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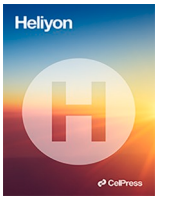

Acaricidal assessment of the fungal extract of *Pleurotus ostreatus* against *Rhipicephalus microplus*: Role of *in vitro* and *in silico* analysis

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A R T I C L E I N F O

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A B S T R A C T

The *Pleurotus ostreatus* mushroom, commonly referred to as the oyster mushroom, is a widely consumed edible mushroom that grows in clusters on dead or dying trees. In addition to its culinary uses, research has found that the mushroom may also have potential medicinal properties. The current study investigated the potential use of a fungal extract from *P. ostreatus* as a natural acaricide against *Rhipicephalus microplus*, a major vector of economically significant infections and one of the most significant bovine ectoparasites. The study used the adult immersion test (AIT) and the larvae packet test (LPT) to evaluate the effectiveness of the extract against ticks. To evaluate the reproductive effect of the fungal extract on the reproduction of *R. microplus* engorged females, the eggs were tested for weight and egg-laying index. The results of the study showed that *P. ostreatus* extract had a significant acaricidal effect, with a 40 mg/mL concentration causing 89 ± 2.64% mortality in *R. microplus* larvae compared to the 91.00 ± 3.60% mortality by the positive control at 48 h interval. Whereas in the AIT, the extract inhibited 39.86 ± 4.13% of oviposition. The study also used *in silico* approach to further examine the binding mechanisms of the compounds in the fungal extract to the target protein *R. microplus* Triosephosphate isomerase RmTIM, using molecular docking in AutoDock Vina software. Docking scores of −8.3, −7.7 and −6.9 kcal/mol, respectively, showed that rutin, naringin and myricitin had significant interactions with the active site residues of the target protein. Our results suggest that *P. ostreatus* extract may be a potential alternative to traditional acaricides for controlling *R. microplus* in livestock.

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1. Introduction

The tick species *Rhipicephalus microplus* is a global parasite that affects cattle, causing extensive losses related to animal mortality, reproductive disorders, and transmission of various diseases such as babesiosis and anaplasmosis, which leads to bovine parasite sadness [1,2]. Ticks and tick-borne illnesses are among the most significant causal diseases for cattle and animal productivity, with an estimated global annual economic loss of around seven billion dollars [3]. This species of ticks is a major concern for the animal breeding sector and causes a significant financial burden globally.

For decades, mankind has employed various synthetic means like repellents, insecticides and acaricides to control insects and arachnids of medicinal and veterinary importance. However, the excessive use of these control agents has resulted in numerous problems such as resistance, environmental pollution, and negative impacts on non-target species, including human beings (Nazeer et al., 2021). In light of these limitations, researchers have been actively seeking alternative control mechanisms. One such alternative is the utilization of natural pest management techniques based on medicinal herbs, as it provides a safe and effective way to avoid vector bites and infections. The practice of utilizing medicinal plants to combat parasitism has been in existence for many years and is still ongoing. The anti-pest effectiveness of several plant extracts has been thoroughly studied and documented [4–6].

The extensive research conducted, has conclusively established the antimicrobial, insecticide, and acaricidal properties of essential oils [7–9]. Subsequent studies [10–13] have further affirmed the impact of herbal remedies on ticks, such as decreased larval mortality, weight and egg-laying and decreased fecundity and egg viability decreased.

For ages, mushrooms have been revered by various cultures for their culinary enhancements and health benefits [14]. These fungi possess a vast array of valuable bioactive compounds giving the fungi various therapeutic properties [15]. One of the most popular and cultivated mushrooms is *Pleurotus ostreatus*, also known as the oyster mushroom. This species is bountiful in trace elements that are vital for human health and is a treasure trove of macromolecules including “alpha-glucans, beta-glucans, lentinan, lipopolysaccharides, resveratrol, Cibacron blue affinity purified protein, concanavalin A, etc. Its bioactive molecules possess various therapeutic properties such as hypocholesterolemia, free radical scavenging activity, and anti-atherogenic, anti-tumor, immunomodulatory, and antibacterial effects [15].

Triosephosphate isomerase (TIM) holds a crucial role in both the glycolytic and gluconeogenesis pathways by catalyzing the conversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. It plays a vital role in energy metabolism within ticks, and research has explored its potential as a therapeutic research target for several endoparasites, such as in the study by Ref. [16]. The TIM protein has been identified as a component of tick saliva and is thought to contribute to the tick’s successful blood-feeding process. The potential of TIM as a drug candidate arises from its critical role in glycolysis. By targeting TIM, it may be possible to disrupt the energy metabolism of ticks, rendering them unable to feed efficiently and survive. Inhibition of TIM activity can impair the tick’s ability to process glucose, leading to a depletion of energy reserves and ultimately causing their demise. Multiple scientific papers have documented the potential of TIM as a drug candidate for combating several parasitic organisms responsible for human diseases. These include *Plasmodium falciparum, Trypanosoma cruzi, Trypanosoma brucei, Fasciola hepatica,* and *Giardia lamblia* [17–19]. Additionally [20], were the first to investigate TIM’s potential as a target for ectoparasites, discovering that certain cysteine residues in *R. microplus* TIM (RmTIM) could be potential targets for designing a drug. Despite the structural similarity of TIM across different species, selective inhibitors may still be developed by targeting the poorly conserved interface of the enzyme [21].

In this research, we delved into the examination of the acaricidal properties of *P. ostreatus* both in the laboratory and through virtual simulation. Through the screening and selection of phytochemicals, the acaricidal effectiveness of the extract was determined. To further establish the anti-tick capabilities of *P. ostreatus*, advanced computational techniques such as molecular docking and MD simulation were utilized, specifically focusing on the docking of the plant’s phytochemical compounds against the Triosisomerase phosphate protein.

