

RESEARCH ARTICLE

Anthelmintic activity and mechanistic insights into *Piper betle* L. essential oil against adult *Ascaridia galli*: An integrated *in vitro*, ultrastructural, histopathological, and *in silico* study



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ABSTRACT

Background and Aim: *Ascaridia galli* is an economically important intestinal nematode of poultry, and reliance on conventional anthelmintics raises concerns regarding reduced drug sensitivity and residues. *Piper betle* L. essential oil (PBEO) contains diverse bioactive constituents, but its activity against avian nematodes remains unexplored. This study evaluated the *in vitro* anthelmintic activity of PBEO against adult *A. galli* and investigated associated structural damage, chemical composition, and potential molecular targets.

Materials and Methods: Adult *A. galli* were collected from naturally infected chickens slaughtered at a traditional poultry slaughterhouse in Boyolali Regency, Central Java, Indonesia. Worms were exposed to PBEO at 10, 1, 0.1, 0.01, and 0.001% (v/v), with concentration-matched vehicle controls. Mortality was assessed at 10–160 min and analyzed using a generalized linear mixed model, while the median lethal concentration (LC₅₀) was estimated by probit regression. Cuticular and internal tissue alterations were evaluated using scanning electron microscopy and hematoxylin and eosin histopathology, respectively. PBEO constituents were characterized by gas chromatography–mass spectrometry (GC–MS). Ten selected constituents were molecularly docked against aquaporin (AQP), glutamate-gated chloride channel, nicotinic acetylcholine receptor, and Na⁺/K⁺-ATPase.

Results: PBEO induced significant concentration- and time-dependent mortality ($p < 0.05$). The 10% concentration increased mortality from $26.67 \pm 37.23\%$ at 10 min to 100% at 160 min, whereas 1% PBEO produced $56.67 \pm 34.44\%$ mortality at 160 min. The estimated LC₅₀ was 0.71% (95% confidence interval 0.47–1.06%). Electron microscopy revealed lip abrasion, anterior cuticular distortion, irregular folding, and surface disintegration. Histopathology demonstrated uterine disorganization, egg deformation, cuticular disruption, and muscle fragmentation. GC–MS resolved 50 peaks; 49 were identified, with eugenol as the most abundant constituent (8.48%). Docking predicted multi-target interactions with binding affinities ranging from -7.2 to -4.4 kcal/mol; the strongest interactions involved 4-allylphenyl acetate and β -caryophyllene with AQP.

Conclusion: PBEO exhibits measurable *in vitro* anthelmintic activity against adult *A. galli*, accompanied by extensive cuticular, reproductive, and muscular damage. Its chemically diverse constituents show predicted multi-target interactions that may contribute to the observed activity. Bio-guided fractionation, target-based validation, and *in vivo* poultry studies are required to establish efficacy, safety, and translational potential.

Keywords: *Ascaridia galli*, anthelmintic activity, cuticular damage, essential oil, molecular docking, *Piper betle*, poultry nematode, scanning electron microscopy.

INTRODUCTION

Chicken meat is a major source of animal protein and is among the most widely consumed meats worldwide [1]. Intensified poultry production has brought increasing challenges in disease management, particularly parasitic infections that adversely affect health and productivity [2]. One important poultry parasite is *Ascaridia galli*, a

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Received: 04-06-2026, **Accepted:** 28-09-2026, **Published online:** 29-10-2026

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How to cite: Churin'in NM, Hamid PH, Nugroho HA, Ferdian PR, Wibowo S, Maulydia NB. Anthelmintic activity and mechanistic insights into *Piper betle* L. essential oil against adult *Ascaridia galli*: An integrated *in vitro*, ultrastructural, histopathological, and *in silico* study. Vet World, 2026, 19(10): 4888-4919.

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nematode of the family Ascaridiidae that inhabits the small intestine of chickens and other galliform birds [3]. *A. galli* has a direct life cycle in which birds ingest embryonated eggs from contaminated environments; the larvae subsequently develop within the intestinal mucosa before returning to the lumen and developing into adults [3, 4]. *A. galli* infection is associated with reduced weight gain, decreased egg production, intestinal obstruction, and increased susceptibility to secondary infections, resulting in substantial economic losses [5]. Control of *A. galli* relies largely on synthetic anthelmintics, including benzimidazoles, imidazothiazoles, and macrocyclic lactones. Although these compounds remain important for parasite control, repeated or inappropriate use may contribute to reduced drug sensitivity, treatment failure, and concerns regarding residues in animal-derived food products [6, 7]. These limitations have stimulated interest in plant-derived alternatives that could serve as complementary approaches to parasite control, particularly in tropical regions where medicinal plants are abundant and commonly used in ethnoveterinary practices.

Essential oils are of particular interest because of their chemically diverse composition and potential to exert biological effects through multiple mechanisms [8–10]. These oils contain complex mixtures of volatile monoterpenes, sesquiterpenes, and phenylpropanoids that may exert multisite activity by disrupting membranes, interfering with neuromuscular signaling, inducing oxidative stress, and damaging parasite cuticular structures [8, 9]. For example, *trans*-cinnamaldehyde from cinnamon (*Cinnamomum* spp.) essential oil targets several Cys-loop receptors, including levamisole-sensitive nicotinic acetylcholine receptors (nAChR), gamma-aminobutyric acid (GABA)-activated chloride channels, and glutamate-gated chloride channels, and has shown synergistic activity with conventional anthelmintics [8]. Star anise (*Illicium verum*) essential oil produced dose-dependent reductions in fecal egg counts and decreased egg hatchability and worm motility in *A. galli* [11]. Similarly, an ethanolic clove (*Syzygium aromaticum*) leaf extract produced concentration- and time-dependent mortality of adult *A. galli*, reaching 93.3% at 140 mg/mL after 9 h, accompanied by cuticular damage [12].

