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RESEARCH ARTICLE

Larvicidal and Repellent Effects of Nano-Encapsulated *Mentha piperita* Essential Oil Against *Rhipicephalus microplus*

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A B S T R A C T

Ticks and tick-borne diseases (TTBDs) pose significant global health challenges. Essential oils (EOs) have emerged as promising alternative to synthetic chemicals, but their volatile nature, low stability, and exposure to extreme conditions limit their efficacy. Encapsulation of phytochemicals/essential oils in suitable polymers is a potential solution. In this study, the acaricidal activity of chitosan (CS) encapsulated *Mentha piperita* essential oil (MPEO) against the cattle tick, *Rhipicephalus* (*R.*) *microplus* along with its inhibitory effect on acetylcholinesterase (AChE) was evaluated. Briefly, MPEO was encapsulated in CS nanoparticles (NPs) using emulsification/ionic gelation method, these formulated NPs were characterized thoroughly with the help of encapsulation efficiency percentage (EE%), loading capacity percentage (LC%), zeta analysis, Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), and Thermogravimetric Analysis (TGA). The acaricidal efficacy of CS/MPEO NPs was evaluated through prolonged Larval Packet Test (LPT) and climbing repellent bioassay. CS/MPEO NPs with a 1:1 (CS:EO) w/v ratio exhibited the best characterization results such as EE% (98.65), LC% (25.5), average size (293.1nm) and zeta potential as 28mV. Furthermore, the CS/MPEO NPs showed superior acaricidal efficacy, achieving 100% larval mortality at the highest concentration (4mg/mL) by day 7 (LC_{50}=0.126, LC_{90}=1.314), whereas non-encapsulated MPEO revealed decreasing efficacy over time, i.e. 6% mortality by day 7 (LC_{50}=63.269, LC_{90}=614.591). Climbing repellent assay (CRA) exhibited similar trend i.e. encapsulated MPEO showed the highest repellence, reaching 100% at 4mg/mL after 6 hours of exposure and MPEO revealed decreasing repellence over time with 15.3% after 6 hours. Nanoformulation exhibited potent AChE inhibitory activity. Stability analysis after 30 days showed that NPs stored at 4°C had a better characterization results (average size 315.7±1.17nm and ZP 26±0.577mV) compared to those stored at room temperature (405.3±0.75nm, 22.7±0.98mV). This study provides the strong evidence of the larvicidal as well as repellent activity of nanoencapsulated MPEO against *R. microplus*, offering an eco-friendly alternative for tick control.

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INTRODUCTION

Ticks are blood-feeding ectoparasites of veterinary and medical importance, acting as second most abundant vectors after mosquitoes, transmitting a variety of pathogens that cause diseases, skin wounds, anemia and economic losses (Torii *et al*., 2019; Rajput *et al*., 2023). Ixodid ticks alone can transmit more than 170 pathogens worldwide, including protozoa, viruses, spirochetes and rickettsiae which can also result in human diseases such as Q fever, Lyme disease, tick-borne forest encephalitis, spotted fever (Antenucci *et al*., 2020; Razzaq *et al*., 2024).

Among them, *R. microplus* is the most widespread and economically significant cattle ticks, particularly in subtropical and tropical regions (Tay *et al*., 2014; Lima *et al*., 2019). It causes substantial losses to livestock industry through skin damage, blood loss, and transmission of pathogens, including different rickettsial species and *Coxiella burnetii*, which also pose serious health issues in humans (Hussain *et al*., 2021; Xu *et al*., 2023). In Pakistan, this tick is a major constraint to cattle health and productivity (Ghafar *et al*., 2020).

Control strategies to combat TTBDs are categorized into two classes, i.e., nonchemical and chemical. The former includes grooming, pasture management, biological control, genetic manipulation and vaccination. While the latter comprises arsenics, carbamates, chlorinated hydrocarbons, pyretheroids and organophosphates providing an effective and rapid response against TTBDs, but their prolonged and indiscriminate use leads to resistance development in various tick species (Singh *et al*., 2019; Sindhu *et al*., 2022). Furthermore, the highly toxic nature of chemical acaricides poses a threat to non-target species (Joshi and Sukumaran, 2019).

In response to the limitations of chemical acaricides, plant-based alternatives like EOs are gaining attention for their efficient and eco-friendly acaricidal properties (Fayaz *et al*., 2019). Likewise, the MPEO has demonstrated promising efficacy against ticks (Chagas *et al*., 2016) but its high volatility and environmental sensitivity hinder practical application (Lima *et al*., 2019). Nanoencapsulation addresses these challenges by improving bioavailability, stability, and sustained release of EOs (Weisany *et al*. 2022; Yammine *et al*., 2024).

Nanotechnology, leveraging the utilization of NPs, offers a targeted approach to overcome issues linked to direct EO use (Fantatto *et al*., 2025). Various encapsulation techniques within nanotechnology are employed for EO encapsulation, with emulsification/ionic gelation techniques receiving considerable attention due to their nontoxic nature, convenience and controllability (Hosseini *et al*., 2013). The acaricidal mechanism of encapsulated EOs includes enhanced penetration through the cuticle, leading to the damage of cellular membranes, interference with neurotransmission, specifically through AChE inhibition and oxidative stress induction. Which cause the tick's neuromuscular incoordination and metabolic function impairment, ultimately leading to mortality (Gamal, 2023).

Despite the strong potential as an acaricide of the MPEO, its practical application is limited by poor stability and rapid degradation under environmental stressors. Nanoencapsulation provides a potential solution, yet no research has explored the efficacy of MPEO loaded NPs against larval stages of *R. microplus*. Therefore, this study was designed to formulate and characterize CS/MPEO NPs, evaluate their stability, analyze their acaricidal and AChE inhibitory potential against *R. microplus*.

MATERIALS AND METHODS

Essential oil extraction and composition analysis: The *M. piperita* herbs were procured from local market of Lahore, then they were taxonomically identified by the Department of Botany, Government College University, Lahore, Pakistan. The dried herbs were steam distilled for EO extraction (Boutekedjiret *et al*., 2003). Gas chromatography mass spectrometry (GC-MS) was performed in Department of Chemistry, COMSATS University, Lahore, Pakistan, by following the protocol of Al-Asmari *et al*. (2017) with slight changes through a GC-MS system (Agilent 7890 series, USA). In this analytical system, Helium served as the carrier gas and flowed at a rate of 1mL/min. The split ratio was consistently maintained at 1:20, while the injector and detector temperature were set at 250 and 300°C, respectively. The column temperature underwent a staged progression. It started at 60°C for duration of 2 minutes, followed by a linear increase from 60 to 250°C at a rate of 2°C/min, subsequently maintaining an isothermal state for 2 additional minutes. Simultaneously, the transfer line was maintained at a temperature of 280°C. Mass spectra were captured using scan mode (at 70eV) across the 50-550m/z range. A minute quantity of the oil sample, specifically two microliters of a 1000µL/L dilution in hexane, was subjected to injection. The constituents were identified using NIST libraries.

Formulation of *Mentha piperita* essential oil loaded chitosan nanoparticles: The CS/MPEO NPs were formulated via a two-step method, oil in water emulsion leading towards ionic gelation (Shetta *et al*., 2019), with minor modifications where required with three ratios (1:0.5, 1:1, 1:1.5) W/V.