Table 1
The average % larval mortality and oviposition inhibition at different concentrations of *P. ostreatus* fungal extract, evaluated *in vitro* against *R. microplus* in LPT and AIT, respectively.

Extract	Concentration	Mean ± S.D.		
		Larval Mortality[a]		% IO*
		24 h	48 h	
Pleurotus ostreatus	40	45.33 ± 2.51^{a}	89 ± 2.64^{a}	39.86 ± 4.13^{b}
	20	38.66 ± 2.08^{b}	80.333 ± 1.52^{ab}	32.64 ± 2.58^{bc}
	10	30.66 ± 1.52^{c}	71.333 ± 5.50^{b}	18.64 ± 8.78^{cd}
	5	26.33 ± 1.52^{c}	56.667 ± 8.50^{c}	14.13 ± 8.07^{de}
	2.5	16.66 ± 2.08^{d}	36.667 ± 2.08^{d}	7.30 ± 4.71^{de}
Control Group	Deltamethrin	46.66 ± 3.51^{a}	91 ± 3.60^{a}	83.15 ± 4.10^{a}
	Distilled water	0^{e}	1 ± 1^{e}	0.08 ± 2.54^{e}

[a] Means not sharing any letters in the same column were significantly different by Tukey’s HSD test at a 5% significance level ($p < 0.05$). S.D.: standard deviation.

2. Results

2.1. Adult immersion test

The *in vitro* effectiveness of *P. ostreatus* methanolic fungal extract against *R. microplus* is presented in Table 1, showcasing the observed percentages. As depicted in Table 1 and Figure 1D, F, the extract's % IO rose as the extract concentration elevated (from 7.30 ± 4.71 d % IO at 2.5 mg/mL to 39.86 ± 4.13% IO at 40 mg/mL). In summary, by utilizing higher concentrations (≥10 mg/mL) and prolonging the treatment time (48 h) (as shown in Fig. 1F), the fungal extract demonstrated the ability to significantly augment larval mortality and potentially impede the egg-laying activity of *R. microplus*.

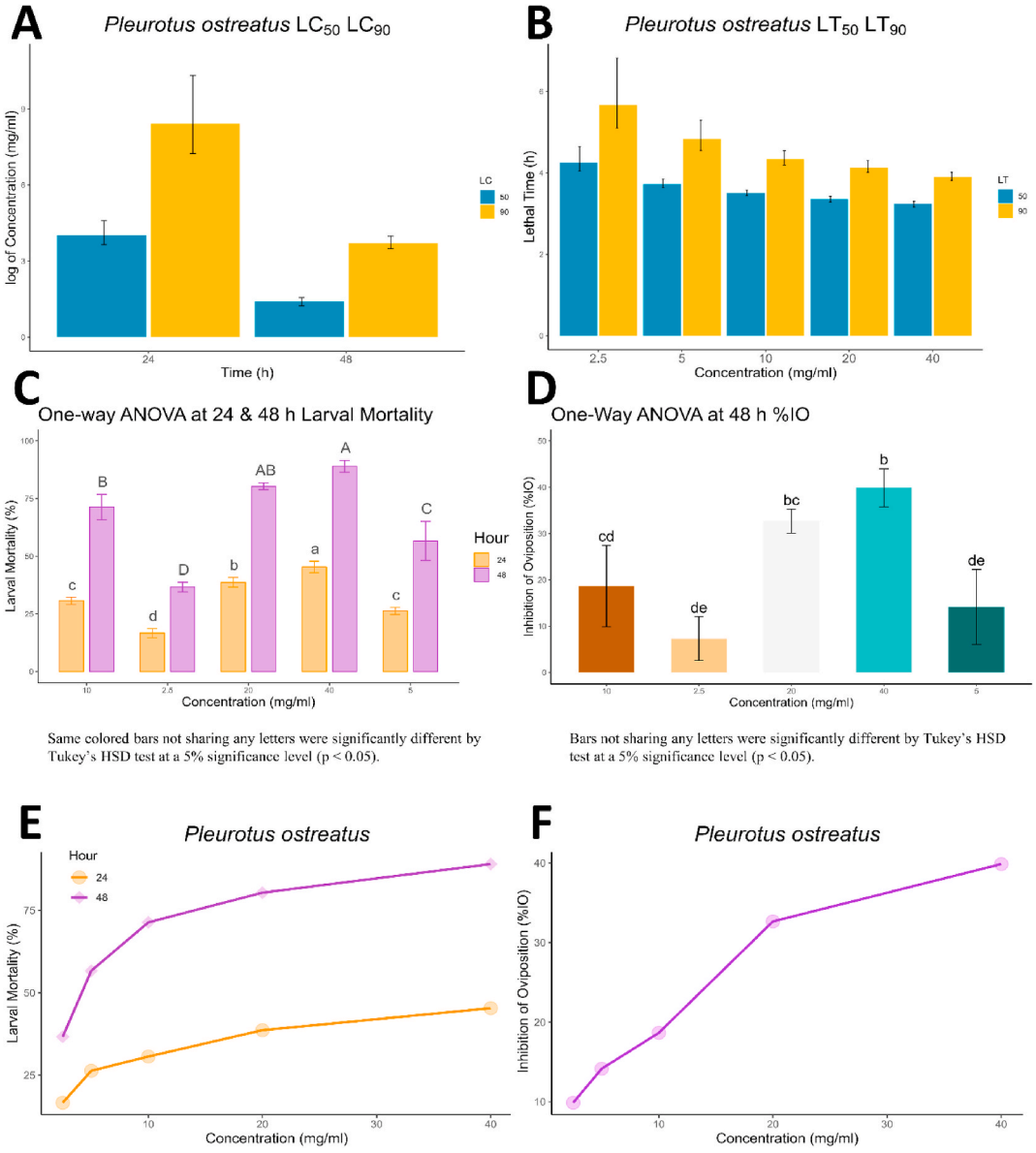

Fig. 1. (A, B) Represent log-transformed LC_{50}, LC_{90}, LT_{50}, and LT_{90} with error bars showing the corresponding log-transformed lower and upper confidence limits values of the LC and LT in LPT; **(C, D)** represent one-way ANOVA with post hoc Tukey HSD test for larval mortality and % IO, for LPT and AIT, respectively; **(E)** concentration–mortality curve for larval mortality at 24 h and 48 h in LPT; **(F)** Response curve graph for the inhibition of oviposition (% IO in AIT).

