistency, emulsification stability, softness, greasiness, and stickiness, following standard pharmacopeial and regulatory guidelines (ICH Q1A(R2); USP, 2025). Spreadability was evaluated using the slip-and-drag method. Briefly, 1 g of each cream was placed between two glass plates (13 × 13 cm), and a 1 kg load was applied for 5 min. The diameter of the spread area was measured to assess ease of application, in accordance with standard testing protocols for topical dosage forms (European Pharmacopoeia, 11th

Edition). The pH of each formulation was determined by dispersing 1 g of cream in 25 mL of deionized water, allowing equilibration for 30 min, and measuring using a calibrated digital pH meter. All measurements were performed in triplicate, consistent with USP and FDA recommendations. Microbial stability was assessed by inoculating cream samples onto Nutrient Agar for bacterial growth and Sabouraud Dextrose Agar for fungal and yeast growth. The inoculated plates were incubated at 37°C and 28°C, respectively, and monitored for microbial growth over a period of 3 days, in accordance with USP < 61 > and < 62 > microbial limits testing. Temperature-dependent stability studies were conducted by storing the selected formulations at 4°C, 25°C, and 40°C for a period of 90 days. Evaluations were performed on Days 7, 15, 30, 60, and 90 to monitor changes in color, odor, texture, phase separation, and pH, following ICH Q1A(R2) guidelines for long-term and accelerated stability studies.

In vitro safety assessment

The cytotoxicity of the *T. erecta* extract-based cream was assessed using the MTT assay in human dermal fibroblast (HDF) cells. Human dermal fibroblast adult (HDFa) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; Catalog No. PCS-201-012). A stock solution of the test product (10 mg/mL) was prepared in DMEM-HG medium (Gibco, USA) supplemented with 2% inactivated FBS (Gibco, USA), followed by serial two-fold dilutions to obtain lower test concentrations. HDF cells were trypsinized, and the cell density was adjusted to 1×10^5 cells/mL. A volume of 0.1 mL of the cell suspension was seeded into each well of a 96-well microtiter plate. After 24 hours of incubation to allow partial monolayer formation, the supernatant was removed, and the cell monolayer was washed with DPBS (HiMedia, India). Different concentrations of the test formulation were added to the wells, with untreated cells maintained as controls. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. After incubation, the test solutions were discarded, and 100 µL of MTT (HiMedia, India) solution diluted in DPBS was added to each well. Plates were incubated for an additional 3 hours under the same conditions. The supernatant was then removed, and 100 µL of DMSO (SRL, India) was added to solubilize the formazan crystals. Absorbance was measured at 570 nm using a microplate reader, and the data were used to determine cell viability and cytotoxicity parameters.

Mechanistic assessment: AChE inhibition

The enzymatic inhibition of acetylcholinesterase (AChE) was evaluated using the colorimetric method described by Ellman et al. (1961), with minor modifications. *H.bispinosa* nymphal ticks were exposed to varying concentrations (62.5, 125, 250, 500, and 1000 µg/mL) of the *T. erecta* ethanolic extract-based cream (diluted in distilled water). Post-exposure, ticks were homogenized using a chilled mortar and pestle in ice-cold sodium phosphate buffer (50 mM, pH 7.4) containing protease inhibitors. The homogenate was incubated for 30 minutes at 4°C, followed by centrifugation at 10,000 rpm for 30 minutes. The supernatant was collected and stored at 4°C for enzyme analysis. For the assay, 100 µL of enzyme extract was pre-incubated with the test cream at each concentration for 30 minutes at 37°C. The reaction mixture included 50 mM Tris–HCl buffer (pH 8.0), 0.5 mM DTNB (5,5'-dithiobis(2-nitrobenzoic acid)), and 0.5 mM acetylthiocholine iodide as substrate. Absorbance was recorded at 412 nm, and the rate of color development was measured to determine enzyme activity. Galanthamine, a known AChE inhibitor, was used as a positive control at the same concentrations as the test samples, prepared in 50 mM Tris–HCl buffer (pH 8.0). The enzyme inhibition (%) was calculated from the rate of absorbance change with time (V = Abs/Δt) the calculation as follows.

$$Inhibition\,(\%) = 100 - \frac{Change\ of\ sample\ absorbance}{Change\ of\ blank\ absorbance} \times 100$$

In Silico Molecular Docking against RmAChE1

Molecular docking was conducted to evaluate the binding affinity of selected phytocompounds from *T.erecta* against the acetylcholinesterase enzyme (RmAChE1) of *R.microplus*. The 3D structure of RmAChE1 was retrieved from the Protein Data Bank, and ligands were obtained from PubChem and energy-minimized using standard molecular mechanics force fields. Docking simulations were performed using a virtual screening platform, with the active site defined based on known catalytic residues. A grid box encompassing the binding pocket was generated, and each ligand was docked individually. Binding affinities (kcal/mol) were recorded, and the stability of docked poses was assessed via root mean square deviation (RMSD) values for lower and upper bounds (rmsd/lb and rmsd/ub). Ligands with RMSD values of 0.0 were considered to have stable, reliable conformations.

Molecular Docking and Dynamics Simulation

The 3D structure of the tick salivary protein BSAP1 (PDB ID: 7NE8) (Ehebauer et al., 2002), was retrieved from the PDB archive of the Research Collaboratory for Structural Bioinformatics (RCSB) (https://www.rcsb.org). Since the 3D structure of the RmAChE1 protein of *R. microplus* and *H. bispinosa* was not reported, the crystal structure of AChE1 was modelled using *swiss model* (https://swissmodel.expasy.org/). The acetylcholinesterase (*R. microplus*) sequence (NCBI Reference: XP_037284828.2) was considered as a target, whereas the Acetylcholinesterase structure (PDB ID: 5DTI) was selected as a template structure (Cerqueira et al. 2022). The Seven bioactive compounds, were sourced from PubChem. Protein-Ligand docking was performed in *Schrodinger Maestro Suite Version 2024-4* at ICMR NIV Pune (HPC NAKSHATRA facility). Proteins were prepared using Schrödinger's Protein Preparation Wizard, including removal of water molecules, addition of hydrogens, and energy minimization with the OPLS4 force field. Seven bioactive compounds were prepared via LigPrep at physiological pH, and were docked into the BSAP1 and AChE1 active site using Glide's Extra Precision (XP) mode. The 3D structure of BSAP1 protein documented was available in the structure databases but its catalytic sites were not reported. Additionally, the 3D structure of RmAChE1 was not available in databases, resulting in the absence of any information related to the catalytic sites. As a result, the potential binding regions of the receptor were anticipated based on the area with the largest cavity for docking compounds (Malak et al. 2023). For this, the site map tool present in the Schrodinger Maestro Suite Version 2024-4was utilized. The Active site residues with the highest druggability scores (D-scores) were selected for BSAP1 a and RmAChE1protein. To validate docking results and assess complex stability, 100 ns molecular dynamics simulations were performed using *Desmond* with TIP3P solvent and 0.15 M NaCl. Systems were equilibrated under NPT conditions (300 K, 1 atm) prior to production runs. The simulation ran under constant temperature and pressure conditions. Trajectory were saved every 100 ns for analysis, the stability of the system was evaluated by calculating the Root Mean Square Deviation (RMSD) of both protein and ligand over time. The Desmond simulation trajectories were analysed to calculate the root mean square deviation (RMSD) and protein ligand interaction contacts, interaction profiles were analysed to assess the stability and nature of the binding.