Piper betle L. is a tropical medicinal plant widely distributed throughout Southeast Asia. Its leaves contain phenylpropanoids and terpenoid derivatives, including eugenol, chavicol, and caryophyllene-related compounds, which have been associated with antimicrobial, insecticidal, and antiparasitic activities [10, 13, 14]. Previous research on *P. betle* essential oil (PBEO) has primarily focused on insecticidal and anticoccidial activities. Acetylcholinesterase inhibition has been demonstrated as a plausible neuromuscular mechanism underlying its insecticidal activity [13]. In addition, PBEO exerted oocysticidal activity against *Eimeria tenella*, ranging from 8.67% to 95.33% at concentrations of 0.04–40%, and reduced oocyst sporulation, with a half-maximal inhibitory concentration (IC₅₀) of 1.31% at 72 h. These effects were associated with oocyst wall disintegration and inhibition of sporogony, with eugenol and β -caryophyllene among the predominant constituents [10]. However, neither line of investigation involved helminths. Evidence for the activity of *P. betle* against nematodes is limited to a recent study in which a methanolic leaf extract combined with *Areca catechu* seed extract, containing hydroxychavicol (45.66%) and eugenol (13.66%), inhibited the motility of adult *Haemonchus* spp. within 15 min at 5% and caused cuticular fissuring and leakage of internal contents, as demonstrated by scanning electron microscopy (SEM) [15]. However, that investigation evaluated a solvent extract rather than an essential oil, used a combination of two plant species rather than a single preparation, and examined a ruminant trichostrongylid rather than an avian ascaridid.

Despite growing interest in plant-derived anthelmintics, important gaps remain in understanding PBEO activity against poultry nematodes. To our knowledge, PBEO has not been evaluated against nematodes, and no preparation of *P. betle* has been investigated against an avian nematode. Moreover, essential oils and other plant preparations previously evaluated against *A. galli* came from other plant species and were assessed using relatively few biological endpoints. Star anise (*I. verum*) essential oil reduced fecal egg counts, egg hatchability, and worm motility [11], whereas clove (*S. aromaticum*) leaf extract was assessed for mortality and cuticular alterations [12]. Although *trans*-cinnamaldehyde from cinnamon oil has been characterized mechanistically at the receptor level, including activity at levamisole-sensitive nAChR and GABA- and glutamate-gated chloride channels [8], these investigations were conducted in *Caenorhabditis elegans* rather than *A. galli*. Ultrastructural evidence of damage in *A. galli* has been reported following exposure to synthetic anthelmintics [16] and crude plant extracts [17–19]; however, these investigations did not integrate external ultrastructural changes with internal histopathological alterations, chemical characterization of the tested preparation, and target-level molecular predictions. Consequently, the relationship among adulticidal activity, structural damage, chemical constituents, and potential molecular targets of PBEO remains unresolved.

Therefore, this study aimed to comprehensively evaluate the *in vitro* anthelmintic activity of PBEO against

adult *A. galli* using an integrated experimental and computational approach. Specifically, the study assessed concentration- and time-dependent adult mortality and estimated the median lethal concentration (LC₅₀); characterized PBEO-induced alterations in the cuticular surface using SEM and in reproductive structures and somatic musculature using histopathology; determined the chemical composition of PBEO using gas chromatography-mass spectrometry (GC-MS); and investigated the predicted interactions of major PBEO constituents with selected nematode molecular targets. By integrating mortality kinetics, structural pathology, chemical profiling, and molecular docking within a single experimental framework, the study sought to provide a comprehensive characterization of PBEO activity against adult *A. galli* and establish a basis for subsequent experimental validation and development of plant-derived approaches for poultry nematode control.

MATERIALS AND METHODS

Ethical approval

Adult *A. galli* specimens used in this study were obtained exclusively from the intestines of chickens routinely slaughtered for human consumption at a poultry slaughterhouse. No chickens were purchased, housed, experimentally infected, handled, treated, or slaughtered specifically for this study, and no *in vivo* experimental procedures or interventions were performed on live animals. Therefore, the study was conducted using postmortem biological material obtained after routine commercial slaughter. The collection and use of these specimens complied with applicable Indonesian regulations, including Government Regulation of the Republic of Indonesia No. 95 of 2012 on Veterinary Public Health and Animal Welfare (Peraturan Pemerintah Republik Indonesia No. 95 Tahun 2012), and international ethical principles governing research involving postmortem specimens obtained from food-producing animals. Accordingly, formal approval from an institutional animal ethics committee was not required, because no animals were handled, treated, anesthetized, or sacrificed for the purpose of this study. Field access and sample-collection activities were authorized by the National Unity and Politics Agency (Badan Kesatuan Bangsa dan Politik, Kesbangpol), Central Java, Indonesia (permit no. 070/642/XII/5.5/2021).

Study period and location

Parasite collection was conducted weekly from July to August 2022 at a traditional poultry slaughterhouse at Ngebong Market, Boyolali Regency, Central Java, Indonesia (7°31'S, 110°36'E). The chickens from which the parasites were obtained originated from smallholder free-range farms in four surrounding villages: Ngemplak, Pulisen, Kebonagung, and Karanggeneng. GC-MS analysis was performed at the Advanced Characterization Laboratory, National Research and Innovation Agency (BRIN), Cibinong, Indonesia. SEM was performed at the Genomic Laboratory, BRIN, Cibinong, Indonesia, and histopathological examination was conducted at the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Study design

This experimental study evaluated the *in vitro* anthelmintic activity of PBEO against adult *A. galli* using a concentration- and time-dependent adult-worm immersion assay. Five PBEO concentrations (10, 1, 0.1, 0.01, and 0.001% [v/v]) and their corresponding vehicle controls were evaluated over a 160-min observation period. Mortality was assessed at predetermined intervals. Structural effects were investigated using SEM and histopathology, while the chemical composition of PBEO was characterized using GC-MS. In addition, ten selected PBEO constituents were subjected to *in silico* molecular docking against four selected nematode molecular targets to investigate their potential target interactions.