Characterization of the nanoparticles: Encapsulation efficiency and LC% were determined through spectrophotometer (UV1100, Robus Technologies, UK) (227nm) with formulae (1) and (2), respectively.

$$\text{EE \%} = \frac{\text{Total amount of loaded EOs}}{\text{Initial amount of Eos}} \times 100 \quad (1)$$

$$\text{LC \%} = \frac{\text{Total amount of loaded EOs}}{\text{Weight of NPs after freeze drying}} \times 100 \quad (2)$$

Mean particle size and ZP of the freshly prepared NPs (1:0.5, 1:1, 1:1.5) were measured using dynamic light scattering (DLS) (Zetasizer, Nano ZSP, Malvern Panalytical, UK) (Shetta *et al*., 2019). Furthermore, the FTIR was performed using a Cary 630 FTIR spectrometer (Agilent Technologies, USA) to study the chemical characteristics (Kustrin *et al*., 2020). The morphology and shape of freshly prepared and lyophilized particles (1mg each) were analysed through FESEM (Zeiss Gemini, Sigma 500VP, Germany) in UET, Lahore (Schneider *et al*., 2012). Their thermal stability was also observed in UET, Lahore, using TGA (Shimadzu, Japan) (Ferreira *et al*., 2019). The Td and derivative thermogravimetry (DTG) thermogram were determined.

Collection and identification of ticks: Engorged female ticks were collected from the infested animals and transported to the Entomology lab, Department of Parasitology, University of Veterinary and Animal Sciences, Lahore, Pakistan in pre-labelled container. After rinsing and drying, ticks were identified using the

taxonomic keys (Walker, 2003). Females of *R. microplus* were separated and divided into aliquots and incubated (26±2°C, 70-80% RH and 12:12 hours of light/dark cycles) for oviposition.

Evaluation of acaricidal activity: *In vitro* acaricidal efficacy of the CS/MPEO NPs was evaluated through LPT and the repellent effect was assessed using the CRA against larvae of *R. microplus*. In acaricidal assay, trichlorphon and distilled water (DW) while in repellent assay N, N-diethyl-meta-toluamide (DEET) were used as control positive and DW along with CNPs as control negative.

Prolonged larval packet test: The prolonged larvicidal effectiveness of CS/MPEO NPs and pure MPEO was assessed on days 1, 3, 5 and 7 through modified LPT technique with a few modifications (Chamuah and Management, 2020; Moudgil *et al*., 2023). Five concentrations (4, 2, 1, 0.5 and 0.25mg/mL) of CS/MPEO NPs and MPEO were prepared.

Triangular packets of 7×7cm Whatman filter paper were prepared. All packets were impregnated with 1mL of formulations and placed in controlled environment (37°C and 80±5% relative humidity) in Climate Chamber (Biobase Bioyu, China). Each time (0, 2, 4 and day 6) new larvae (approximately 100) were placed in impregnated filter papers and their mortality was observed after 24 hrs. Three replicates were run for each concentration accordingly**.** The LC_{50} and LC_{90} were calculated to determine lethal concentrations against larvae. The mortality was expressed as a percentage following the undermentioned formula (3).

$$\text{Percentage mortality} = \frac{\text{Total dead larvae } \times 100}{\text{Total larvae}} \quad (3)$$

Climbing repellent assay against *Rhipicephalus microplus* Larvae: The repellent efficacy of the CS/MPEO NPs was evaluated following the modified protocols of Lima *et al*. (2019). A 7×4cm filter paper marked into three pencil-drawn zones (two zones of 1×4cm at either end and a 4×5cm zone between them). Afterwards, 5 serial dilutions (4-0.25mg/mL) were applied to the middle zone. 30 larvae (each time fresh) per petri dish were used and attached to the lower untreated end of the filter paper. After 5 minutes of attaching the ticks each time, their locations were spotted at 1, 3 and 6 hours. Larvae in the lower zone/dish were considered repelled; those in middle/upper zones/clip were not. Three replicates were observed for each treatment. The RC_{50} and RC_{90} were calculated for each concentration. The percentage repellency was calculated using following formula (4).

$$\text{Percentage repellency} = 100 - \frac{\text{Mean number of larvae not repelled}}{\text{Mean number of ticks not repelled on control}} \times 100 \quad (4)$$

Determination of enzyme activity and stability analysis: The biochemical assay was performed by utilizing the Ellman's method (Ellman *et al*., 1961; Santos *et al*., 2021) for assessing AChE inhibition in tick larvae with minor changes. AChE inhibition percentage was determined through the following formula (5).

$$\text{AChE inhibition} = \frac{\text{Optical density of Blank} - \text{Optical density of Treatment}}{\text{Optical density of Blank}} \times 100 \quad (5)$$

Furthermore, the shelf life of CS/MPEO NPs was assessed by key characterization tools (ZP and average dynamic size) placed at two different temperatures (room temperature and 4°C) for 1 month (Boyás *et al*., 2017).

Statistical analysis: Results are reported as the mean±SE for triplicate measurements. The EE and LC% were calculated using formulae (1) and (2) respectively. Morphology, individual sizes and average sizes of NPs achieved from SEM were examined through ImageJ software. Moreover, the LC_{50} and LC_{90} values in prolong LPT and RC_{50} and RC_{90} in CRA were measured through probit analysis using SPSS (20.0). Percentage AChE inhibition was calculated using formula (5).

RESULTS

Identification and chemical composition analysis of MPEO: Procured herbs were identified as *M. piperita* L. (Voucher No: GC.Herb.Bot.3808). Subsequently, GC-MS analysis revealed the presence of more than 190 compounds in the extracted MPEO, among which 4 were major constituents accounted for 89.16% of total composition: menthone (28.56%), limonene (26.21%), carvone (23.09%), and menthol (11.3%). The remaining detected components constituted about 10.84% of the total compounds.

Determination of encapsulation efficiency and loading capacity: The EE% of the CS/MPEO NPs for all formulated ratios (1:0.5, 1:1 and 1:1.5) ranged from 98.60 to 98.65 with values of 98.60, 98.65 and 98.63%, respectively. The LC% values were 12.2, 25.5 and 18.8%. Whereas, the highest EE and LC% were observed for EO:CS (1:1) ratio, hence chosen for further investigation in the study.

Particle size, ZP and chemical interaction: The average size for all formulated ratios of CS/MPEO NPs was ranged between 293.1±0.64nm to 744.2±1.05nm. Whereas, the size of CSNP was recorded as 278.9±1.10nm (Fig. 1a). Likewise, the ZP of all ratios of CS/MPEO NPs was recorded between 24±1 to 28±0.577mV. However, the ZP of the CSNPs was 22±0.577mV (Fig. 1b). Furthermore, it was revealed that the formulated nanoparticles for CS:EO (1:1) had shown smallest particle size with highest ZP hence another reason to choose the ratio for further biological efficacy analysis in the study.