Results

Compound identification

The ethanol extraction of *T. erecta* flowers yielded a maximum of 64%, indicating efficient recovery of the phytochemical constituents. GC-MS analysis of the *T.erecta* flower ethanolic extract revealed the presence of multiple phytochemical constituents with retention times ranging from 4.160 to 47.860 minutes. A total of 7 major compounds were identified, varying in relative abundance (Fig. 1). Yangambin was the most abundant constituent, accounting for 30.81% of the extract at a retention time of 45.925 minutes, followed by 1H,3H-furo[3,4-c]furan at 29.78%, indicating these as the primary bioactive components. Other significant compounds included gamma-sitosterol, phenol, 2,6-dimethoxy-, and stigmasterol (stigmasta-5), all known for various pharmacological activities. Minor constituents detected included 2-butanone (1.93%), 4H-pyran-4-one, 2,3- (1.20%), 1-butanol (0.85%), beta-D-glucopyranose (0.69%), 2-pyrrolidineacetic acid (0.54%), nonylamine, N,N-di(allyl)- (0.51%), and n-hexadecanoic acid (0.28%).

1H NMR spectra showed signals for yangambin at δ ppm: 6.51 (4H, s, H-2,20 ,6,60), 4.8 (2H, d, J = 4.0 Hz, H-1,10), 4.33 (2H, dd, J = 9.0 Hz, J = 7.0 Hz, H-9α,90α), 3.96 (2H, dd, J = 9.0 Hz, J = 3.5 Hz, H-9β,90β), 3.90 (12H, s, 3,30 ,5,50 -OCH3), 3.86 (6H, s, 4,40 -OCH3), 3.13 (2H, m, H-8,80). (Fig. 2A). 13CNMR showed peaks at different ppm, δ ppm: 153.5 (C-3,30 ,5,50), 137.5 (C-4,40), 130.08 (C-1,10), 102.4 (C-2,20 ,6,60), 82.3 (C-7,70), 72.4 (C-9,90), 60.0 (4,40 -OCH3), 56.5 (3,30 ,5,50 -OCH3), 54.4 (C-8,80). (Fig. 2B)

Repellency evaluation

The tick-repellent efficacy of a 1% *T. erecta* extract-based cream was assessed against reference products, Benzyl benzoate (BB) and lemongrass oil (LG), using a filter paper bioassay. For each replicate, 200 larvae and 50 adults *H. bispinosa* ticks were tested, and repellency was monitored over a 6-hour period post-application. The extract-based formulation demonstrated superior and sustained repellency compared to the reference products. Maximum relative repellency for the extract-based cream was observed at 1- and 3-hours post-application, reaching 97.5% in larvae and 87.5% in adults. In contrast, BB showed lower repellency after 1 hour (71.1% in larvae and 26% in adults), while LG exhibited minimal effect (45.1% in larvae and 6.4% in adults). Importantly, the *T. erecta* extract maintained its repellent activity for the entire 6-hour observation period, whereas the efficacy of the reference products declined rapidly after the first hour (Fig. 3).

Optimized Cream Formulation

All formulations exhibited a yellowish hue with a uniform texture, attributed to the presence of lutein - a carotenoid pigment in *T. erecta* extract. To improve emulsification and spreadability, five selected prototypes (P11–P15) were further optimized by reducing stearic acid concentrations and increasing levels of glycerol and triethanolamine (TEA). The concentration of *T. erecta* extract in the cream was varied from 0.5% to 2% for efficacy testing. At 0.5%, the cream showed repellent activity for only 1 hour, with reduced effectiveness thereafter. Increasing the extract concentration to 1.0% significantly extended repellency up to 6 hours. However, no substantial improvement was observed when increasing the concentration to 2%, and this higher dose resulted in an undesirable deep yellow to brownish coloration. Based on performance, aesthetics, and stability, the optimized cream formulation was finalized with 1% *T.erecta* extract, offering the best balance of repellency, consistency, emulsification, and visual appeal (Table 2).

Stability profiling

Colour: The freshly prepared cream was yellow due to the presence of lutein, while the control (base cream without extract) appeared white. At 40°C, a slight shift in color from yellow to pale yellow was observed by Day 90, likely due to partial phase separation or degradation of thermosensitive compounds. **Phase separation**: No signs of phase separation were detected in samples stored at 4°C and 25°C throughout the 90-day period. However, slight phase separation was noted in the cream stored at 40°C from Day 30 onward, indicating reduced emulsion stability under elevated temperatures. **pH stability**: The initial pH of the cream was adjusted to 5.5–6.4 using lactic acid, maintaining skin-friendly acidity (skin pH range: 4.5–7.0). The pH remained relatively stable across all storage conditions, with minor fluctuations observed particularly at 40°C. **Microbiological evaluation**: All formulations tested negative for *Escherichia coli*, *Pseudomonas spp.*, and *Staphylococcus aureus*. Additionally, no yeast or mold growth was detected in any sample throughout the study period, indicating good microbial stability (Table 2).

Table 2
Physical stability profile of the formulated *Tagetes erecta* cream stored at different temperatures over 90 days.