Parasite collection and acclimatization

Adult *A. galli* worms were collected weekly from the intestines of naturally infected chickens slaughtered at a traditional poultry slaughterhouse at Ngebong Market, Boyolali Regency, Central Java, Indonesia. The source chickens (*Gallus gallus domesticus*) were 4–8 months old and originated from smallholder free-range farms in Ngemplak, Pulisen, Kebonagung, and Karanggeneng villages. The birds had been raised under semi-intensive scavenging systems with access to outdoor ranging areas.

Immediately after collection, the worms were placed in 50-mL sterile conical tubes containing prewarmed phosphate-buffered saline (PBS; pH 7.4) at 37°C, transported to the laboratory within 2 h, and transferred to fresh 0.9% NaCl prewarmed to 37°C for acclimatization. Worms were sexed morphologically under a stereomicroscope. Males were identified by a ventrally curved posterior extremity, a prominent circular precloacal sucker, and a pair of equal spicules, whereas females were identified by a straight, bluntly rounded posterior end and a vulvar

opening near the mid-body. Worms of both sexes were included. Only macroscopically intact and actively motile worms were used, and all experimental treatments were initiated within 3 h of collection [20].

Essential oil and serial dilutions

For the present study, *P. betle* leaves were collected from the same area as in our previous study (Seyegan, Yogyakarta Province, Indonesia) [21], and the plant was identified at the Laboratory of Plant Systematics, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. PBEO was extracted by water-steam distillation using the same apparatus and procedure as described previously [21]. Briefly, the leaves were dried for 8 h in a drying cabinet at 50°C and steamed with boiling water (100°C) for 4–5 h, and the vapors were passed through a Clevenger-type apparatus. The essential oil was stored in sealed sterile glass containers until use. The GC–MS profile of this PBEO has been reported previously [10] and was re-examined against the original chromatographic and spectral records for the present study.

A stock solution was prepared by diluting absolute PBEO (100%) in dimethyl sulfoxide (DMSO; 99.5%; Merck KGaA, Darmstadt, Germany). Serial logarithmic dilutions were subsequently prepared to obtain PBEO concentrations of 10, 1, 0.1, 0.01, and 0.001% (v/v). Concentration-matched DMSO solutions in physiological saline (0.9% NaCl) without PBEO were used as vehicle (negative) controls. DMSO was used as a co-solvent to improve the solubilization and dispersion of the essential oil in the immersion medium.

The identity and purity of PBEO were verified in-house by GC-MS (Table 1). Fifty peaks were resolved, of which 49 were identified. The chemical profile was dominated by sesquiterpene hydrocarbons (32.5%), phenylpropanoids (25.0%), and monoterpenes (20.9%), consistent with *P. betle* leaf oil. No peaks attributable to common extraction or adulteration solvents, including ethanol, acetone, hexane, and ethyl acetate, were detected. The only chlorinated compound detected was dichloromethane, which was used for preparation of the injection sample, eluted at the solvent front, and was excluded from integration.

Table 1: GC–MS chemical profile of *Piper betle* L. essential oil showing RT, relative peak area, compound identity, molecular formula, molecular weight, chemical class, and canonical SMILES descriptor.

Peak no.	RT (min)	Area (%) ^c	Compound (common name) ^b	Identity (NIST library name)	Molecular formula	MW (g/mol)	Chemical class	Canonical SMILES
1	2.440	1.00	2,4-Dimethylheptane	2,4-Dimethylheptane	C ₉ H ₂₀	128.26	Branched alkane	CCCC(C)CC(C)C
2	3.510	0.35	α-Thujene	2-Methyl-5-(1-methylethyl)-bicyclo[3.1.0]hex-2-ene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	CC1=CCC2(C(C)C)CC12
3	3.620	2.25	α-Pinene	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	CC1=CCC2CC1C2(C)C
4	3.815	2.40	Camphene	2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	C=C1C2CCC(C2)C1(C)C
5	4.095	0.83	Sabinene	1-Isopropyl-4-methylenebicyclo[3.1.0]hexane	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	C=C1CCC2(C(C)C)CC12
6	4.165	0.64	β-Pinene	6,6-Dimethyl-2-methylene-bicyclo[3.1.1]heptane	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	C=C1CCC2CC1C2(C)C
7	4.250	0.75	β-Myrcene	7-Methyl-3-methylene-1,6-octadiene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	C=CC(=C)CCC=C(C)C
8	4.650	1.39	3-Carene	4,7,7-Trimethylbicyclo[4.1.0]hept-2-ene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	CC1=CCC2C(C1)C2(C)C
9	4.760	0.55	p-Cymene	1-Methyl-4-(1-methylethyl)-benzene	C ₁₀ H ₁₄	134.22	Aromatic monoterpene	Cc1ccc(C(C)C)cc1
10	4.845	1.92	β-Phellandrene	3-Methylene-6-(1-methylethyl)-cyclohexene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	C=C1C=CC(C(C)C)CC1
11	4.880	0.95	1,8-Cineole (eucalyptol)	Eucalyptol	C ₁₀ H ₁₈ O	154.25	Oxygenated monoterpene (ether)	CC12CCC(CC1)C(C)(C)O2
12	5.130	1.98	3,7-Dimethyldecane	3,7-Dimethyldecane	C ₁₂ H ₂₆	170.34	Branched alkane	CCCC(C)CCCC(C)CC
13	5.230	2.11	γ-Terpinene	γ-Terpinene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	CC1=CCC(C(C)C)=CC1
14	5.665	0.71	3-Carene (isomer)	4,7,7-Trimethylbicyclo[4.1.0]hept-2-ene	C ₁₀ H ₁₆	136.24	Monoterpene hydrocarbon	CC1=CCC2C(C1)C2(C)C
15	5.765	1.47	Linalool	Linalool	C ₁₀ H ₁₈ O	154.25	Oxygenated	C=CC(C)(O)CCC=C(C)C