The FTIR spectrum of MPEO showed several characteristic peaks at 3417 (OH stretching), 2909 and 2870 (CH stretching), 1699 (C=O stretching in menthone), 1371 (isopropyl group) and 1043 (C-O-C in menthofuran). Characteristic peaks corresponding to the CS powder were observed at 3432 (OH and NH_2 stretching), 2862 (CH

stretching), 1587 (C-O stretching of amide I) and 1028 (C-O-C stretching). Surprisingly, upon encapsulation of MPEO in CSNP, all the characteristic peaks appeared in the spectra obtained for MPEO and CS for all formulated ratios of CS/MPEO NPs. However, minor changes were observed at different peaks, i.e., at 3417cm−1 (OH stretching) and 1371cm−1 (isopropyl group), for all ratios of CS/MPEO NPs (Fig. 2).

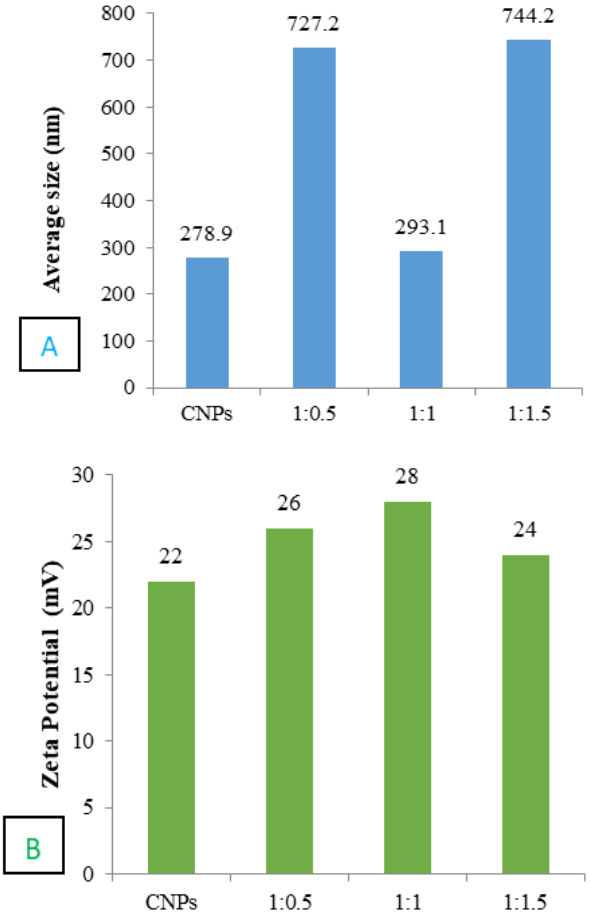

Fig. 1: Average Size and zeta potential of CS fabricated NPs with and without MPEO (a) Average Size (b) Zeta potential.

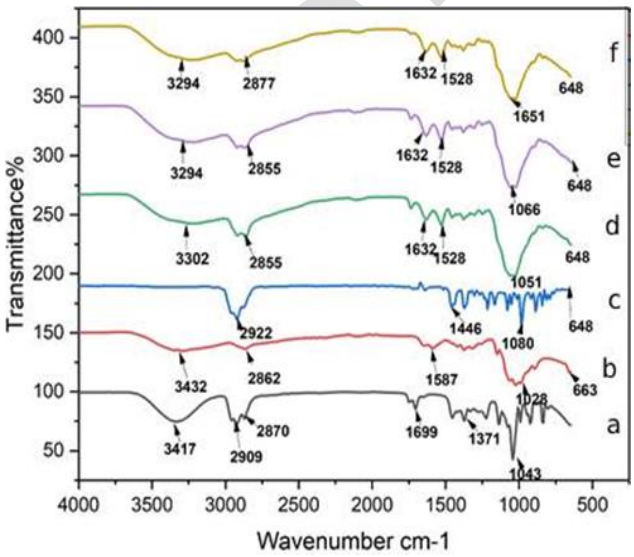

Fig. 2: FT-IR spectra of (a) MPEO, (b) CS, (c) CSNP, (d) CS/MPEO NP (1:0.5), (e) CS/MPEO NP (1:1), (f) CS/MPEO NP (1:1.5).

Morphology and surface study: Freshly prepared CS/MPEO (1:1) NPs exhibited spherical morphology during FESEM analysis. The individual particle size ranged from 44.4 to 116.7nm, with an average size of 82.86±15.69nm (Fig. 3a). In contrast, the individual particle size of lyophilized NPs, ranged from 57.33 to 386.08nm, with an average size of 149.9±42.2nm which has shown a slight increase in the size of particles after freeze drying (Fig. 3b). Moreover, no aggregation among the NPs was observed.

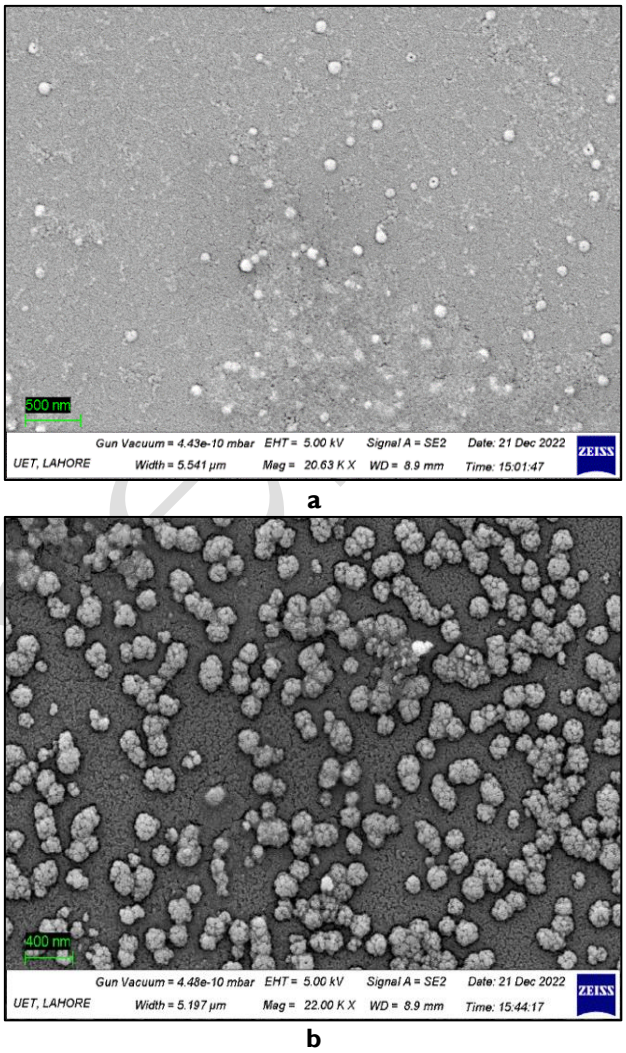

Fig. 3: FESEM images of CS fabricated MPEO NPs of **(a)** fresh sample and **(b)** lyophilized sample.

Thermal stability study: The TGA thermogram of pure MPEO showed a one-step mass loss that began at 50°C (Fig. 4Ac), with a Td value of 161°C (Fig. 4Ba). In contrast, the CSNPs exhibited a three-step weight loss, with three Td values ranged from 43 to 266°C (Fig. 4Bb). Similarly, the weight loss of the CS/MPEO nanoparticles (1:1) was comparable to that of the CSNP, with Td values ranged between 41 to 273°C (Fig. 4Bc).