Day	Temp	pH	Emulsification	Appearance	Consistency	Color	Odor	Texture	Phase separation
7	40	7	+++	Soft	+++	+++	+++	+++	No
	25	7	+++	Soft	+++	+++	+++	+++	No
	4	7	+++	Soft	+++	+++	+++	+++	No
15	40	6.7	+++	Soft	+++	+++	+++	+++	No
	25	6.6	+++	Soft	+++	+++	+++	+++	No
	4	6.8	+++	Soft	+++	+++	+++	+++	No
30	40	6.7	+ - -	Rough	+ - -	+ --	+++	- - -	Yes
	25	6.6	+++	Soft	+++	+++	+++	+++	No
	4	6.8	+++	Soft	+++	+++	+++	+++	No
60	40	6.7	+ - -	Rough	+ - -	+ --	+++	- - -	Yes
	25	6.4	+++	Soft	+++	+++	+++	+++	No
	4	6.8	+++	Soft	+++	+++	+++	+++	No
90	40	6.7	+ - -	Rough	+ - -	+ --	+++	- - -	Yes
	25	6.4	+++	Soft	+++	+++	+++	+++	No
	4	6.7	+++	Soft	+++	+++	+++	+++	No

+++ = excellent; ++ = good; + = fair; +- – = reduced/weak; – – – = poor;

Phase separation: Yes = visible separation; No = stable emulsion.

Cytotoxicity Assessment in Human Dermal Fibroblasts

The cytotoxic potential of the 1% *T. erecta* extract-based cream was evaluated in human dermal fibroblast (HDF) cells to determine its suitability for topical application. HDF cells were treated with a range of extract concentrations (7.8–1000 µg/mL), and cell viability was measured after exposure. The formulation exhibited high cell viability (> 80%) across all tested concentrations, including the highest concentration of 1000 µg/mL (88.18 ± 2.41%). The cytotoxic concentration for 50% cell viability (CTC_{50}) was determined to be > 1000 µg/mL, confirming that the cream is non-toxic to human skin cells. As shown in Fig. 4, cell viability remained above the 80% threshold across all doses, underscoring the cream's safety and compatibility for dermal application. A paired Wilcoxon signed-rank test revealed a statistically significant difference between the formulated cream and control groups ($p < 0.05$), indicating that the formulated cream maintained comparable or improved cellular compatibility relative to the control.

Ache enzyme inhibition activity

The AChE enzyme inhibition activity of the formulated *T.erecta*-based cream was analysed in comparison with galanthamine, used as a standard, and the percentage inhibition was evaluated and tabulated. The formulated cream showed above 60% inhibition (61.56 ± 2.5%) at the 1000 µg/ml concentration when compared with galanthamine (85.62 ± 3.2%), as shown in Fig. 5. Inhibitory concentrations (IC_{50}) data of the formulated cream in different concentrations showed 267.6 µg/ml when compared to Galanthamine (IC_{50} = 234.2 µg/ml).

Molecular Docking against tick BSAP1 and RmAChE1

Among the compounds, Yangambin demonstrated the strongest binding affinity with a docking score of -4.055 kcal/mol, suggesting a stable interaction with the active site of BSAP1 (Table 3). BSAP1–1H,3H-furo[3,4-c] furan complex and BSAP1- pyrrolidineacetic acid complex showed lower docking scores, indicating comparatively weaker binding. In case of RmAChE1, Nonylamine N N di(allyl) showed strongest interaction, with a docking score of-9.296 kcal/mol. The docking scores for the remaining compounds were as follows: 1H,3H-furo[3,4-c]furan (-7.092 kcal/mol), Yangambin (-6.539 kcal/mol), Stigmasta (-5.945 kcal/mol), Pyrrolidineacetic acid (-5.911 kcal/mol), 4H-Pyran-4-one,2–3 (-3.863 kcal/mol), and Gamma-sitosterol (-3.669 kcal/mol).

To evaluate the structural stability of these complexes, the molecular dynamics simulation, was performed over the course of a 100 ns trajectory for each ligand using Root Mean Square Deviation (RMSD). The BSAP1–Yangambin complex maintained RMSD values within 3 Å, indicating a stable protein–ligand interaction with minor fluctuations at the beginning and in between 70 ns to 85 ns (Fig. 6). BSAP1–Yangambin complex achieve stable RMSD values after initial fluctuations, suggesting that the protein-ligand complex is reasonably stable over the sampled timeframe. In contrast, the other two complexes (BSAP1–1H,3H-furo[3,4-c]furan, and BSAP1–pyrrolidineacetic acid) exhibited greater RMSD deviations, suggesting lower conformational stability as shown in Supplementary Fig. 1.

For the RmAChE1(Swiss-model) - Yangambin complex was stabilized after 40 ns at approximately 2 Å, suggesting that the ligand remained within the binding site. RmAChE1- Nonylamine N N di(allyl) docked complex, was stabilised at ~ 2 Å till 30ns, after those slight fluctuations were observed. The complex was then stabilized after 40ns within 3 Å over and remained steady throughout the 100ns of simulation, demonstrating a well-defined stable complex of the ligand and binding site. RmAChE1 and 1H,3H-furo[3,4-c] furan complex also maintained RMSD values within 3 Å, throughout the 100 ns simulation period indicating a stable and dynamically suitable interaction with the active site. Similarly, RmAChE1-Stigmata complex also showed RMSD stabilization around 2 Å, reflecting better stability. RmAChE1-Gamma-sitosterol complex achieved stable RMSD value of 2.3 Å. Finally, theRmAChE1–4H-Pyran-4-one,2–3 complex stabilised between 20-60ns within 3 Å, experienced minor fluctuations afterwards, and then re-stabilized near 2.5 Å by the end of 100ns simulations. In contrast, RmAChE1-Pyrrolidineacetic acid complex observed unstable complex over 100 ns of simulation as shown Supplementary Fig. 1.

Table 3
Molecular docking and simulation results of selected phytochemicals from *T.erecta* against the $BaSO_4$-adsorbing protein 1 (BSAP1) and acetylcholinesterase enzyme (RmAChE1) of ticks

Compound (abundance %) [PubChem ID]	Binding affinity (kcal/mol) (BSAP1 PDB: 7NE8) (RmAChE1 model structure of Rm tick)	Stability (RMSD)	Interactions
Yangambin (30.81%) [443028]	BSAP1: -4.055	3A with minor fluctuations at 70-80ns	Hbond (70%), pi-cation bond
	RmAChE1: -6.53	2A after 40ns	pi-pi stacking (63%)
stigmasterol [12303924]	BSAP1: -2.9	Not stable	No interactions
	RmAChE1: -5.94	2A stable	H bond (91%)
1H,3H-furo[3,4-c] (29.78%) [101612]	BSAP1: -2.9	Not stable	No interactions
	RmAChE1: -7.09	3A Stable	Hydrophobic (39%)
4H-pyran-4-one, 2,3 [7968]	BSAP1: -3.02	Not stable	No interactions
	RmAChE1: -3.86	3A between 20-60ns	No interaction
Gamma-sitosterol [457801]	BSAP1: – 2.7	Not stable	No interactions
	RmAChE1: -3.66	2.3 A Stable	No interaction
2-pyrrolidineacetic acid [123784]	BSAP1: -3.2	Not stable	Hbond (34%)
	RmAChE1: -5.91	Not stable	Pi-cation (36%)
nonylamine, N,N-di(allyl) [87723842]	BSAP1: – 0.16	Not stable	No interactions
	RmAChE1: -9.29	2A till 30ns and 3A after 40ns	Pi-cation (74%) Hbond (51%)