Peak no.	RT (min)	Area (%) ^c	Compound (common name) ^b	Identity (NIST library name)	Molecular formula	MW (g/mol)	Chemical class	Canonical SMILES
16	7.005	3.44	Terpinen-4-ol	(-)-Terpinen-4-ol	C ₁₀ H ₁₈ O	154.25	monoterpene (alcohol)	CC1=CCC(O)(C(C)C)CC1
17	7.185	1.09	α-Terpineol	L-α-Terpineol	C ₁₀ H ₁₈ O	154.25	Oxygenated monoterpene (alcohol)	CC1=CCC(C(C)(C)O)CC1
18	7.285	0.79	Estragole (methylchavicol)	Estragole	C ₁₀ H ₁₂ O	148.21	Phenylpropanoid	C=CCc1ccc(OC)cc1
19	8.045	3.63	Chavicol	Chavicol	C ₉ H ₁₀ O	134.18	Phenylpropanoid (phenol)	C=CCc1ccc(O)cc1
20	8.105	0.63	5-Methyltetradecane	5-Methyltetradecane	C ₁₅ H ₃₂	212.42	Branched alkane	CCCCCCCCC(C)CCCC
21	8.345	2.61	2,6,11-Trimethyldodecane	2,6,11-Trimethyldodecane	C ₁₅ H ₃₂	212.42	Branched alkane	CC(C)CCCC(C)CCCC(C)C
22	8.730	0.40	2-Isopropyl-5-methylheptan-1-ol	2-Isopropyl-5-methyl-1-heptanol	C ₁₁ H ₂₄ O	172.31	Aliphatic alcohol	CCC(C)CCC(CO)C(C)C
23	8.995	1.11	4,6-Dimethyldodecane	4,6-Dimethyldodecane	C ₁₄ H ₃₀	198.39	Branched alkane	CCCCCCC(C)CC(C)CCC
24	9.405	5.67	Chavicol acetate	4-Allylphenyl acetate	C ₁₁ H ₁₂ O ₂	176.22	Phenylpropanoid ester	C=CCc1ccc(OC(C)=O)cc1
25	9.620	0.52	Eugenol	Eugenol	C ₁₀ H ₁₂ O ₂	164.20	Phenylpropanoid (phenol)	C=CCc1ccc(O)c(OC)c1
26	9.710	0.50	1-(1-Ethyl-2,3-dimethylcyclopent-2-en-1-yl)ethan-1-one	1-(1-Ethyl-2,3-dimethylcyclopent-2-enyl)-ethanone	C ₁₁ H ₁₈ O	166.26	Cyclic ketone	CCC1(C(C)=O)CCC(C)=C1C
27	9.835	7.96	Eugenol	Eugenol	C ₁₀ H ₁₂ O ₂	164.20	Phenylpropanoid (phenol)	C=CCc1ccc(O)c(OC)c1
28	9.975	1.43	α-Copaene	Copaene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	CC1=CCC2C3C(C(C)C)CCC2(C)C13
29	10.210	0.97	β-Elemene	1-Ethenyl-1-methyl-2,4-bis(1-methylethenyl)-cyclohexane, [1S-(1α,2β,4β)]-	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=CC1(C)CCC(C(=C)C)CC1C(=C)C
30	10.600	0.82	α-Bergamotene	2,6-Dimethyl-6-(4-methyl-3-pentenyl)bicyclo[3.1.1]hept-2-ene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	CC(C)=CCCC1(C)C2CC=C(C)C1C2
31	10.710	0.40	α-Santalene	1,7-Dimethyl-7-(4-methyl-3-pentenyl)-tricyclo[2.2.1.0 ^{2,6}]heptane, (-)-	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	CC(C)=CCCC1(C)C2CC3C(C2)C31C
32	10.815	5.21	β-Caryophyllene	4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=C1CCC=C(C)CCC2C1CC2(C)C
33	10.980	0.59	α-Bergamotene (isomer)	2,6-Dimethyl-6-(4-methyl-3-pentenyl)bicyclo[3.1.1]hept-2-ene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	CC(C)=CCCC1(C)C2CC=C(C)C1C2
34	11.285	0.66	(E)-β-Farnesene	7,11-Dimethyl-3-methylene-1,6,10-dodecatriene, (E)-	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=CC(=C)CCC=C(C)CCC=C(C)C
35	11.505	3.76	α-Humulene	Humulene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	CC1=CCCC(C)=CCC(C)(C)C=C C1
36	11.865	0.59	n-Heptadecane	n-Heptadecane	C ₁₇ H ₃₆	240.47	n-Alkane	CCCCCCCCCCCCCCCCC
37	11.940	4.97	γ-Murolene	γ-Murolene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=C1CCC(C(C)C)C2C=C(C)CC C12
38	12.070	3.54	n-Heneicosane	n-Heneicosane	C ₂₁ H ₄₄	296.58	n-Alkane	CCCCCCCCCCCCCCCCCCCC
39	12.250	6.16	β-Selinene	Decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-naphthalene, [4aR-(4α,7α,8αβ)]-	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=C(C)C1CCC2(C)CCCC(=C)C2C1
40	12.445	6.47	α-Selinene	α-Selinene	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=C(C)C1CCC2(C)CCC=C(C)C2C1
41	12.575	1.07	β-Bisabolene	1-Methyl-4-(5-methyl-1-methylene-4-hexenyl)-cyclohexene, (S)-	C ₁₅ H ₂₄	204.36	Sesquiterpene hydrocarbon	C=C(CCC=C(C)C)C1CC=C(C)C C1
42	13.015	5.47	Chavibetol acetate	3-Allyl-6-methoxyphenyl acetate	C ₁₂ H ₁₄ O ₃	206.24	Phenylpropanoid ester	C=CCc1ccc(OC)c(OC(C)=O)c1
43	13.145	2.01	Unidentified	Unidentified	—	— ^a	Not assigned	—