Prolonged larval packet test: The larval mortality % showed dose and time dependent results. A concentration of 4mg/mL of CS/MPEO NPs resulted in 100% larval mortality by day 7. While, MPEO concentrations were associated with a greater death rate, which peaked at 100% mortality at 4mg/mL on day 1 and then gradually declined.

Notably, from day 3 to 7, the mortality (%) decreased when trichlorfon was administered (Fig. 5). LC_{50} and LC_{90} values of CS/MPEO NPs are consistently decreasing with respect to days i.e. (1.009 to 0.126) and (73.761 to 1.314), respectively. In comparison, LC_{50} and LC_{90} values of pure MPEO are increasing day wise i.e. (0.160 to 63.269) and (1.059 to 614.519), respectively (Table 1).

Climbing repellent assay against *Rhipicephalus microplus* larvae: The mean repellent efficacy of formulations also showed dose and time dependent trends (Fig. 6). At a concentration of 4mg/mL, the CS/MPEO NPs achieved 100% mean larval repellence after 6 hrs. Higher concentrations of MPEO correlated with increased repellent rates, reaching 100% repellence for pure MPEO at 0 hrs, which then gradually decreased. In contrast, the repellent percentage for DEET decreased from 0 to 6 hours. The DW and CSNPs had no effect on repellence. The RC_{50} and RC_{90} values of all the treatments are shown in (Table 2).

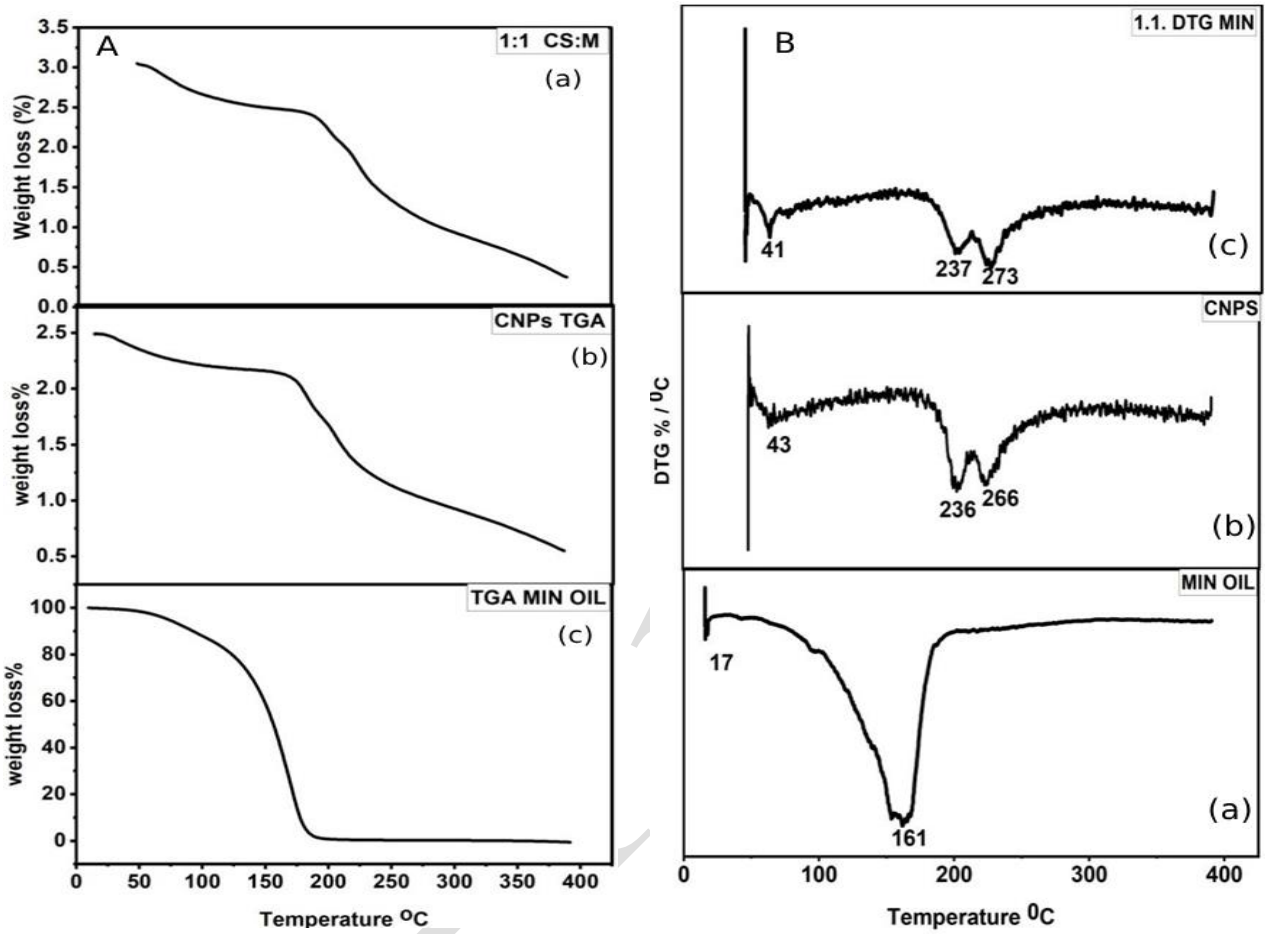

Fig. 4: (A) Thermogravimetric Analysis thermogram representing TGA of (c) MPEO, (b) Chitosan NPs, (a) CS/MPEO NPs (1:1) and (B) Differential Thermogravimetry Analysis thermogram representing DTG of (a) MPEO, (b) Chitosan NPs, (c) CS/MPEO NPs (1:1).

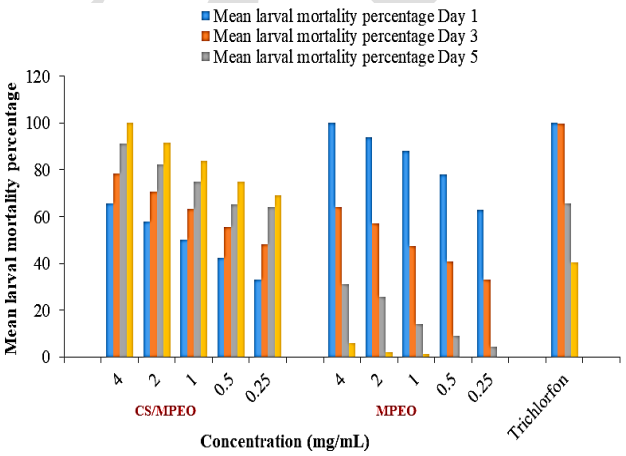

Fig. 5: Mean larval mortality percentage of different concentrations of MPEO NPs, MPEO, trichlorfon and DW against *Rhipicephalus microplus* larvae.

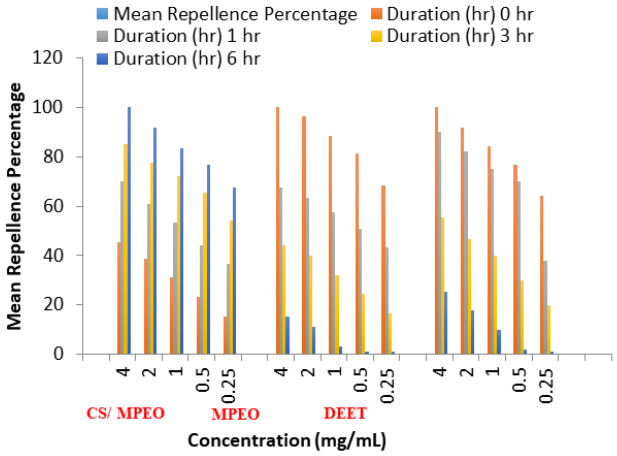

Fig. 6: Percent repellence of different concentrations of MPEO NPs, MPEO, DEET, CNPS and DW against *R. microplus* ticks according to duration.