2.2. Larval packet test

The *P. ostreatus* fungal extract revealed remarkable larvicidal activity, with over 50% larval mortality at concentrations ≥5 mg/mL after 48 h. This is on par with the mortality caused by the positive control, deltamethrin. The extract's LC_{50} value at 48 h of treatment is 4.08 (3.44–4.72) mg/mL, while its LC_{90} value is 40.75 (32.65–53.89) mg/mL (Table 2, Fig. 1A). Additionally, its LT_{50} value is 25.49 h (23.65–27.14) and its LT_{90} value is 49.38 h (45.18–55.60) at 40 mg/mL concentration (Table 3 and Fig. 1B). To further compare the mortality between different concentrations, a "post-hoc Tukey's HSD" test was used and the results are illustrated in Fig. 1C. Moreover, Fig. 1E the concentration response curve graph for the larval mortality in LPT at different concentrations.

2.3. Protein identification and preparation

Creating an accurate representation of a protein's tertiary structure is vital in computational biology, as it enables conversion of macromolecular structures into forms that are better suited for docking approaches. To accomplish this, the chosen protein molecules were first meticulously prepared and optimized using the "protein preparation wizard" from "BIOVIA Discovery Studio Visualizer". Additionally, the 3D structural conformation of the protein Triosephosphate isomerase was obtained from the protein data bank (PDB ID: 3TH6), which had undergone experimental validation and were determined using X-ray diffraction with resolutions of 2.40 Å. The PDB structure of the selected protein was subsequently fine-tuned by following these steps.

1. Elimination of "water, bound complex molecules, and all heteroatoms",
2. Addition of "polar hydrogen bonds" and "non-polar H atoms" were merged,
3. "Gasteiger–Marsili" charges were assigned.

Once the protein structure was prepared, it was ready to be used for further analysis.

2.4. Binding sites prediction

To evaluate catalytic residues, a computational tool such as CASTp [22] was employed. This server utilizes "weighted Delaunay triangulation" and the "alpha complex" to calculate shape measurements. It offers the capability to identify and measure surface accessible pockets as well as interior inaccessible cavities in proteins and other molecules. The tool analytically calculates the area and volume of each pocket and cavity on both the solvent accessible surface ("SA, Richard's surface") and molecular surface ("MS, Connolly's surface"). From the perspective of the CASTp server, it was observed that catalytic site residues including ASN11, LYS13, MET14, PRO44, GLN64, ASN65, HIS95, GLU97, ARG98, VAL101 and PHE102 were present in the first predicted site of volume 160 A^3 (Figuer 2).

2.5. Analysis of molecular docking and post docking investigation

The AutoDock Vina software was utilized to prepare all the ligands and proteins. The process of molecular docking for the chosen ligand molecules onto the binding site (pocket) of the protein was performed using the same software. For docking, AutoDock Vina was employed, utilizing the protein and ligand information along with the properties of the grid box specified in the configuration file. ADT performed the assignment of polar hydrogens and united atom Kollman charges, as well as solvation parameters and fragmental volumes to the protein. The prepared file was saved in PDBQT format by AutoDock. AutoGrid was utilized to generate a grid map, where a grid box with dimensions of 60 × 60 × 60 xyz points and a grid spacing of 0.375 Å was employed. The center of the grid was defined as (−13.82, −3.62, 1.34) in the x, y, and z dimensions, respectively. The docking procedure assumed both the protein and ligands to be rigid during the simulation. Following docking, the results with a positional root-mean-square deviation (RMSD) of less than 1.0 Å were clustered together, and the representation with the most favorable free energy of binding was chosen.

Out of the selected 22 ligands, the compounds rutin, naringin and myricitin displayed the greatest binding affinity with docking scores of −8.3 kcal/mol, −7.7 kcal/mol and −6.9 kcal/mol, respectively. To investigates the interactions between the protein and ligand post-docking, the "BIOVIA Discovery Studio Visualizer version 21.1.0.20298" software was employed. After the completion of the docking process, the output files were visualized using the Receptor-ligand interaction segment of the "Discovery Studio" software, and the interactions were analyzed. In the case of the protein RmTIM, rutin (Fig. 3A) was found to produce three hydrogen bonds - two

Table 2
Medium lethal concentration causing 50% and 90% mortalities (LC_{50} and LC_{90} values) of *P. ostreatus* fungal extract at 24 and 48 h against *R. microplus in vitro*.

Time (h)	LC_{50} (mg/ml)	Confidence Limits (95%)		LC_{90} (mg/ml)	Confidence Limits (95%)		Chi Square	Slope ± S.E.	Intercept ±S.E.	*p*-value
		LCL	UCL		LCL	UCL				
24	55.73	38.28	98.66	4528.99	1394.86	30432.14	3.36	0.67 ± 0.08	−1.17 ± 0.09	0.99
48	4.08	3.44	4.72	40.75	32.65	53.89	13.33	1.28 ± 0.08	−0.78 ± −.08	0.43

LC: Lethal concentration, S.E.: standard error, LCL: lower confidence limit, UCL: Upper confidence limit.