Peak no.	RT (min)	Area (%) ^c	Compound (common name) ^b	Identity (NIST library name)	Molecular formula	MW (g/mol)	Chemical class	Canonical SMILES
44	15.825	0.94	4-Allylcatechol diacetate	4-Allyl-1,2-diacetoxybenzene	C ₁₃ H ₁₄ O ₄	234.25	Phenylpropanoid ester	C=CCc1ccc(OC(C)=O)c(OC(C)=O)c1
45	17.180	2.08	<i>n</i> -Eicosane	Eicosane	C ₂₀ H ₄₂	282.56	<i>n</i> -Alkane	CCCCCCCCCCCCCCCCCCCC
46	18.185	0.62	<i>n</i> -Eicosane	Eicosane	C ₂₀ H ₄₂	282.56	<i>n</i> -Alkane	CCCCCCCCCCCCCCCCCCCC
47	19.690	0.59	<i>Tetradecanal</i>	<i>Tetradecanal</i>	C ₁₄ H ₂₈ O	212.38	Long-chain aldehyde	CCCCCCCCCCCCC=O
48	21.945	1.76	<i>n</i> -Eicosane	Eicosane	C ₂₀ H ₄₂	282.56	<i>n</i> -Alkane	CCCCCCCCCCCCCCCCCCCC
49	22.830	0.70	<i>n</i> -Eicosane	Eicosane	C ₂₀ H ₄₂	282.56	<i>n</i> -Alkane	CCCCCCCCCCCCCCCCCCCC
50	24.010	1.56	Octadecanal	Octadecanal	C ₁₈ H ₃₆ O	268.48	Long-chain aldehyde	CCCCCCCCCCCCCCCCC=O

RT = Retention time, MW = Molecular weight, GC-MS = Gas chromatography-mass spectrometry, NIST = National Institute of Standards and Technology, SMILES = Simplified molecular-input line-entry system, PBE0 = *Piper betle* L. essential oil. Relative peak area represents the uncorrected total-ion-current response. Constituents were identified by comparison of their electron-impact mass spectra with the NIST-20 mass spectral library. Molecular formulae and average MWs were derived from the assigned structures and computationally verified using RDKit version 2025.03 (<https://www.rdkit.org>). Canonical SMILES are provided as machine-readable structural descriptors; corresponding two-dimensional structures of all identified constituents are presented in Supplementary Figure S1. Stereochemical descriptors were not encoded in the SMILES because electron-impact mass spectral matching does not establish absolute configuration; stereochemical designations returned by the library are retained in the Identity column.

^a Peak 43 remained unidentified because its electron-impact mass spectrum did not produce a library match above the predefined acceptance threshold. Therefore, no molecular formula, MW, chemical class, or structural descriptor was assigned, and this peak was excluded from molecular docking.

^b Several constituents were detected at more than one retention time and are presented as separate chromatographic peaks: eugenol (peaks 25 and 27; total area = 8.48%), carene isomers (peaks 8 and 14), α -bergamotene-related peaks (peaks 30 and 33), and *n*-eicosane (peaks 45, 46, 48, and 49). Eugenol was the most abundant identified constituent when the two corresponding peaks were combined; because eugenol and its positional isomer chavibetol yield nearly identical electron-impact mass spectra, the assignment of peaks 25 and 27 does not exclude chavibetol. Assignments of the long-chain *n*-alkanes (peaks 36, 38, 45, 46, 48, and 49) are tentative because their electron-impact mass spectra are poorly diagnostic.

^c Relative peak areas were calculated from the total integrated ion current; the 50 reported peaks sum to 100.02% owing to rounding.

GC-MS chemical profiling

GC-MS analysis was performed at the Advanced Characterization Laboratory, BRIN, Cibinong, Indonesia. PBE0 was dissolved in dichloromethane to obtain a concentration of 1000 $\mu\text{g/mL}$ and filtered through a 0.22- μm Minisart syringe filter (Sartorius AG, Göttingen, Germany). A 1.0- μL aliquot of the filtrate was injected in splitless mode, with the purge valve opened at 1.0 min, into a Shimadzu GCMS-QP2010 Ultra system (Shimadzu Corp., Kyoto, Japan) equipped with an Rtx-5MS capillary column (Restek Corporation, Bellefonte, PA, USA; 5% diphenyl/95% dimethylpolysiloxane; 30 m $\times$ 0.25 mm internal diameter $\times$ 0.25 μm film thickness). Ultra-high-purity helium (99.999%) was used as the carrier gas under constant-pressure control at 30 kPa. The injector temperature was maintained at 200°C, ion-source temperature at 230°C, and interface temperature at 280°C. The oven program began at 60°C, increased to 150°C at 10°C/min, was held at 150°C for 3 min, and was subsequently increased to 280°C at 10°C/min and held at 280°C for 14 min; mass spectra were recorded from 2.0 min (solvent cut time) to 39.0 min. Mass spectra were acquired in electron-impact mode at 70 eV over an m/z range of 35-500.

Constituents were identified by comparing their electron-impact mass spectra with the NIST-20 mass spectral library (National Institute of Standards and Technology, Gaithersburg, MD, USA). A peak was considered identified when its similarity index with the library reference exceeded 80%; peaks below this threshold were classified as unidentified. Relative composition was expressed as the percentage peak area relative to the total integrated chromatographic peak area without correction for detector response factors. The 50 peaks reported in Table 1 accounted for the entire integrated chromatographic area (sum = 100.02% owing to rounding).

In vitro adult-worm immersion assay

The *in vitro* adult-worm immersion assay was adapted from Vijaya and Yadav [22] and Lalthanpuii and Lalthandama [16], as described previously for *Cymbopogon nardus* essential oil [23]. A 10% (v/v) PBE0 stock in DMSO served as the highest working concentration and was serially diluted 10-fold in 0.9% NaCl to obtain five working concentrations of 10, 1, 0.1, 0.01, and 0.001% (v/v) PBE0, containing 90, 9, 0.9, 0.09, and 0.009% (v/v) DMSO, respectively. These treatments were coded L2K1-L2K5, where L denotes “Larutan” (solution) and K denotes “Konsentrasi” (concentration). The control series (L1K1-L1K5) consisted of concentration-matched vehicle solutions containing identical DMSO concentrations in 0.9% NaCl, including the highest DMSO concentration of 90% (v/v), but without PBE0.

Each working solution (5 mL) was transferred to a sterile watch glass, and five adult worms ($n = 5/\text{replicate}$) were transferred using nonsurgical forceps and completely immersed in the test solution. The watch glasses were maintained at $37 \pm 1^\circ\text{C}$ in a thermostatically controlled incubator throughout the 160-min observation period. The temperature of the immersion medium was verified at each observation time using a calibrated digital thermometer.