Table 1: LC_{50} and LC_{90} with 95 % confidence intervals of each formulation evaluated on *R. microplus* larvae.

Compound	Days	LC_{50} value with 95% confidence interval			LC_{90} CI value with 95% confidence interval		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
MPEO NPs	1	1.009	0.674	1.515	73.761	21.391	1140.591
	3	0.304	0.127	0.483	22.584	8.626	194.082
	5	0.119	0.034	0.220	4.847	2.739	15.281
	7	0.126	0.058	0.196	1.314	0.988	1.998
MPEO	1	0.160	0.094	0.224	1.059	0.837	1.463
	3	1.142	0.767	1.781	93.389	24.866	1872.906
	5	10.958	6.068	33.868	204.243	56.042	2717.396
	7	63.269	15.243	343112.530	614.591	59.228	320628377.9

Determination of enzyme activity and stability analysis: The CS/MPEO showed dose dependent AChE inhibition through Ellman's assay in encapsulated particles treated *R. microplus* larvae (Fig. 7). During the stability analysis, after one month, the ZP of the CSNPs and CS/MPEO NPs kept at 4°C were noted as 21.5 and 26mV, respectively. Whereas, the ZP of the CSNP and CS/MPEO NPs kept at room temperature were recorded as 16.6 and 22.7mV, respectively. The average sizes of the CSNPs and CS/MPEO NPs placed at 4°C were measured as 295.5 and 315.7nm, respectively. The average sizes of the CSNP and CS/MPEO NPs kept at room temperature were noticed as 370.8 and 405.3nm, respectively (Table 3).

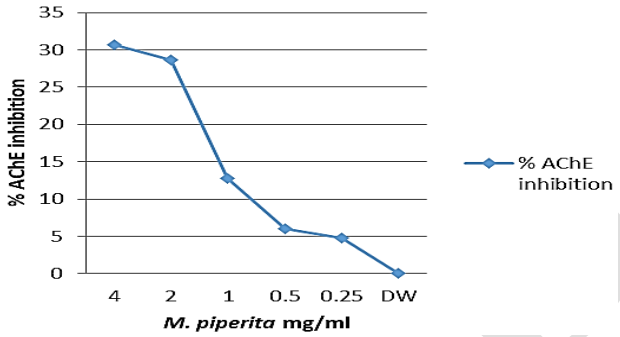

Fig. 7: Effect of different concentrations of CS/MPEO NPs (mg/mL) on the acetylcholinesterase activity of *R. microplus* larvae expressed as percentage of inhibition (%).

Table 2: Repellent Concentration of MPEO NPs, MPEO, DEET, CNPS and DW against *R. microplus*

Compound	Hours	RC_{50}	CI 95%	RC_{90}	CI 95%
MPEO NPs	0	5.214	3.073-14.881	283.418	59.792-8763.463
	1	0.773	0.498-1.111	47.702	15.944-494.655
	3	0.168	0.055-0.292	8.783	4.323-38.992
	6	0.130	0.063-0.200	1.293	0.979-1.943
MPEO	0	0.131	0.069-0.193	0.886	0.699-1.215
	1	0.472	0.174-0.783	136.180	24.717-22224.497
	3	5.420	3.073-17.828	396.776	70.541-22539.690
	6	23.598	10.583-155.482	242.419	55.978-8391.999
DEET	0	0.150	0.081-0.219	1.270	0.976-1.850
	1	0.306	0.272-0.503	3.432	2.406-5.914
	3	2.498	1.723-4.554	106.501	31.536-1261.788
	6	10.861	6.558-27.509	93.972	34.570-643.063

Table 3: Zeta Potential and average size of CSNPs and MPEO NPs stored at different temperatures for 1 month

ZP (mV) of NPs		
Day 0	Day 30 at 4°C	Day 30 at 25°C
CSNPs	CSNPs	CSNPs
22±0.577	21.5±0.89	16.6±0.923
CS/MPEO NPs	CS/MPEO NPs	CS/MPEO NPs
28±0.577	26±0.577	22.7±0.98
Average Size (nm) of NPs		
CSNPs	CSNPs	CSNPs
278.9±1.10	295.5±0.866	370.8±1.04
CS/MPEO NPs	CS/MPEO NPs	CS/MPEO NPs
293.1±0.64	315.7±1.17	405.3±0.75

DISCUSSION

Mentha piperita is a medicinal plant that has been evaluated in extensive detail across the world (Hudz *et al*., 2023). It is rich in many physiologically active chemicals which has been tested for insecticidal and acaricidal properties (Kalemba and Synowiec, 2019; Rajkumar *et al*., 2020; Tourabi *et al*., 2023; Ahmad *et al*., 2024). Moreover, the direct MPEO has revealed notable acaricidal efficacy against various ixodid tick species (Chagas *et al*., 2016; Voronova *et al*., 2022). But issues related to direct EOs application provided research gap to encapsulate the MPEO which presented numerous advantages over its direct application, particularly against *R. microplus*, including prolonged acaricidal efficacy, enhanced stability, targeted delivery and reduced impact on non-target species.

The chemical components of MPEO explored through GC-MS align with previous studies (Taherpour *et al*., 2017; Rajkumar *et al*., 2020) with minor variations. The composition of EO is influenced by multiple factors including plant species, environmental conditions (climate, soil, and altitude), and harvesting methods. Moreover, extraction techniques and storage conditions play vital roles in evaluating the chemical profile of EOs (Dhifi *et al*., 2016; Khan *et al*., 2023).

The results revealed higher EE and LC% of the CS/MPEO NPs. The results of both readings depicted maximum values at a 1:1 (CS:MPEO) concentration, which was constant with the findings of previous studies (Shetta *et al*., 2019; Lee *et al*., 2021). Optimizing polymer-drug ratios and compatibility enhances their interaction, maximizing EE and LC% (Kumari *et al*., 2014; Lee *et al*., 2021). The other reason behind the higher EE% of MPEO may be due to the characteristic of Lamiacae family herbs, i.e., having oxygenated monoterpene phytochemicals, which are smaller in size (Karageçili and Gülçin 2025).