Detailed protein–ligand interaction analysis highlighted key residues contributing to binding

Interaction analysis revealed that, in the BSAP1 active site, Asn66 formed hydrogen bond with Yangambin (70% interaction), while Ala56 contributed 61% and 74% interactions with two oxygen atoms of the compound, and Arg71 formed a 39% pi-cation bond (Fig. 3C and 3E). Similarly, Yangambin stabilized through a 63% pi–pi stacking interaction with Tyr161 with RmAChE1 (Fig. 3D and 3F). In contrast, 1H,3H-furo[3,4-c]furan showed no interaction above 30% with BSAP1.Pyrrolidineacetic acid formed hydrogen bonds with Arg8 (34% and 33% interactions with two oxygen atoms) and with Asp84 (32% interaction via the NH2 + group). For RmAChE1, the Nonylamine N N di(allyl) complex showed strongest interactions: Phe369(74% pi cation), Tyr 372 (36%pi cation 36%) and Gly 324 (51% hydrogen bonds). The RmAChE1-1H,3H-furo[3,4-c]furan complex involved Tyr372 (39% hydrophobic interaction) and Val 108 (36%) with oxygen atom. The Stigmasta complex showed strong hydrogen bonding with Thr110 and Val108 (91% each). Pyrrolidineacetic acid formed a pi-cation bond with Phe369 (36%). Gamma-sitosterol and 4H-Pyran-4-one,2-3showed no interactions above 30%, indicating unstable complexes (Supplementary Fig. 2).

Discussion

The search for effective and sustainable alternatives to chemical acaricides remains a pressing priority in tick management and the control of tick-borne diseases, particularly in the face of rising resistance and environmental concerns (Rosado-Aguilar et al. 2017; Guilherme et al. 2021). Plant-derived compounds, with their complex mixtures of bioactive metabolites, offer a promising avenue to develop multi-target interventions that are both effective and environmentally benign (Al-Rajhy et al. 2003; Vázquez et al. 2015; Sanda et al. 2021). In this study, we evaluated the potential of *T. erecta* flower extract as a source of tick-repellent and bioactive

molecules, integrating phytochemical profiling, topical formulation, bioassays, and computational analyses to elucidate potential mechanisms of action.

GC-MS analysis revealed that the extract contains diverse phytochemicals, including lignans (yangambin), furofuran derivatives, sterols (gamma-sitosterol, stigmasterol), and phenolics. The presence of yangambin—previously reported for anti-inflammatory, antioxidant, and insecticidal activities (Felix et al. 2021)—and furofuran derivatives, supports the pharmacological potential of Tagetes species against arthropods. Phytosterols have also been reported to exert broad-spectrum insecticidal and enzyme-inhibitory effects (Li et al. 2025), supporting the rationale for exploring multi-target mechanisms against ticks.

Topical formulation trials identified a 1% extract cream as optimal, combining high repellency with acceptable physicochemical properties and stability under typical storage conditions. Microbiological evaluation confirmed the formulation's safety for dermal application. In comparative bioassays, the extract-based cream outperformed commonly used botanical repellents, providing long-lasting protection against both larval and adult ticks. This aligns with prior studies on Tagetes essential oils, which have demonstrated potent repellency and acaricidal activity through volatile compounds such as limonene, ocimenes, and tagetones (Salman et al. 2020).

Many plant-derived repellents exert their bioactivity through neurotoxic mechanisms, particularly via acetylcholinesterase (AChE) inhibition, which disrupts normal cholinergic signaling in arthropods (Selles et al. 2021). Essential oil constituents, especially monoterpenes and phenolic compounds, have demonstrated potent AChE inhibitory and acaricidal activity. For instance, thymol and carvacrol—major components of thyme and oregano oils—exhibited potent inhibition of *R. microplus* AChE, with IC_{50} values of approximately 0.93 mg/mL and 0.04 mg/mL, respectively (Temeyer et al. 2013). Moreover, even in acaricide-resistant tick strains, carvacrol and eucalyptol inhibited AChE with IC_{50} values ranging from 0.28 to 0.36 mg/mL, demonstrating efficacy across resistant phenotypes (Jyoti et al. 2025). In *R.annulatus*, treatment with 10% thymol or eucalyptus oil resulted in 93–97% mortality among resistant adults and significantly suppressed AChE activity, supporting a neurotoxic mode of action (Valcárcel et al. 2021).

In the current study, in vitro AChE inhibition assays revealed significant enzyme suppression by the *T. erecta*-based cream, supporting a possible neurotoxic mechanism. The combination of molecular docking results highlighting high binding affinities of Nonylamine (–9.29 kcal/mol), followed by 1H,3H-furo[3,4-c]furan (–7.09 kcal/mol), Yangambin (–6.53 kcal/mol), and stigmasterol (–5.94 kcal/mol) to tick AChE—with potent in vivo repellency and enzyme inhibition suggests that both volatile cues and interference with tick neural function likely mediate the cream's effectiveness. This dual-action strategy aligns with broader botanical pest management approaches, which typically leverage complex mixtures of secondary metabolites for multi-target disruption, reducing the risk of resistance development and offering safer alternatives to synthetic repellents.

Molecular docking and simulation analyses further reinforced the biochemical findings. Nonylamine and furofuran derivatives emerged as strong binders to RmAChE1, indicating potential drivers of acute AChE inhibition (Tran et al. 2020). Yangambin, although the most abundant constituent, showed moderate binding, suggesting a possible supporting or synergistic effect rather than a dominant inhibitory action alone. These data emphasize the poly pharmacological nature of plant extracts, where collective minor and major phytochemicals contribute to bioactivity (Vázquez et al. 2015; Srivastava et al. 2023). Docking studies targeting the tick salivary gland protein BSAP1 highlighted yangambin as the top-binding ligand (–4.055 kcal/mol). Molecular dynamics (MD) simulations further demonstrated that the yangambin–BSAP1 complex retained backbone and ligand RMSD values below 3 Å over 100 ns, signaling sustained conformational stability and persistent interaction. This is noteworthy, as salivary proteins like BSAP1 are pivotal for ticks in subverting host defenses during feeding (Francischetti et al., 2009), and their disruption could reduce tick viability and pathogen transmission - a novel target rarely explored in botanical acaricide research (Sonenshine et al. 2022).