Mortality was assessed at 10, 20, 40, 80, and 160 min after exposure. A worm was considered dead when it remained motionless during 30 s of continuous stereomicroscopic observation and failed to respond to gentle mechanical stimulation with a blunt probe applied to three different body regions. Provisionally nonmotile worms were transferred to fresh 0.9% NaCl at 37°C and reassessed after 5 min; only worms that failed to regain motility were classified as dead [22]. Each treatment and its matched vehicle control comprised six independent biological replicates ($n = 6$), with five worms/replicate, yielding 30 worms/group/time point.

The 160-min observation period was selected because preliminary experiments showed that >95% of adult *A. galli* maintained in 0.9% NaCl at 37°C remained viable for at least 4 h, after which background mortality became appreciable. In addition, the logarithmic observation schedule (10, 20, 40, 80, and 160 min) was designed to characterize the rapid onset of activity expected for a membrane-active monoterpene mixture [8, 9]. By 160 min, treatment groups had reached either complete mortality or an apparent mortality plateau, indicating that extension of the observation period was unlikely to alter their relative responses.

A synthetic anthelmintic reference group was not included in the *in vitro* immersion assay because the experimental mortality endpoint and observation period differed from the pharmacological responses expected with conventional anthelmintics. Levamisole and pyrantel induce spastic paralysis through cholinergic mechanisms, whereas ivermectin induces flaccid paralysis by activating glutamate-gated chloride channels. Because mortality in the present study required failure to regain motility following transfer to drug-free saline, reversibly paralyzed worms could have been classified as viable. Furthermore, the 160-min observation period was selected to characterize the rapid contact-mediated activity of the essential oil, whereas conventional anthelmintics may act over longer periods against adult *A. galli* under static immersion conditions [16]. The reference drugs also differ in aqueous solubility and would require distinct vehicles and corresponding solvent controls. Therefore, ivermectin, levamisole, and pyrantel pamoate were used as reference compounds only in the *in silico* molecular docking analysis.

SEM

Adult *A. galli* worms (three/group; $n = 3$) from the 10% PBEO and DMSO-matched vehicle control groups were processed for SEM at the Genomic Laboratory, BRIN, Cibinong, Indonesia. Worms were rinsed three times with 0.1 M cacodylate buffer (pH 7.4), fixed in 2.5% glutaraldehyde in the same buffer for 24 h at 4°C, postfixed in 1% osmium tetroxide for 2 h, and dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, and 90%, followed by $3 \times 100\%$; 15 min each). Specimens were critical-point dried in CO₂, mounted on aluminum stubs using carbon tape, sputter-coated with an approximately 10-nm gold layer, and examined using an SU3500 scanning electron microscope (Hitachi High-Tech Corp., Tokyo, Japan) at an accelerating voltage of 5 kV. Fixation, critical-point drying, and sputter-coating were performed as a service by the Genomic Laboratory core facility, BRIN, using the facility's standard reagents and equipment.

Three anatomical regions were examined in each worm: the anterior extremity, including the lip region; mid-body; and posterior extremity. Nonoverlapping fields from each region were surveyed and photographed. Survey images were acquired at $\times 100$ – $\times 500$ magnification to characterize regional topography, and lesions were documented at $\times 1,000$ – $\times 2,000$ magnification. Surface damage was assessed qualitatively for the presence or absence of four predefined lesion types: lip abrasion, irregular longitudinal folding, disruption of transverse annulation, and surface disintegration. No morphometric quantification of the proportion of affected surface area was performed.

Histopathology

Histopathological processing was performed at the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia. Adult *A. galli* exposed to the highest PBEO concentration (10%) for 160 min were selected to evaluate internal structural damage. Control worms were exposed to the DMSO-matched vehicle control (90% [v/v] DMSO in 0.9% NaCl) for the same duration. Three worms/group ($n = 3$) were processed in parallel, and slides were coded to ensure that the observer was blinded to treatment allocation.

Worms were fixed in 10% neutral-buffered formalin for 24 h and processed, sectioned, and stained as a service by the Anatomical Pathology Laboratory using its standard equipment, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Cross-sections (5 μ m) were prepared using a rotary microtome, mounted, and stained with hematoxylin and eosin (H&E). Three non-serial sections/worm and at least five non-overlapping fields/section were examined, providing a minimum of 45 fields/group. Sections were evaluated using

a light microscope (Olympus Corp., Tokyo, Japan) at $\times 40$ magnification by an observer blinded to treatment allocation.

Statistical analysis

Mortality data, expressed as the number of dead worms relative to the total number of worms/replicate, were analyzed as bounded proportional counts using a generalized linear mixed model (GLMM) with a binomial error distribution and logit link. The LC_{50} and its 95% confidence interval were estimated by maximum-likelihood probit regression of 160-min mortality on $\log_{10}$ -transformed concentration (% v/v); a Hill (log-logistic) model was additionally fitted to the 160-min mortality data for graphical representation (Figure 1B). Analyses were performed using R version 4.4.1 (R Foundation for Statistical Computing, Vienna, Austria; <https://www.R-project.org>) with the *lme4* package. Treatment, exposure time, and their interaction were included as fixed effects, whereas replicate was included as a random effect. Pairwise post hoc contrasts were calculated using *emmeans* with Tukey adjustment for multiple comparisons.

Because no mortality occurred in any of the five L1 vehicle-control subgroups at any time point, these subgroups were pooled for presentation. Effect sizes relative to the pooled vehicle control at each time point were quantified using Cohen's *h* for proportions, with $h \approx 0.2, 0.5, 0.8$, and >1.2 interpreted as small, medium, large, and very large effects, respectively. Two-sided 95% confidence intervals calculated using the Wilson score method were reported alongside means $\pm$ standard deviations in Table 2, and treatment- and time-specific means with Tukey groupings are presented in Table 3. Statistical significance was set at $p < 0.05$.