The GC-MS results in this study expressed that among the primary constituents, MPEO had 62.95% oxygenated monoterpenes and 26.21% non-oxygenated monoterpenes. Both are small enough in size to be efficiently encapsulated in polymers (Pateiro *et al*., 2021). Moreover, the 1:1 concentration of CS:MPEO showed minimum average sizes of NPs compared to the other concentrations. The reason behind this difference is the optimal balance of CS-Oil interactions that promotes smaller nanoparticle sizes, while at other ratios, there are fewer favourable interactions that lead to larger sizes (Mikušová and Mikuš, 2021). Comparable results have been reported in various studies (Shetta *et al*., 2019; Rajkumar *et al*., 2020). The best ZP was also shown by the 1:1 (CS:MPEO) compared to the other ratios. Results were in line with the previous study (Nallamuthu *et al*., 2015; Shetta *et al*. 2019). Furthermore,

it has been reported that the stability of NPs is directly proportional to the ZP, i.e., 30mV indicate long-term stability (Wissing *et al*., 2004; Gumustas, 2017). The NPs exhibited good stability (SEM images) despite having ZP values less than 30mV, showing stability is not solely ZP dependent. Tween 80 provided steric stabilization, helping stable nanoformulations (Pochapski, 2021). The lower ZP values of the CS/MPEO NPs might be due to the shielding effect of the protonated NH2 group of MPEO on the CS layer of the NPs (Keawchaoon and Yoksan, 2011) and the highly charged nanoformulations may also show low ZP due to the shear plane shifts from adsorbed polymer layers (Li, 2021).

In FTIR analysis, no significant chemical interaction between polymer and MPEO was found in all formulated ratios (CS:MPEO). However, a more pronounced change in C-O-C stretching was observed for 1:1.5/CS:MPEO. This difference may be due to the greater quantity of MPEO in the CS NPs. Post-encapsulation changes in the spectrum suggest a potential alteration or chemical interaction between MPEO and CS. These results were in agreement with those of a previous study (Shetta *et al*., 2019).

The SEM images of the CS/MPEO NPs (1:1) showed spherical particles and particles with rough surfaces are seen in lyophilized samples. The surface roughness observed may be attributed to the lyophilization process, which can lead to the formation of cracks, ruptures, splits, and some particle aggregation during the preservation stage (Das *et al*. 2020). Additionally, the high stability of the NPs was depicted in images with no aggregation despite having a ZP less than 30mV. Furthermore, the measurement of NPs sizes were found to be smaller than DLS, in line with findings from prior studies (Keawchaoon and Yoksan, 2011; Hosseini *et al*., 2013; Esmaeili and Asgari, 2015; Shetta *et al*., 2019). The possible reason for this fluctuation must be due to the change of state of particles during measurement (Keawchaoon and Yoksan, 2011).

The TGA thermogram of pure MPEO revealed a one-step mass loss with a Td value. In contrast, the CSNPs expressed a three-step mass loss, with three Td values which are linked with dehydration and decomposition of water and CS. Similarly, the weight loss of the CS/MPEO NPs was analogous to that of the CSNPs, with three Td values, which are associated with dehydration and decomposition of water, CS and oil, respectively (Keawchaoon and Yoksan 2011). Compared with those of pure MPEO, the Td values of CS/MPEO NPs show an increase in the thermal stability of encapsulated MPEO by approximately 1.66-fold. These results were also in good agreement with the findings of previous studies (Keawchaoon and Yoksan, 2011; Shetta *et al*., 2019).

The larvicidal and repellent efficacies of CS/MPEO NPs and pure MPEO against R. microplus are comparable to earlier findings (Mkolo et al., 2011). Results indicate a significant difference in the larvicidal activity between the nanoencapsulated and the pure MPEO. Acaricidal potential of MPEO may be due to the menthol and other active compounds that may cause nervous system disruption of ticks by disturbing the ion channels (TRPM8 receptors) and inhibiting AChE, causing acetylcholine accumulation, nerve stimulation, paralysis, and death (Isman, 2006; Tong and Coats, 2010). It may penetrate the cuticle easily due to the lipophilic nature, damaging membranes, leaking cellular contents, and dehydration leading towards the death of ticks (Roger et al., 2012). Furthermore, nano-encapsulated MPEO revealed time and dose dependent response, with larval mortality increasing steadily across all tested concentrations, confirming its sustained release and prolonged effectiveness (Gupta and Variyar, 2016). The superior performance is linked to nanoencapsulation, which ensures controlled release, stability, and increased penetration into the larval cuticle due to smaller particle size (Weisany et al., 2022; Gamal et al., 2023). In contrast, pure MPEO expressed a rapid decline in efficacy over the seven days, likely due to fast evaporation or degradation (Bilia et al. 2014).

The repellent efficacy of CS/MPEO and pure MPEO revealed patterns similar to that of their larvicidal effects. Repellence may occur through disruption of olfactory receptors, reducing the tick ability to sense host cues i.e. heat or CO_2 (Benelli *et al*., 2016). Secondly, menthol may disturb potassium and sodium channels, inducing paralysis, reducing mobility and disorientation of ticks. Furthermore, volatile compounds repel ticks by induction of avoidance, and exert anti-feedant effects due to strong aroma preventing ticks from feeding and biting (Priestley, *et al*. 2003; Benelli *et al*., 2016; Gupta, *et al*. 2019).

Moreover, Mkolo *et al*., (2011) reported highest level of MPEO repellency against Ixodid ticks for an hour. The reason for this difference must be due to the volatile nature of the EOs. The CS/MPEO NPs effectively enhanced repellence against *R. microplus* in present study, aligning with previous research outcomes (Lima *et al*., 2019).

The findings from the study on CS/MPEO NPs demonstrate a clear dose-dependent inhibition of AChE activity, as evidenced by Ellman's assay results, highlighting the potential of MPEO as a natural AChE inhibitor, which can be used to control ticks by interfering AChE activity. The results align with existing literature, emphasizing the relevance of AChE inhibition in therapeutic contexts, particularly through the utilization of plant-derived compounds (Ferreira *et al*., 2020; Patel, 2023). This inhibition is because of terpenes, most of the plants have these compounds in EOs. In this study, GC-MS analysis revealed the presence of terpenes in the EO composition.

Findings of stability analysis in this study suggest that storing CS nanoparticles, whether loaded with MPEO or not, at lower temperatures (4°C) enhances their stability in terms of both ZP and particle size. These results are in accordance with previous study (Teng *et al*., 2024) where average dynamic size of NPs increases with the passage of time and ZP decreases. Storing NPs at lower temperatures enhances their stability due to reduced molecular motion, which minimizes the likelihood of aggregation and degradation (Katas *et al*., 2013).

Thus, nanoencapsulation of MPEO into CSNPs enhanced stability, sustained release and acaricidal efficacy compared to pure oil. These findings highlight its potential as a natural, eco-friendly alternative for controlling *R. microplus*.

Conclusions: In conclusion, EO nanoformulations are receiving huge attention as alternate acaricides and repellents for the control of insects. In this study, the CS/MPEO NPs

with a 1:1 (CS:Oil) w/w ratio showed the best results, as they had the maximum EE and LC% and good characterization results. Additionally, CS/MPEO NPs revealed to possess stronger acaricidal and repellent activity against *R. microplus* larvae. It revealed potent AChE inhibitory activity. Furthermore, stability analysis showed temperature dependent response i.e. at low temperature more stable product after one month was found. This study enhances the understanding of the development of an innovative and safer MPEO-based nanoacricidal and nanorepellent for *R. microplus* tick control.

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Declarations

Competing Interests: The authors declare that they have no conflict of interest.

Ethics approval: The study was approved by the Research Ethics Committee of University of Veterinary and Animal Sciences, Lahore, Pakistan (DR. Number 400, September 30, 2021). All applicable International, National, and/or Institutional guidelines for the care and use of animals have been followed.