Although Yangambin has not yet being investigated in context of tick-borne diseases, it is known to exhibit anti-inflammatory, anti-platelet, and some anti-parasitic activities in preclinical studies [PMID: 7753914,9225600]. In addition to BSAP1, Yangambin also binds to RmAChE1 and the complex was stabilized after 40 ns at approximately 2 Å, supporting its potential as a promising candidate. Interaction energy also validates the observation that Yangambin form stable complexes with both BSAP1 and RmAChE1. Moreover, the interacting residues identified in the present study are in concordance with earlier studies on human acetylcholinesterase (AChE) proteins, which also reported the active amino acids Phe, Tyr, Gly, Val and Thr involved in the hydrogen bonds, pi-cation and Pi-Pi stacking formed between the ligands and the protein residues (Jang et al. 2018).

Simulation studies with RmAChE1 and Seven ligand phytocompounds dock complex reveal that 1h,3h-furo[3,4-c] furan, Yangambin, Stigmata, Pyrrolidineacetic acid and Nonylamine N N di (allyl) forms stable stronger binding affinity.Whereas in the case of RmAChE1–gamma-sitosterol Complex and RmAChE1– 4H Pyran4 one,2–3 Complex RMSD values found to be stable within range but no significant interactions more than 30% found in the protein-ligand complex. Polarity and the ring structure were key features for docking at the AChE binding site. Pharmacophore based 3D QSAR models for human acetylcholinesterase (AChE) inhibitors were generated, the virtual screening approach, in the combination with pharmacophore modeling, molecular docking and consensus scoring function can be used to identify and design novel AChE inhibitors with higher selectivity as reported in previous study (Soares et al. 2010; Jang et al. 2018).

While this study demonstrates the promising anti-tick potential of *T. erecta* extract and its topical cream formulation, several limitations warrant consideration. First, the bioassays were conducted under controlled laboratory conditions; field-based evaluations are needed to assess efficacy under variable environmental and ecological contexts. Second, although in vitro AChE inhibition and molecular docking studies provide mechanistic insights, confirmation through in vivo neurophysiological assays in ticks would strengthen causal inference. Third, the long-term safety, skin sensitization potential, and formulation stability under extreme climatic conditions remain to be fully evaluated. Finally, while molecular dynamics simulations suggest stable interactions of key phytochemicals with tick proteins, the contribution of minor compounds and potential synergistic effects in vivo require further investigation. Addressing these gaps is essential for advancing *T. erecta*-based formulations toward practical vector control applications.

Conclusion

This study establishes *T. erecta* as a potent source of multi-target bioactive compounds capable of repelling and potentially impairing ticks through both neural (AChE inhibition) and immune-interference (BSAP1 inhibition) pathways. The optimized 1% extract cream demonstrated sustained repellency against larval and adult ticks, robust physicochemical stability, low cytotoxicity, and superior efficacy compared to conventional botanical and synthetic reference products. Molecular docking and dynamics analyses revealed stable interactions of major constituents, particularly Yangambin and Nonylamine, with key tick proteins, providing mechanistic insight into the observed repellency and neurotoxic effects. Collectively, these findings highlight the potential of *T. erecta* -derived formulations as environmentally safe, multi-target alternatives to chemical acaricides. Future studies should focus on field validation, large-scale formulation, and comprehensive safety assessments to enable their integration into sustainable tick and tick-borne disease management programs.

Declarations

Ethical approval and consent to participate

Not applicable

Consent for publication

Not applicable

Clinical trial number

Not applicable

Competing interests

The authors declare that they have no competing financial or non- financial interests that could have influenced the work reported in this manuscript. The authors declare no conflicts of interest related to this study. All analyses and interpretations were conducted independently and without influence from any external party. The authors report no proprietary or commercial interest in any product, service, or company mentioned in this article.

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Author Contribution

This work was carried out in collaboration by seven authors. Author SS designed and carried out the experimental studies, wrote the protocol, and the first draft of the manuscript. VS, lead the in-silico work, headed the molecular docking studies with support from AG, HH, VV and IK. RB supervised the overall study and provided critical guidance. All authors contributed to the manuscript preparation and approved the final version.

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Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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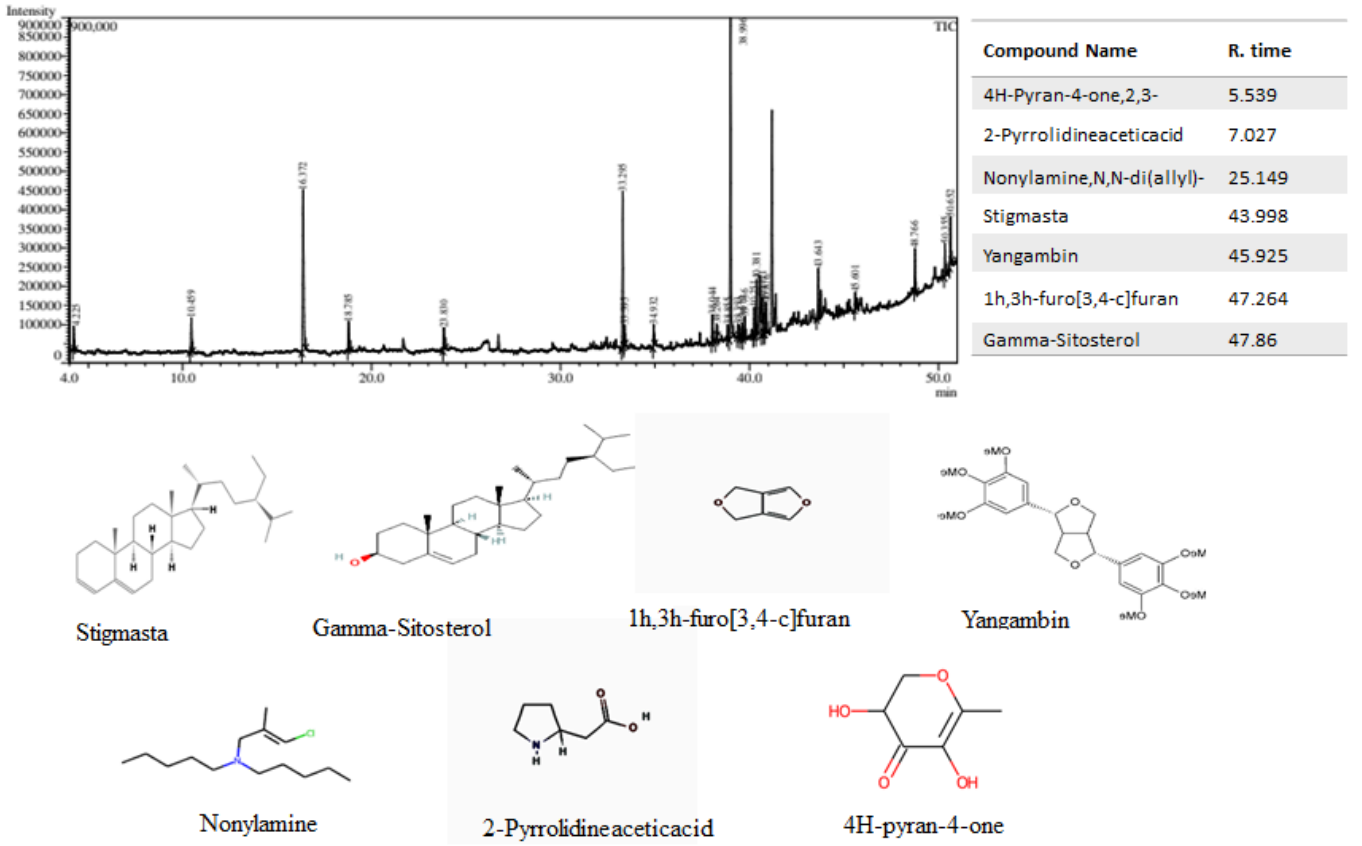
Figures