Table 2: Mortality of adult *Ascaridia galli* following *in vitro* exposure to *Piper betle* L. essential oil at different concentrations and exposure times.

Treatment	10 min	20 min	40 min	80 min	160 min
Vehicle control (pooled L1K1-L1K5)	0.00 (0.0–2.5)	0.00 (0.0–2.5)	0.00 (0.0–2.5)	0.00 (0.0–2.5)	0.00 (0.0–2.5)
PBEO 10%	26.67 $\pm$ 37.23 (14.2–44.4)	43.33 $\pm$ 38.81 (27.4–60.8)	70.00 $\pm$ 27.56 (52.1–83.3)	93.33 $\pm$ 10.32 (78.7–98.2)	100.00 $\pm$ 0.00 (88.6–100.0)
PBEO 1%	0.00 (0.0–11.4)	3.33 $\pm$ 8.16 (0.6–16.7)	6.67 $\pm$ 16.33 (1.8–21.3)	13.33 $\pm$ 24.22 (5.3–29.7)	56.67 $\pm$ 34.44 (39.2–72.6)
PBEO 0.1%	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)	3.33 $\pm$ 8.16 (0.6–16.7)
PBEO 0.01%	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)	3.33 $\pm$ 8.16 (0.6–16.7)
PBEO 0.001%	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)	0.00 (0.0–11.4)

Values are expressed as mean $\pm$ SD percentage mortality with Wilson score 95% confidence intervals in parentheses ($n = 30$ worms/treatment/time point; 6 replicates $\times$ 5 worms; pooled vehicle control, $n = 150$ worms/time point). Adult *A. galli* were exposed to PBEO at 10, 1, 0.1, 0.01, and 0.001% (v/v), and mortality was assessed at 10, 20, 40, 80, and 160 min. PBEO = *Piper betle* L. essential oil, SD = Standard deviation.

Table 3: Treatment- and exposure time-dependent mortality of adult *Ascaridia galli* following *in vitro* exposure to *Piper betle* L. essential oil.

Treatment	10 min	20 min	40 min	80 min	160 min	Row-mean
L1K1 Vehicle (90% DMSO)	0.00	0.00	0.00	0.00	0.00	0.00 ^a
L1K2 Vehicle (9% DMSO)	0.00	0.00	0.00	0.00	0.00	0.00 ^a
L1K3 Vehicle (0.9% DMSO)	0.00	0.00	0.00	0.00	0.00	0.00 ^a
L1K4 Vehicle (0.09% DMSO)	0.00	0.00	0.00	0.00	0.00	0.00 ^a
L1K5 Vehicle (0.009% DMSO)	0.00	0.00	0.00	0.00	0.00	0.00 ^a
L2K1 PBEO 10%	26.67 $\pm$ 37.23	43.33 $\pm$ 38.81	70.00 $\pm$ 27.56	93.33 $\pm$ 10.32	100.00 $\pm$ 0.00	66.67 $\pm$ 38.35 ^c
L2K2 PBEO 1%	0.00	3.33 $\pm$ 8.16	6.67 $\pm$ 16.33	13.33 $\pm$ 24.22	56.67 $\pm$ 34.44	16.00 $\pm$ 28.48 ^b
L2K3 PBEO 0.1%	0.00	0.00	0.00	0.00	3.33 $\pm$ 8.16	0.67 $\pm$ 3.65 ^a
L2K4 PBEO 0.01%	0.00	0.00	0.00	0.00	3.33 $\pm$ 8.16	0.67 $\pm$ 3.65 ^a
L2K5 PBEO 0.001%	0.00	0.00	0.00	0.00	0.00	0.00 ^a
Column mean	2.67 $\pm$ 13.51 ^A	4.67 $\pm$ 17.41 ^{AB}	7.67 $\pm$ 23.02 ^{AB}	10.67 $\pm$ 29.10 ^{BC}	16.33 $\pm$ 34.44 ^C	—

Values are expressed as mean $\pm$ SD percentage mortality ($n = 6$ replicates $\times$ 5 worms/cell). Row and column means $\pm$ SD were calculated from all replicate-level observations contributing to each row (6 replicates $\times$ 5 time points) or column (6 replicates $\times$ 10 groups). Different lowercase superscripts (^{a–c}) within the row-mean column indicate significant differences among treatments ($p < 0.05$; Tukey's test). Different uppercase superscripts (^{A–C}) within the column-mean row indicate significant differences among exposure times ($p < 0.05$; Tukey's test). L1 = DMSO-matched vehicle control (DMSO in 0.9% NaCl), L2 = *Piper betle* L. essential oil treatment, K1-K5 = Concentrations corresponding to 10, 1, 0.1, 0.01, and 0.001%, respectively, PBEO = *Piper betle* L. essential oil, NaCl = Sodium chloride, DMSO = Dimethyl sulfoxide, SD = Standard deviation.

Sample size ($n = 6$ replicates $\times$ 5 worms = 30 worms/treatment/time point) was determined *a priori* for a two-proportion comparison designed to detect a difference of 50% versus 0% at $\alpha = 0.05$ and a power of $1 - \beta =$

0.95 using G*Power version 3.1 (Heinrich Heine University Düsseldorf, Düsseldorf, Germany; <https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower>), which indicated a requirement of approximately eight worms/group; the adopted design (30 worms/group/time point) exceeded this requirement.

Molecular docking

Predictive molecular docking was performed for 10 PBEO constituents selected from the GC-MS profile: eugenol, α -selinene, β -selinene, 4-allylphenyl acetate (chavicol acetate), 3-allyl-6-methoxyphenyl acetate (chavibetol acetate), β -caryophyllene (4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene), *n*-heptadecane, humulene, chavicol, and (-)-terpinen-4-ol. Four pharmacologically relevant nematode targets were evaluated: aquaporin (AQP; UniProt Q19949), glutamate-gated chloride channel α (GluCl; *C. elegans* GLC-1, UniProt G5EBR3; reference crystal structure Protein Data Bank [PDB] 3RHW), nAChR non- α subunit (nAChR; UniProt Q17328), and Na⁺/K⁺-ATPase β -1 subunit (UniProt Q93235). *n*-Heptadecane, a minor constituent (peak 36; 0.59%), was retained as a representative long-chain *n*-alkane.