REFERENCES

Ahmad S, Oneeb M, Lateef M, *et al*., 2024. Acaricidal efficacy of nano-encapasulated *Mentha piperita* essential oil against *Rhipicephalus microplus*: eco-friendly alternative for tick control in livestock. J Popul Ther Clin Pharmacol 31(5):1224-1235.

Al-Asmari AK, Athar MT, Al-Faraidy AA, *et al*., 2017. Chemical composition of essential oil of *Thymus vulgaris* collected from Saudi Arabian market. Asian Pac J Trop Biomed 7(2):147-150.

Antenucci SM, Kramer LD, Gebhardt LL, *et al*., 2020. Emerging tick-borne diseases. Clin Microbiol Rev 33(2):10-11.

Benelli G, Pavela R, Canale A, *et al*., 2016. Tick repellents and acaricides of botanical origin: a green roadmap to control tick-borne diseases. Parasitol Res 115:2545-2560.

Bilia AR, Guccione C, Isacchi B, *et al*., 2014. Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach. Evid Based Complement Alternat Med 1:651593.

Boutekedjiret C, Bentahar F, Belabbes R, *et al*., 2003. Extraction of rosemary essential oil by steam distillation and hydrodistillation. Flavour Fragr J 18(6):481-484.

Boyás MS, Pacheco ZC, Baños SB, *et al*., 2017. Release study and inhibitory activity of thyme essential oil-loaded chitosan nanoparticles and nanocapsules against foodborne bacteria. Int J Biol Macromol 103:409-414.

Chagas ACDS, Oliveira MCDS, Giglioti R, *et al*., 2016. Efficacy of 11 Brazilian essential oils on lethality of the cattle tick *Rhipicephalus (Boophilus) microplus*. Ticks Tick Borne Dis 7(3):427-432.

Chamuah JK, 2020. Occurrence of acaricidal resistance on ticks and their detection measures. Int J Stress Manag 11(3):240-245.

Das S, Singh VK, Dwivedy AK, *et al*., 2020. Fabrication, characterization and practical efficacy of *Myristica fragrans* essential oil nanoemulsion delivery system against postharvest biodeterioration. Ecotoxicol Environ Saf 189:110000.

Dhifi W, Bellili S, Jazi S, *et al*., 2016. Essential oils' chemical characterization and investigation of some biological activities: A critical review. Med 3(4):25.

Ellman GL, Courtney KD, Andres JV, *et al*., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7(2):88-95.

Esmaeili A and Asgari A, 2015. *In vitro* release and biological activities of *Carum copticum* essential oil (CEO) loaded chitosan nanoparticles. Int J Biol Macromol 81:283-290.

Fantatto RR, Constantini JV, Politi FA, *et al*., 2025. Current tick control strategies and prospects for using nanotechnology as an efficient alternative—a review. Vet Sci 12(2):163.

Fayaz MR, Abbas RZ, Abbas A, *et al*., 2019. Potential of botanical driven essential oils against *Haemonchus contortus* in small ruminants. Bol Latinoam Caribe Plantas Med Aromat 18(6):533-543.

Ferreira J, Santos S and Pereira H, 2020. *In vitro* screening for acetylcholinesterase inhibition and antioxidant activity of *Quercus suber* cork and corkback extracts. Evid Based Complement Alternat Med 1:3825629.

Ferreira PT, Haddi K, Corrêa FTR, *et al*., 2019. Prolonged mosquitocidal activity of *Siparuna guianensis* essential oil encapsulated in chitosan nanoparticles. PLoS Negl Trop Dis 13(8).0007624

Gamal A, Aboelhadid SM, Abo El-Ela FI, *et al*., 2023. Synthesis of carvacrol-loaded invasomes nanoparticles improved acaricide efficacy, cuticle invasion and inhibition of acetylcholinestrase against hard ticks. Microorganisms 11(3):733.

Ghafar A, Gasser RB, Rashid I, *et al*., 2020. Exploring the prevalence and diversity of bovine ticks in five agro-ecological zones of Pakistan using phenetic and genetic tools. Ticks Tick Borne Dis 11(5):101472.

Gumustas M, Turk SCT, Gumustas A, Ozkan SA, Uslu B. 2017. Effect of polymer-based nanoparticles on the assay of antimicrobial drug delivery systems. In: Multifunctional systems for combined delivery, biosensing and diagnostics. Elsevier, Amsterdam, Netherlands, pp:67-108.

Gupta RC, Doss RB, Srivastava A, *et al*., 2019. Nutraceuticals for control of ticks, fleas, and other ectoparasites. In: Nutraceuticals in veterinary medicine. Springer, London, UK, pp:625-633.

Gupta S and Variyar PS, 2016. Nanoencapsulation of essential oils for sustained release: application as therapeutics and antimicrobials. In: Nanotechnology in the Agri-Food Industry: Vol 2: Encapsulations (Grumezescu AM): Academic Press, San Diego, USA, pp:641-672.

Hosseini SF, Zandi M, Rezaei M, *et al*., 2013. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study. Carbohydr Polym 95(1):50-56.

Hudz N, Kobylinska L, Pokajewicz K, *et al*., 2023. Mentha piperita: essential oil and extracts, their biological activities, and perspectives on the development of new medicinal and cosmetic products. Molecules 28(21):7444.

Hussain S, Saqib M, Ashfaq K, *et al*., 2021. First molecular evidence of *Coxiella burnetii* in ticks collected from dromedary Camels in Punjab, Pakistan. Pak Vet J 42(2):276-280.

Isman MB, 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu Rev Entomol 51(1):45-66.

Joshi AKR and Sukumaran BO, 2019. Metabolic dyshomeostasis by organophosphate insecticides: insights from experimental and human studies. EXCLI J 18:479-484.

Kalemba D and Synowiec AJM, 2019. Agrobiological interactions of essential oils of two menthol mints: *Mentha piperita* and *Mentha arvensis*. Molecules 25(1):59.

Karageçili H and Gülçin İ, 2025. The Lamiaceae family plants ethnobotanical properties, ethnopharmacological uses, phytochemical studies and their utilization in public or current clinical practices: a review. Rec Nat Prod 19:466-487.

Katas H, Raja MAG and Lam KL, 2013. Development of chitosan nanoparticles as a stable drug delivery system for protein/siRNA. Int J Biomater 1:146320.

Keawchaoon L and Yoksan R, 2011. Preparation, characterization and in vitro release study of carvacrol-loaded chitosan nanoparticles. Colloids Surf B Biointerfaces 84(1):163-171.

Khan MH, Dar NA, Alie BA, *et al*., 2023. Unraveling the variability of essential oil composition in different accessions of *Bunium persicum* collected from different temperate micro-climates. Molecules 28(5):2404.

Kumari A, Singla R, Guliani A, *et al*., 2014. Nanoencapsulation for drug delivery. EXCLI J 13:265.

Kustrin SA, Ristivojevic P, Gegechkori V, *et al*., 2020. Essential oil quality and purity evaluation via ft-ir spectroscopy and pattern recognition techniques. Appl Sci 10(20):7294.

Lee KH, Khan FN, Cosby L, *et al*., 2021. Polymer concentration maximizes encapsulation efficiency in electrohydrodynamic mixing nanoprecipitation. Front Nanotechnol 3: 719710.