Compound Name	R. time
4H-Pyran-4-one,2,3-	5.539
2-Pyrrolidineaceticacid	7.027
Nonylamine,N,N-di(allyl)-	25.149
Stigmasta	43.998
Yangambin	45.925
1h,3h-furo[3,4-c]furan	47.264
Gamma-Sitosterol	47.86

Figure 1

GasChromatography–Mass Spectrometry (GC-MS) analysis of *T.erecta* flower extract. The chromatogram shows retention times of major phytocompounds, including 4H-Pyran-4-one, 2-Pyrrolidineacetic acid, Nonylamine, Stigmasta, Yangambin, 1h,3h-furo[3,4-c]furan, and Gamma-Sitosterol. Structural representations of these compounds are shown below the chromatogram, highlighting bioactive constituents with potential acaricidal and repellent properties

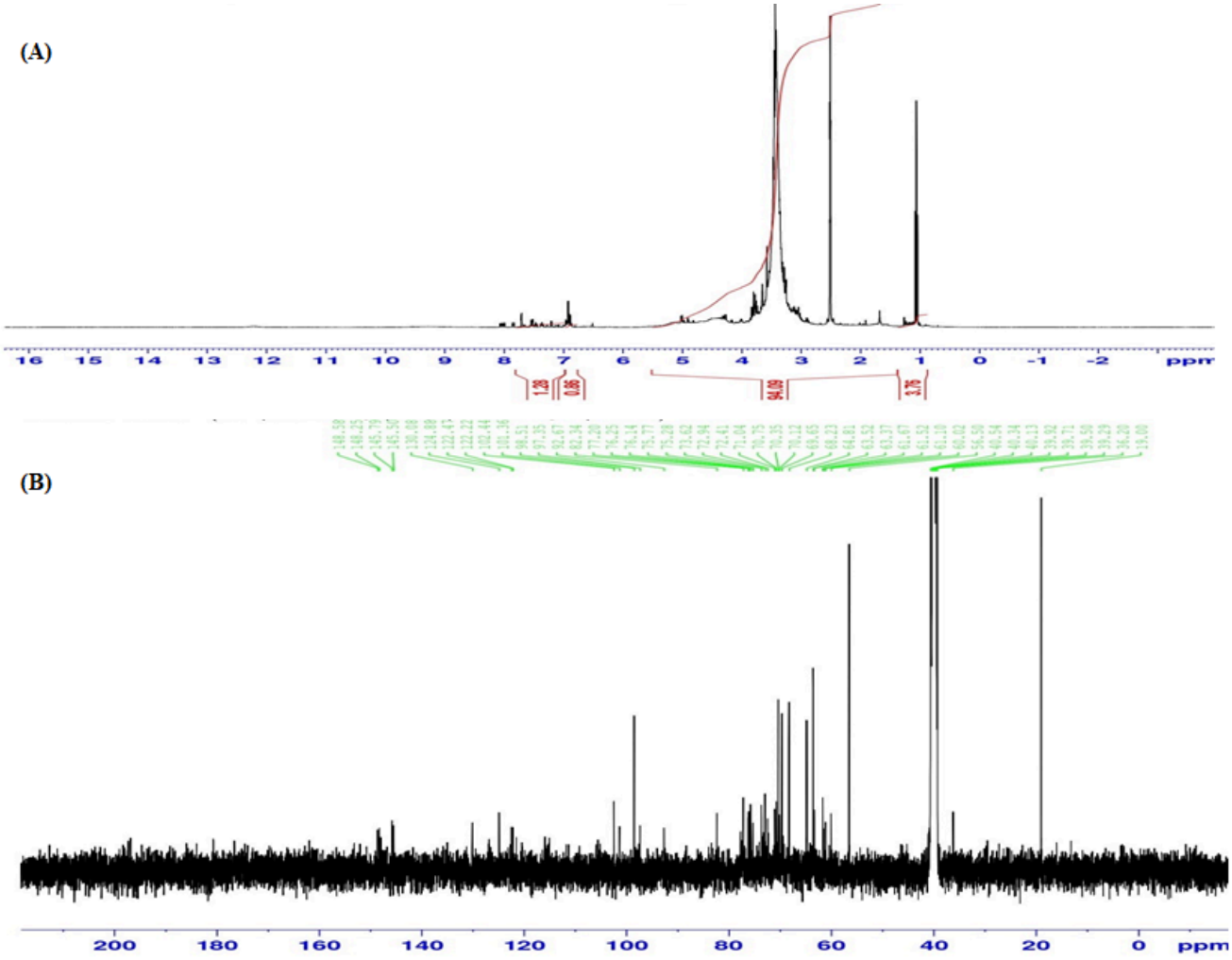


Figure 2

GasChromatography–Mass Spectrometry (GC-MS) analysis of *T.erecta* flower extract. The chromatogram shows retention times of major phytocompounds, including 4H-Pyran-4-one, 2-Pyrrolidineacetic acid, Nonylamine, Stigmasta, Yangambin, 1h,3h-furo[3,4-c]furan, and Gamma-Sitosterol. Structural representations of these compounds are shown below the chromatogram, highlighting bioactive constituents with potential acaricidal and repellent properties