Table 3
Medium lethal time causing 50% and 90% mortalities (LT_{50} and LT_{90} values) of *P. ostreatus* fungal extract at different concentrations against *R. microplus in vitro*.

Concentration (mg/ml)	LT_{50} (h)	95% Confidence Limits		LT_{90} (h)	95% Confidence Limits		Chi Square	Slope ± S.E.	Intercept ±S.E.	*p*-value
		LCL	UCL		LCL	UCL				
2.5	69.96	56.98	104.21	288.69	165.42	919.72	0.99	2.08 ± 0.37	−3.84 ± 0.58	0.91
5	41.50	37.82	46.74	125.82	95.28	200.46	6.13	2.66 ± 0.35	−4.30 ± 0.54	0.19
10	33.31	31.04	35.70	76.49	65.91	94.87	3.18	3.54 ± 0.35	−5.40 ± 0.55	0.53
20	28.58	26.48	30.55	62.24	55.21	73.65	0.66	3.79 ± 0.36	−5.52 ± 0.55	0.96
40	25.49	23.65	27.14	49.38	45.18	55.60	1.94	4.46 ± 0.40	−6.27 ± 0.59	0.75

LT: Lethal time, S.E.: standard error, LCL: lower confidence limit, UCL: Upper confidence limit.

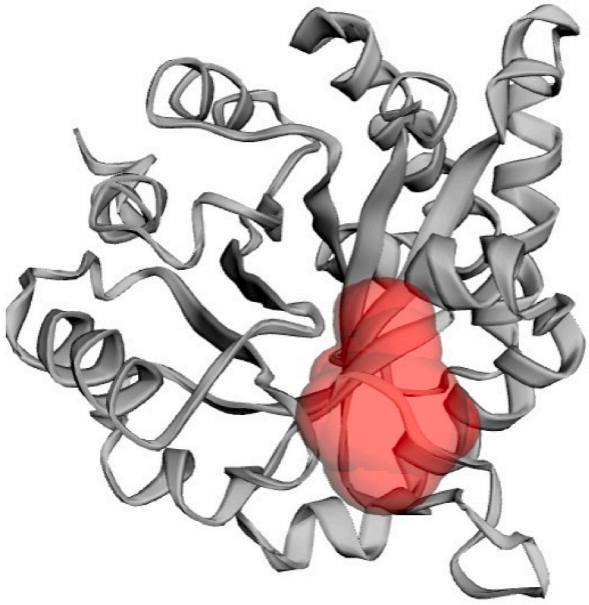

Fig. 2. Active sites determinations of the RmTIM using CASTp server.

with the amino acid ARG98 and one with the amino acid ASN11 - as well as hydrophobic bonds, specifically Pi-cation, Pi-alkyl, and Pi-suphur bonds with the amino acids LYS13, MET14, PHE102 and ARG98. On the other hand, naringin (Fig. 3B) formed three hydrogen bonds with the amino acids ARG98, GLN64, and ASN65 and hydrophobic bonds, including Pi-alkyl, alkyl, and Pi-sigma bonds, with the amino acids MET14, PRO46, ILE78 and PRO44. Lastly, myricitin also exhibited H-bonds and hydrophobic bonds, involving the amino acids GLU77, ASN65 and ARG 98 (Fig. 3C).

2.6. Analysis of molecular dynamic simulation

The MD simulation has been demonstrated to be a useful tool for examining internal and exterior motions, as well as conformational changes in relation to the average positions of all atoms. Thus, a simulation study was performed in order to capture the time-dependent motion of the selected ligand in the binding pocket of the protein. The toolset for analyzing molecular dynamics simulations includes the root mean square deviation (RMSD) and the root mean square fluctuation (RMSF). In general, root mean square divergence (RMSD) conveys information on overall protein complex stability by calculating divergence from the initial structure. The ligand-free state of the target protein (apo system) fluctuated up to 50ns, then showed a stable configuration up to 70ns, while a higher departure was found in the backbone, as shown in Fig. 4A. The system was significantly stable with varying deviation in the case of complicated, as shown in Fig. 4B. Initially, the compound fluctuated between 20 and 50ns, and subsequently between 60 and 80ns, but towards the conclusion of the MD, the RMSD had become relatively stable.

The RMSF represented the average displacement of each residue following ligand binding. To assess the dynamics of residue side chains, the RMSF of the protein residues was determined as a function of time for both the apo system and the complex, as shown in Fig. 5(A and B respectively).