Li J, Wang Z, Zhang H, *et al*., 2021. Progress in the development of stabilization strategies for nanocrystal preparations. Drug Deliv 28(1):19-36.

Lima AdS, Landulfo GA and Junior LMG, 2019. Repellent effects of encapsulated carvacrol on the *Rhipicephalus* (Boophilus) *microplus* (Acari: Ixodidae). J Med Entomol 56(3):881-885.

Mikušová V and Mikuš P, 2021. Advances in chitosan-based nanoparticles for drug delivery. Int J Mol Sci 22(17):9652.

Mkolo NM, Olowoyo J, Sako K, *et al*., 2011. Repellency and toxicity of essential oils of *Mentha piperita* and *Mentha spicata* on larvae and adult of *Amblyomma hebraeum* (Acari: Ixodidae). Sci J Microbiol 1(1):1-7.

Moudgil AD, Daundkar PS, Nehra AK, *et al*., 2023. Molecular characterization of *Hyalomma dromedarii* and evaluation of acaricidal potential of herbal methanolic extracts against *H. dromedarii* larvae in comparison to synthetic acaricides. Exp App Acarol 89(1):141-156.

Nallamuthu I, Devi A and Khanum F, 2015. Chlorogenic acid loaded chitosan nanoparticles with sustained release property, retained antioxidant activity and enhanced bioavailability. Asian J Pharm Sci 10(3):203-211.

Pateiro M, Gómez B, Munekata PE, *et al*., 2021. Nanoencapsulation of promising bioactive compounds to improve their absorption, stability, functionality and the appearance of the final food products. Molecules. 26(6):1547.

Patel AB, 2023. Simple and rapid colorimetric method for determination of erythrocyte and plasma cholinesterase activities and comparison with the standard Ellman's method. Public Health Toxicol 3(3):1-10.

Pochapski DJ, Santos CCD, Leite GW, Pulcinelli SH, Santilli CVJL. 2021. Zeta potential and colloidal stability predictions for inorganic nanoparticle dispersions: Effects of experimental conditions and electrokinetic models on the interpretation of results. Langmuir 37(45):13379-13389.

Priestley CM, Williamson EM, Wafford KA, *et al*., 2003. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human GABA receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. Br J Pharmacol 140(8):1363-1372.

Rajkumar V, Gunasekaran C, Paul CA, *et al*., 2020. Development of encapsulated peppermint essential oil in chitosan nanoparticles: Characterization and biological efficacy against stored-grain pest control. Pestic Biochem Physiol 170:104679.

Rajput M, Sajid MS, Imran M, *et al*., 2023. A participatory approach in assessing the knowledge, attitude, and practices (KAP) of stakeholders and livestock owners about ticks and tick-borne diseases from Sindh, Pakistan. Pathogens 12:800.

Rajput S, Sharma P and Malviya R, 2023. Utilization of Herbal Components as Insecticidal and Repellent Effects. Recent Adv Food Nutr Agric 14(3):144-154.

Razzaq MA, Imran M, Atif FA, et al., 2024. Molecular surveillance based on anaplasmosis in domestic small ruminants: First report on zoonotic *Anaplasma capra* and phylogenetic insights from Faisalabad, Pakistan. PLoS One 19(9):e0305412.

Roger CR, Vincent C and Arnason JT, 2012. Essential oils in insect control: low-risk products in a high-stakes world. Annu Rev Entomol 57(1):405-424.

Santos EGGd, Bezerra WAdS, Temeyer KB, León AA, *et al*., 2021. Effects of essential oils on native and recombinant acetylcholinesterases of *Rhipicephalus microplus*. Rev Bras Parasitol Vet 30:2221.

Schneider CA, Rasband WS and Eliceiri KW, 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671-675.

Shetta A, Kegere J and Mamdouh W, 2019. Comparative study of encapsulated peppermint and green tea essential oils in chitosan nanoparticles: Encapsulation, thermal stability, in-vitro release, antioxidant and antibacterial activities. Int. J Biol Macromol 126:731-742.

Sindhu ZuD, Naseer MU, Raza A, *et al*., 2022. Resistance to cypermethrin is widespread in cattle ticks (*Rhipicephalus microplus*) in the province of Punjab, Pakistan: in vitro diagnosis of acaricide resistance. Pathogens 11:1293.

Singh NK, Abhijit N and Harkirat S, 2019. Detection of multi-acaricide resistance in *Rhipicephalus* (Boophilus) *microplus* (Acari: Ixodidae). Explor Anim Med Res 9:24-28.

Taherpour AA, Khaef S, Yari A, *et al*., 2017. Chemical composition analysis of the essential oil of *Mentha piperita* L. from Kermanshah, Iran by hydrodistillation and HS/SPME methods. J Anal Sci Technol 8:1-6.

Tay S, Koh F, Kho K, *et al*., 2014. Molecular survey and sequence analysis of Anaplasma spp. in cattle and ticks in a Malaysian farm. Trop Biomed 31(4):769-776.

Teng Q, Li Y, Cai Y, Guo J, *et al*., 2024. A potential acaricide of *Moutan Cortex* essential oil encapsulated in nanoemulsion and mesoporous silica nanoparticles against the house dust mite Dermatophagoides farinae. J Pest Sci:1-19.

Tong F and Coats JR, 2010. Effects of monoterpenoid insecticides on [3H]-TBOB binding in house fly GABA receptor and 36Cl− uptake in American cockroach ventral nerve cord. Pestic Biochem Phys 98(3):317-324.

Torii S, Matsuno K, Qiu Y, *et al*., 2019. Infection of newly identified phleboviruses in ticks and wild animals in Hokkaido, Japan indicating tick-borne life cycles. Ticks Tick Borne Dis 10(2):328-335.

Tourabi M, Nouioura G, Touijer H, *et al*., 2023. Antioxidant, Antimicrobial, and Insecticidal Properties of Chemically Characterized Essential Oils Extracted from *Mentha longifolia*: In Vitro In Silico Anal 2(21):3783.

Voronova N, Horban V and Bohatkina V, 2022. The effectiveness of acaricidic drugs based on herbal raw material. Ecol Quest 33(1):55-71.

Walker AR. 2003. Ticks of domestic animals in Africa: a guide to identification of species. Vol 74: Edinburgh, Bioscience Reports.

Weisany W, Yousefi S, Tahir NA-r, *et al*., 2022. Targeted delivery and controlled released of essential oils using nanoencapsulation: A review. Adv Colloid Interface Sci 303:102655.

Wissing S, Kayser O and Müller R, 2004. Solid lipid nanoparticles for parenteral drug delivery. Adv Drug Deliv Rev 56(9):1257-1272.

Xu J, Gu X-L, Jiang Z-Z, *et al*., 2023. Pathogenic *Rickettsia, Anaplasma*, and *Ehrlichia* in *Rhipicephalus microplus* ticks collected from cattle and laboratory hatched tick larvae. PLoS Negl Trop Dis 17(8):e0011546.

Yammine J, Chihib NE, Gharsallaoui A, *et al*., 2024. Advances in essential oils encapsulation: development, characterization and release mechanisms. Polym Bull 81(5):3837-3882.