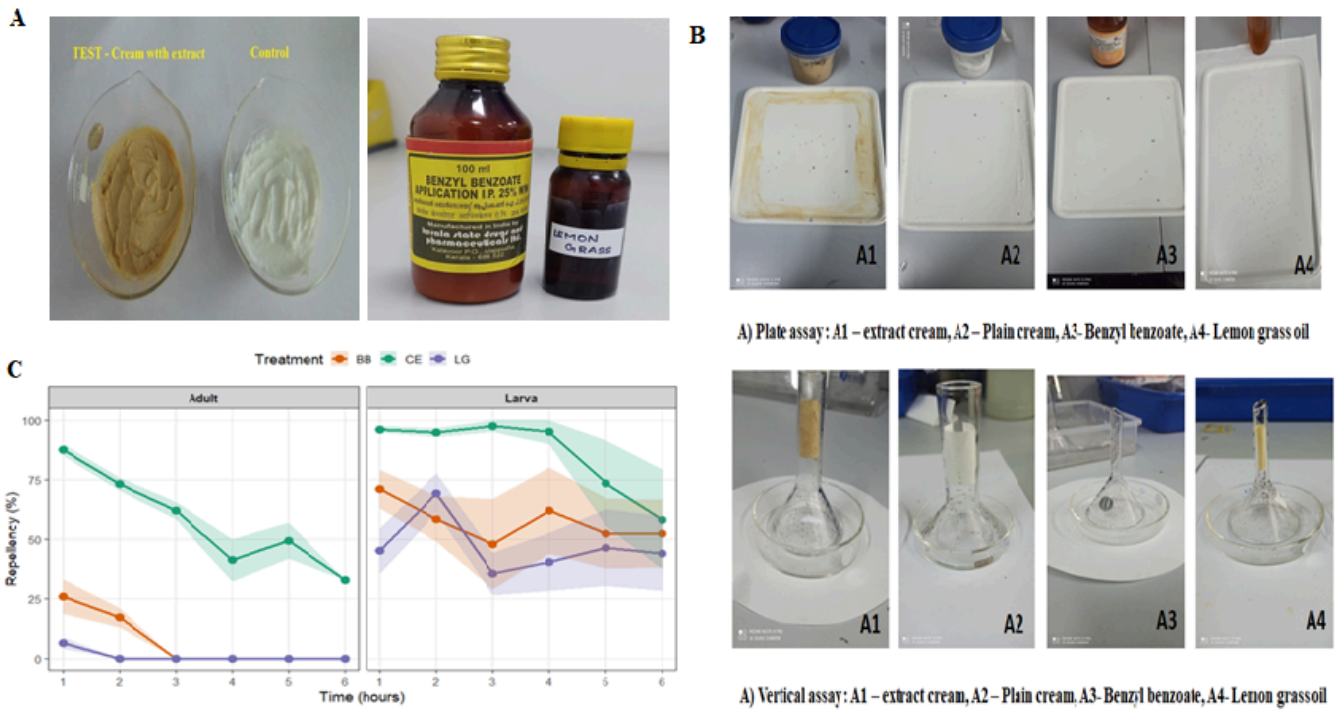


Figure 3

(A) Images of the formulated creams and reference products used in the study. (B) Schematic representation of the tick repellency assays conducted using the plate and vertical cone methods. (C) Time-dependent percent repellency of Rhipicephalus microplus adult and larval ticks for cream with Tagetes erecta extract (CE), Benzyl benzoate (BB), and Lemon grass (LG). Data are presented as mean ± standard error of the mean (SEM).

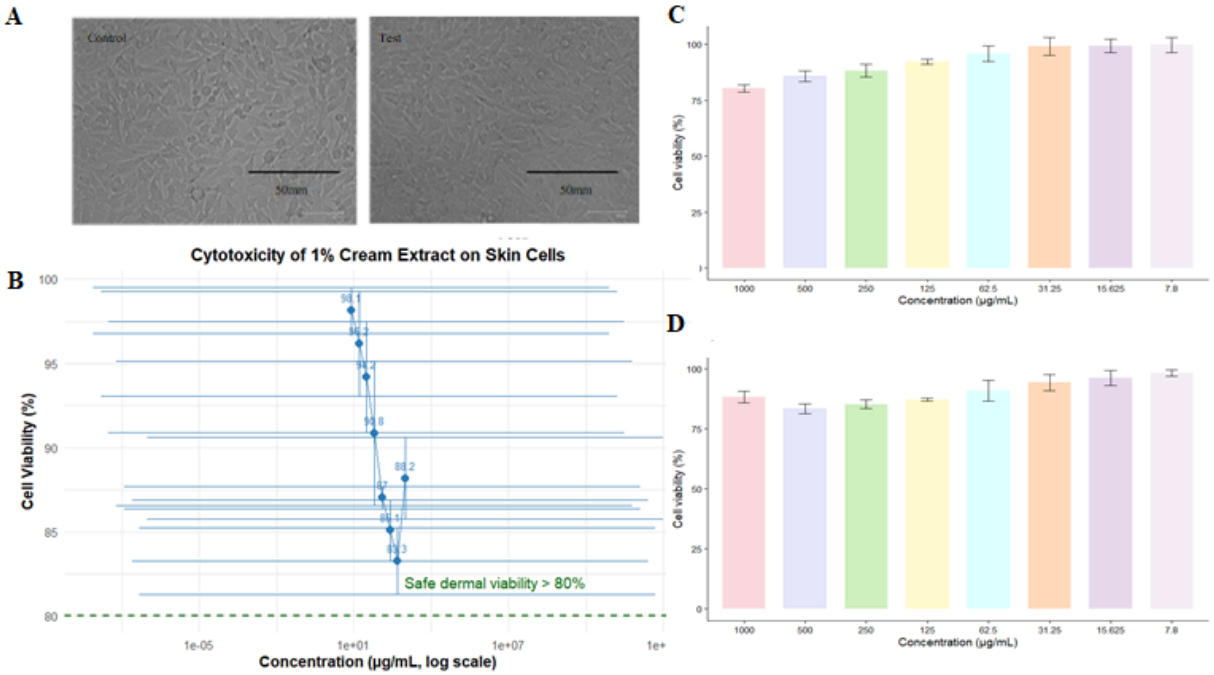


Figure 4

A) Microscopic images of skin cells treated with control (without extract) and test cream (with Tagetes erecta extract). (B) Cytotoxicity of 1% cream on skin cells, showing cell viability (%) across log_{10} concentrations (µg/mL); viability >80% indicates safety. (C, D) Bar graphs representing cellular responses at 0–1000 µg/mL concentrations for control (C) and test (D) treatments.