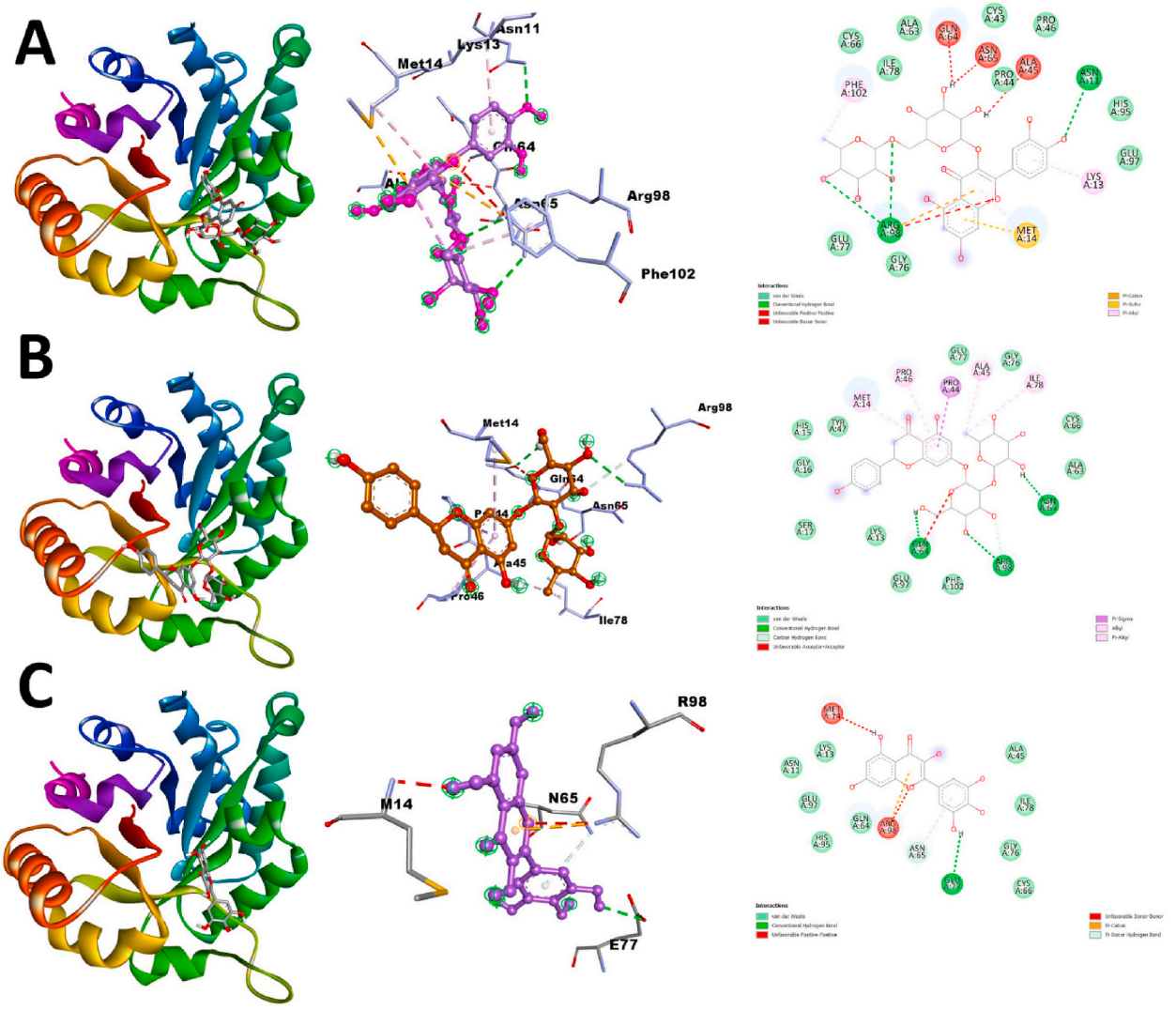

Fig. 3. Complex three-dimensional interaction of **(A)** rutin; **(B)** naringin and **(C)** myricitin with the RmTIM and its corresponding 2D structures.

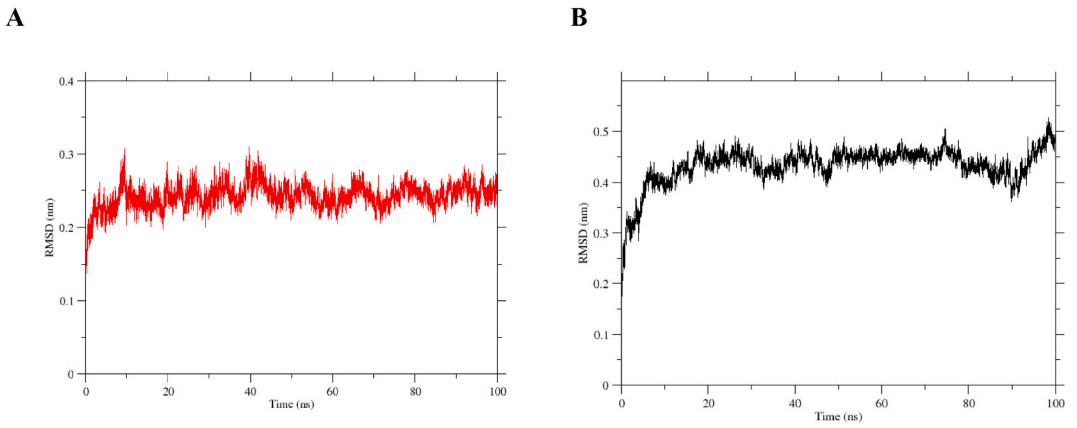

Fig. 4. The RMSD calculated over time (A) Apo protein (B) ligand-based complexes.

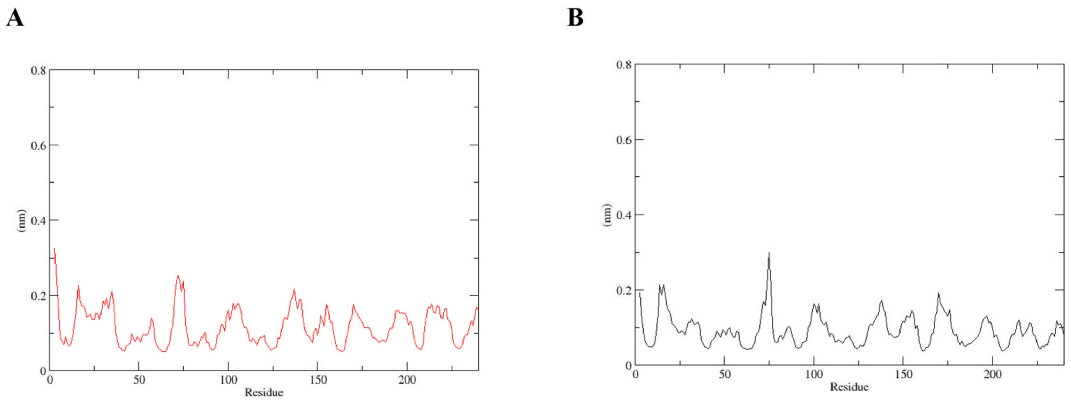

Fig. 5. The RMSF graph (A) Apo protein (B) ligand-based complexes.

2.7. Analysis of molecular mechanics-Poisson Boltzmann surface area (MMPBSA)

The study of molecular mechanics Because of its less demanding nature in terms of computer labour and higher accuracy, Poisson-Boltzmann surface area (MM/PBSA) is likely the most preferred approach for performing such evaluations. To get insight into the forces involved in the of binding protein ligand, MM(GB/PB) SA free-energy was studied. To measure the complex's binding ability, 10 ns of MD trajectory was taken from a conformation of 100 ns? The compound's calculated binding free energy was found to be −115.974 19.62 KCal/mol. The observations revealed that energies of polar solvation and van der Waals interactions were crucial for the burial of the inhibitor's hydrophobic groups, laying the groundwork for favorable binding (Table 4).

3. Discussion

Rhipicephalus microplus is highly prevalent and causes significant economic damage by infesting beef and dairy cattle. To address this problem, various methods have been proposed, such as the use of phyto-acaricides as a viable solution [23]. Plants contain a variety of natural compounds that can disrupt the biological processes of insects and interrupt their life cycle, and are commonly used in ethno-veterinary practices [24,25]. Compared to synthetic acaricides, botanicals are typically less toxic to mammals, do not leave residual effects, and have a lower likelihood of developing resistant tick populations [26]. As far as we know, there is no existing literature on the effect of the fungal extract of *P. ostreatus* against *R. microplus*. The White oyster mushrooms chosen for this study were selected based on reported activities and contain various secondary metabolites with pharmacological effects [27,28]. Among natural products, the extracts demonstrated notable activity against larvae and adults of *R. microplus* ticks.

Pleurotus ostreatus is a highly consumed and cultivated mushroom worldwide, known for its rich content of trace elements and macromolecules essential to human health. To date, research on the acaricidal effects of *P. ostreatus* has been limited. However, studies have shown that it possesses a variety of therapeutic properties, such as reducing cholesterol levels, scavenging free radicals, and possessing "antioxidant, anti-atherogenic, anti-tumor, immunomodulatory, and antibacterial" properties [15]. The current study employed both *in vitro* and *in silico* methods, offering a unique opportunity to explore the acaricidal effects through a multidisciplinary approach. The results of this study demonstrate the potential use of *P. ostreatus* extracts as acaricidal agents against *R. microplus*. At a concentration of "40 mg/mL", the methanolic extract of *P. ostreatus* resulted in a 39.86 ± 4.13 inhibition of oviposition in adult *R. microplus* and a mean mortality of 45.33 ± 2.51 at 24 and 89 ± 2.64 at 48 h in larvae. In a similar study by da Costa, Martins [29] the hydroethanolic extract of *Ximenia americana* bark demonstrated a comparable effect against *R. microplus.* At concentrations of 20 and 40 mg/mL, the extract resulted in 80% and 100% mortalities, respectively. This mortality rate was similar to the 100% mortality observed in the control group (cypermethrin) at higher concentrations. Similarly, Nasreen, Niaz [30], reported that in-silico molecular docking analysis identified 27 compounds from *Allium sativum* and *Cannabis sativa* with inhibitory activity against the *R. microplus* acetylcholinesterase enzyme. This provides insight into the potential of botanical extracts in addressing the problem of acaricide resistance in tick populations.

During the crucial embryogenesis stage of cattle tick development, the maintenance of energy metabolism is vital. Studies have

Table 4
Binding free energies.

Energy Component	Energy (KJ/mol)
Van der waal energy	– 141.379 ± 20.828
Electro static energy	– 298.98 ± 19.62
Polar Solvation energy	343.740 ± 25.88
SASA	– 19.42 ± 1.367
Binding Energy	– 115.974 ± 19.62

shown that marker enzymes of metabolic pathways, such as "hexokinase and pyruvate kinase", exhibit high activity levels prior to the formation of the cellular blastoderm [31]. Conversely, enzymes responsible for gluconeogenesis display increased activity later on and play a key role in the accumulation of glycogen as an energy source, until the eclosion of the embryos [31]. TIM is a noteworthy enzyme involved in both processes and may serve a diverse range of metabolic functions [16]. Given these findings, the molecules rutin, naringin, and myricitin (with docking scores of −8.3 kcal/mol, −7.7 kcal/mol, and −6.9 kcal/mol respectively) are considered promising lead compounds in the search for effective drugs to combat these parasites. Moreover, the compound rutin has been reported [32] in *Berberim lyceum* plant leaves extract to have a promising inhibitory potential against ticks with a docking score of −8.7013 against the *R. microplus* acetylcholinesterase (RmAChE1) protein. These compounds may have the potential to be developed as new acaricides.