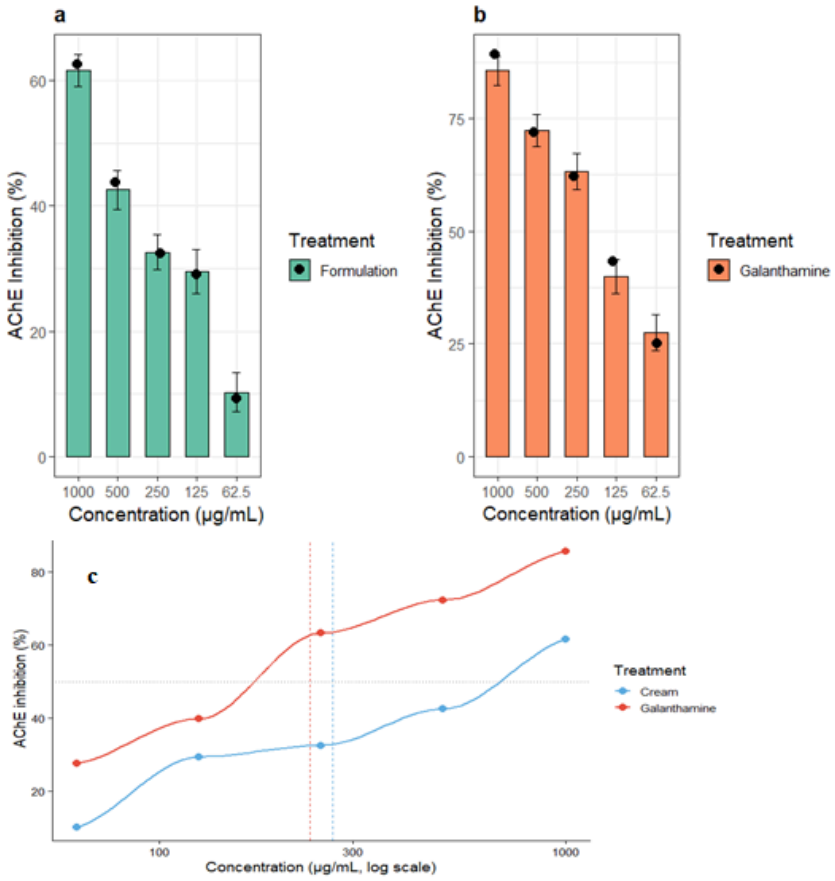


Figure 5

A, B) Percentage inhibition of acetylcholinesterase (AChE) by the formulated cream compared with the standard inhibitor galantamine. (C) IC_{50} values of the formulated cream and galantamine.

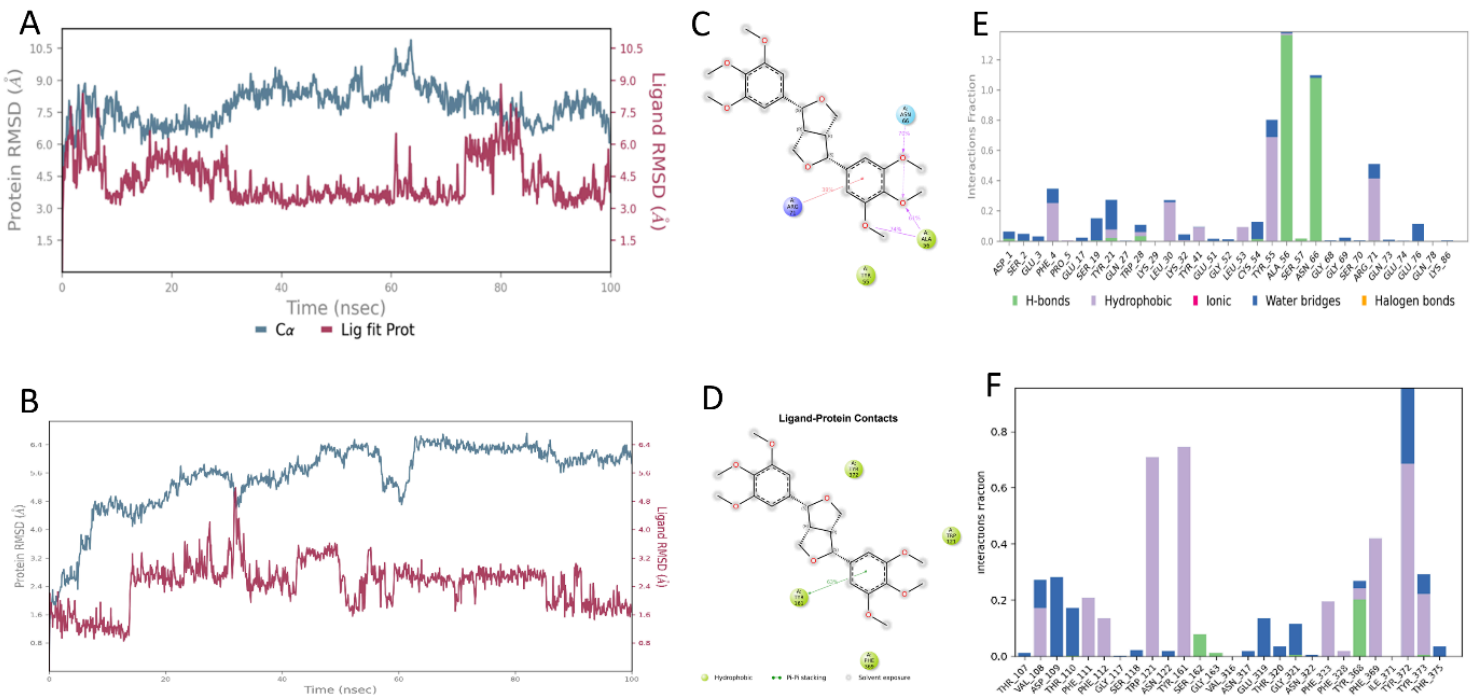


Figure 6

RMSD Profiles of Protein–Ligand Complexes During 100 ns Molecular Dynamics Simulation [RMSD plots depicting the structural stability of tick salivary protein BSAP1 (PDB ID: 7NE8) in complex with (A) Yangambin (PubChem ID: 443028), and tick acetylcholinesterase enzyme model RmAChE1(Swiss-model) in complex with (B) Yangambin (PubChem ID: 443028), over a 100 ns molecular dynamics simulation. The backbone RMSD of the protein Cα atoms is shown in blue, while the protein-ligand complex RMSD relative to the protein is shown in magneta color. Molecular interactions of Yangambin with C) BSAP1 and D) RmAChE1. Bar plot showing interacting residue fractions of Yangambin and E) BSAP1 and F) RmAChE1

Supplementary Files

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