Fungi (*P. ostreatus)* have never been interpreted in earlier reported research by integrated approach. This is the first study against ticks using novel computational approaches. The phytochemicals were chosen based on thorough literature research and their reported presence in *P. ostreatus* alcoholic extract prepared in a similar way by Mishra, Tomar [33], Mohamed and Farghaly [34] which increases the possibility of the reported compounds in our extract, but there is still a need to characterize the fungal extract for its phytochemicals to know the exact values of the chemicals present in this fungus. This information is providing new insight for researchers to explore compounds (rutin, naringin, and myricitin) on the *R. microplus* tick. These compounds may have the potential to be developed as new acaricides. In future studies it will be necessary to study the in-situ effect produced by the rutin, naringin, and myricitin molecules on the R. microplus tick, by searching for damage through electron microscopy as well as characterizing the fungal extract for its phytochemicals through HPLC and or GCMS to offer a more solid platform for docking tests. Based on the results obtained, it can be inferred that the extracts of *P. ostreatus* exhibit exceptional acaricidal properties in laboratory conditions, targeting one or more life stages of ticks. However, to fully understand the impact of this extract on living organisms, further *in vivo* and filed research must be conducted involving this mushroom species. These studies will aid in identifying specific factors within crude fungal extracts that boost the acaricidal property. The mushroom species' anti-ovipositional and acaricidal properties could prove to be a crucial element in the creation of sustainable strategies for managing tick populations in livestock production.

4. Conclusion

This study revealed a good ability of *P. ostratus* extract to counteract tick's larvae, as well as its ability to target various stages of *R. microplus*. Through screening of 22 compounds, three were identified as being particularly effective and specific inhibitors of RmTIM. Of these, the rutin compound demonstrated outstanding inhibitory activity against the RmTIM protein, with a docking score of −8.3 kcal/mol. These findings suggest that the rutin compound could be a valuable acaricide, as well as a novel structure for the development of new acaricides with a distinctive mode of action.

5. Materials and methods

5.1. Preparation of fungal extract

After scouring the lush landscapes of District Mardan (latitude: 34.1986° N, longitude: 72.0404° E) in Khyber Pakhtunkhwa, Pakistan, the mushroom materials of *Pleurotus ostreatus* were meticulously harvested, cleaned, and subsequently deposited at the herbarium of the "Department of Botany, Abdul Wali Khan University, Mardan", where they were given the distinguished accession number of awkum.bot-001. To extract the crude methanolic essence, the maceration method as outlined by Ref. [35] was employed. The mushroom matter was then delicately dried under natural shade at room temperature. The dried mushroom material was then finely ground to a powder using a plant grinder (YUEYUEHONG Model: HC-3000 A Zhejiang, China). Subsequently, the pulverized fungus was soaked in 80% concentrated methanol at a ratio of 1:10 (w/v) to create an extract, which was agitated using an orbital shaking incubator (labForce Model 1165U07, Thomas Scientific, Swedesboro, New Jersey, United States) at 200RPM. The solution was then filtered three times using filter paper and concentrated by evaporating it in a rotary evaporator (BUCHI Rotavapor, Model: R-300 Flawil, Switzerland) at 40 °C in a vacuum to create a stock solution. From this, further dilutions were made in methanol to concentrations of 2.5, 5, 10, 20, and 40 mg/mL (w/v) which were subsequently used for the bioassay against ticks. Deltamethrin (1.25%) was used as positive control as described by Singh, Jyoti [36] whereas, distilled water served as negative control.

5.2. Collection of R. microplus ticks

Ticks were manually collected as recommended by the World Association for the Advancement of Veterinary Parasitology guidelines [37]. With care, the team carefully collected fully engorged *R. microplus* ticks from the bodies of cattle and buffaloes, in 10 farms located in various locations across Mardan (coordinates: 34.1986° N, 72.0404° E) Khyber Pakhtunkhwa, Pakistan. These female ticks were then meticulously cleaned in tap water and dried on absorbent paper. The ticks were then morphologically identified as *R. microplus* using standard ticks identification keys under a microscope [38]. A total of 300 adult engorged female ticks were utilized for this study. A selection of these engorged female ticks was housed in a labeled bottles at 28 ± 1 °C and 85 ± 5% relative humidity, with muslin cloth covering the bottle for eggs hatching. The eggs were allowed to hatch and develop into larvae within 18–25 days under similar incubation conditions. These larvae were then utilized for the larval packet test (LPT). The remaining ticks were divided into seven groups (five treatments and two controls) with 10 ticks in each group per replicate. These groups of ticks were used to measure the acaricidal effects of the respective concentrations of fungal extract through the adult immersion test.

5.3. Adult immersion test (AIT)

The experiment was conducted using the investigational procedure [39] and later modified by the [40]. Five test tubes, each containing different concentrations of crude extract (40, 20, 10, 5, and 2.5 mg/mL), were prepared in triplicate. The ticks in each group were submerged in the extract concentration for a duration of 5 min. Afterwards, the ticks were removed from the extract solutions and delicately dried on filter paper before being placed in individual Petri dishes filled with filter paper at the bottom. These Petri dishes were covered with sterile gauze and placed in an incubator maintained at a temperature of 28 ± 1 °C and a relative humidity of 85 ± 5%. After a week of incubation, the ticks in each Petri dish were observed for egg laying and the eggs produced by each group were weighed. The triplicates were made on separate days employing a fresh extract concentration each time. The effectiveness of the crude extracts was then evaluated by calculating the percent inhibition of oviposition (% IO) using the following formula:

$$\%\ IO = \frac{\text{Egg laying Index (control)} - \text{Egg layingIndex (treated)}}{\text{Egg laying Index control}} \times 100\%$$

whereas "egg-laying index = mean weight of eggs laid ÷ mean weight of engorged females".

5.4. Larval packet test (LPT)

The LPT was implemented with a modification by Ref. [41]. Utilizing a brush, a group of approximately 100, ten-day-old larvae were meticulously placed at the center of 7 × 7 cm filter papers. Subsequently, 100 μL of the experimental solutions were carefully added to each packet, which were then sealed shut. As a benchmark, a control group was treated with Deltamethrin A total of three replicates were carried out for each concentration. The packets were carefully observed after 24 and 48 h to document the mortality rates. The larvae that were observed to have no movement were considered to have succumbed to the treatment.

5.5. Preparation of triosephosphate isomerase protein structure

After retrieving the three-dimensional crystal structure of Triosephosphate isomerase (TIM) from *R. microplus* from the "RSC Protein Data Bank" [PDB: 3TH6], all crystal water molecules were eliminated to ready the target protein structure for molecular docking procedures. In this process, both small molecules and water molecules were excised, resulting in the RmTIM protein being saved as a distinct pdb file for regulated docking.

5.6. Ligand selection and preparation

The fungal phytochemicals were chosen after conducting a comprehensive review of the existing literature. Specifically, articles were selected that investigated the phytochemical composition of *Pleurotus ostreatus* using GC-MS and HPLC analysis methods [33,34]. The chemical composition of small molecules was extracted from the PubChem databases as described by Ref. [42]. Utilizing ChemDraw software, a selection of 3D structures was carefully crafted. Subsequently, the ligand molecules underwent a process of energy minimization through the utilization of PRODRG (a tool that employs Gromos 96 force field for energy minimization) [43]. Finally, AutoDock Tools were employed to add Gasteiger charges to the small molecules, with Torsdof being set accordingly.

5.7. Active site analysis

In order to identify potential catalytic sites, the Computed Atlas of Surface Topology of Proteins (CASTp) program as outlined by Ref. [22], was utilized to examine and investigate a variety of binding sites. After careful examination, the active site was ultimately chosen based on its impressive surface area and volume. Additionally, key residues that play a role in the active site were pinpointed and evaluated against the modeled proteins.

5.8. Molecular docking

AutoDock Vina [44] was chosen for its precision and speed, outpacing AutoDock 4 b y a factor of two orders of magnitude [45] when performing molecular docking. The input "pdbqt" file for Triosephosphate isomerase protein [PDB: 3TH6] was prepared and the grid box dimensions and center were set using AutoDock Tools. To enhance the three-dimensional structure of RmTIM, polar hydrogen atoms and "Kollman" charges were added. The docking process findings, expressed as binding affinity (kcal/mol) with a negative value, were accompanied by the top 10 docking models with binding affinity scores for each procedure. The data of binding affinities were stored in CSV format, while the docked models were in PDBQT format. To gain a deeper understanding of the ligand-protein complex interaction, the output docked model was examined and scrutinized using "BIOVIA Discovery Studio Visualizer version 21.1.0.20298" software, by utilizing the Receptor-ligand interaction segment which offered an in-depth look into 2D and 3D structures of the complex, complete with information on interacting bonds, bond categories, bonding distances, and more.

5.9. Molecular dynamic simulation

Using GROMOS96 force, the protein's topology was created, and the ligand's topology was created using the methods described by Contreras Esquivel and Voget [46], Sousa da Silva and Vranken [47], respectively. The developed systems (compound + protein) were solvated in a cubic box with periodic boundary conditions using the TIP3P water model, and the neutral state was then achieved by adding counterions. After that, energy clashes were eliminated by putting all of the systems through energy minimization. In the second phase, systems were first brought into equilibrium using the NVT ensemble (300 K), and then the NPT ensemble at 1 bar reference pressure. The systems were ultimately put through a production run lasting 100 ns while being kept at a consistent temperature and pressure. The temperature and pressure were managed, respectively, by the velocity-rescale thermostat and the Parrinello-Rahman barostat.

5.10. Molecular mechanics-Poisson Boltzmann surface area (MMPBSA)

To comprehend molecular recognition, precise knowledge of the binding affinities of protein ligand/cognate complexes is crucial. The binding affinities determined by docking scores are not always precise, and they are not good at differentiating between molecules with binding affinities that differ by an order of magnitude or more. As a result, the g_MMPBSA approach, which is compatible with GROMCS, was also used in the current experiment to calculate the Gibbs free energy Joseph and Georgios [48], Kumari, Kumar [49]. For each simulated system, 1000 frames from the last 10 ns of the MD trajectory were captured. Additionally, the specific energy elements that make up the overall binding free energy was also identified.

5.11. Statistical analysis

The data related to IO % and larval mortality were first recorded in excel sheets (MS Excel Version: 2211). "R version 4.2.2 and RStudio version (2022.12.0 + 353)" programming software were used for analysis relating to statistics [50,51]. Different packages were utilized in the in the R environment to perform One-way ANOVA and Tukey's Test for larval mortality and % IO for different concentrations at different time intervals [52]. The 50 and 90% mortality causing concentrations (LC_{50} and LC_{90} at 24 and 48 h) and time (LT_{50} and LT_{90} at different concentrations) for the methanolic extract were calculated based on probit analysis [53–55], using the ecotox R package where it calculates the lethal concentration and or time along with its confidence limits, chi square, intercept and its standard error (SE) and slope and its SE. The heterogeneity significance (p-value) was kept at 0.05, and the fiduciary confidence limit was set at 95%". The results from the analysis were graphed by the "ggplot2" package [56].

Ethical statement

Ticks were manually collected as recommended by the World Association for the Advancement of Veterinary Parasitology guidelines (Holdsworth et al., 2006). The study was approved by the ethical committee of Abdul Wali Khan University Mardan.

Author contribution statement

Bader S. Alotaibi; Nasreen; Sadaf Niaz: Conceived and designed the experiments. Nosheen Malak; Afshan Khan; Adil Khan: Performed the experiments. Bader S. Alotaibi; Afshan Khan; Nosheen Malak: Analyzed and interpreted the data. Imtiaz Ahmad; Roger I. Rodriguez-Vivas; Chien-Chin Chen: Contributed reagents, materials, analysis tools or data. Adil Khan; Nosheen Malak; Chien-Chin Chen: Wrote the paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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