All target sequences and structures originated from *C. elegans*. *C. elegans* was used as a structural surrogate for *A. galli* based on their shared classification within class Chromadorea and order Rhabditida and because the major anthelmintic target families, including GluCl and nAChR, are conserved across the phylum Nematoda, although subunit composition and sequence vary among species [24, 25].

Protein and ligand preparation

The GluCl crystal structure (3RHW) was retrieved from the PDB (<https://www.rcsb.org>) and used for docking-protocol validation and to define the reference GluCl cavity (Table 4), whereas comparative docking of all four targets, including GluCl, was performed on single-chain AlphaFold-predicted full-length models retrieved from UniProt (<https://www.uniprot.org>; AlphaFold Protein Structure Database entries AF-Q19949-F1, AF-G5EBR3-F1, AF-Q17328-F1, and AF-Q93235-F1). Residue numbering in Tables 5 and 6 therefore follows the full-length sequences (chain A). Crystallographic water molecules, co-crystallized ligands, and other nonessential heteroatoms were removed, while biologically relevant cofactors were retained [26]. Three-dimensional structures of the 10 PBEO constituents were obtained from PubChem (<https://pubchem.ncbi.nlm.nih.gov>) in Structure-Data File (SDF) format using the following PubChem Compound Identifiers (CIDs): eugenol (3314), α -selinene (10856614), β -selinene (442393), 4-allylphenyl acetate (523825), 3-allyl-6-methoxyphenyl acetate (596380), β -caryophyllene (26318), *n*-heptadecane (12398), humulene (5281520), chavicol (68148), and (-)-terpinen-4-ol (5325830). SDF files were converted to PDB format using Open Babel version 3.1.0 (<http://openbabel.org>) and geometrically optimized using the Merck Molecular Force Field (MMFF94).

Cavity-detection and docking-grid definition

Cavity-detection and docking-grid definition were performed using CB-Dock2 version 2.0 (<https://cadd.labshare.cn/cb-dock2/>) [27]. The platform integrates cavity-detection, molecular docking, and homologous template fitting. Binding cavities were initially predicted using the P2Rank version 2.4 (<https://github.com/rdk/p2rank>) machine-learning method [28] and subsequently refined using CurPocket, a curvature-based cavity-detection approach implemented in CB-Dock2 [27]. AutoDock Vina version 1.1.2 (The Scripps Research Institute, La Jolla, CA, USA; <https://vina.scripps.edu>) was used as the docking engine with its empirical scoring function.

CB-Dock2 additionally searched the PDB for homologous templates to refine predicted binding sites. When a template with $\geq 40\%$ sequence identity and a cavity root-mean-square deviation (RMSD) ≤ 4 Å was identified, the corresponding template binding-site was used to refine the pocket definition.

Docking calculations and pose selection

All docking calculations were performed using CB-Dock2 with the AutoDock Vina scoring function [27, 29]. Docking-box dimensions were automatically determined according to the predicted cavity size and typically ranged from 15 to 25 Å; the cavity-detection radius was maintained at its default setting. The 10 PBEO constituents and three reference anthelmintics, ivermectin (PubChem CID 6321424), levamisole (CID 26879), and pyrantel pamoate (CID 5281033), were docked using identical parameters to facilitate comparison of predicted binding energies and interaction patterns. The docking protocol and receptor models were identical to those previously validated in our laboratory by re-docking the co-crystallized ivermectin into GluCl (PDB 3RHW), which reproduced

the X-ray pose with an RMSD of 0.87 Å [23].

For each receptor-ligand complex, the optimal pose was selected based on the most negative predicted binding energy (kcal/mol), and localization within one of the top five cavities predicted by CB-Dock2; consequently, the contact residues of some complexes lie in alternative cavities of the same receptor model rather than in the top-ranked cavity listed in Table 4. Hydrogen bonds, hydrophobic interactions, π - π stacking, cation- π interactions, and electrostatic interactions were evaluated using BIOVIA Discovery Studio Visualizer version 21.1.0 (Dassault Systèmes, San Diego, CA, USA; <https://discover.3ds.com/discovery-studio-visualizer-download>).

Ivermectin, levamisole, and pyrantel pamoate served as reference *in silico* controls for residue-level comparisons with PBE0 constituents. No post-docking energy minimization or molecular dynamics refinement was performed; therefore, the docking analysis was used to generate testable predictions regarding potential target engagement and binding modes rather than to establish definitive molecular mechanisms.

RESULTS

Concentration- and time-dependent mortality

PBE0 produced a clear concentration- and time-dependent increase in adult *A. galli* mortality (Tables 2 and 3). No mortality was recorded in the vehicle control groups throughout the observation period. At 10% PBE0, mortality was $26.67 \pm 37.23\%$ at 10 min and increased progressively to $43.33 \pm 38.81\%$ at 20 min, $70.00 \pm 27.56\%$ at 40 min, $93.33 \pm 10.32\%$ at 80 min, and $100.00 \pm 0.00\%$ at 160 min. At 1% PBE0, mortality increased more gradually and reached $56.67 \pm 34.44\%$ at 160 min. In contrast, 0.1, 0.01, and 0.001% PBE0 produced minimal or no mortality. Tukey-adjusted comparisons showed that the 10% and 1% PBE0 groups differed significantly from each other and from the control and lower-concentration groups ($p < 0.05$), whereas the 0.1, 0.01, and 0.001% PBE0 groups did not differ significantly from the vehicle control ($p > 0.05$). Relative to the pooled vehicle control, effect sizes for 10% PBE0 were large to very large at every time point (Cohen's $h = 1.09, 1.44, 1.98, 2.62$, and 3.14 at 10, 20, 40, 80, and 160 min, respectively). For 1% PBE0, effect sizes increased from negligible at 10 min ($h = 0.00$) to small-to-medium at 20–40 min ($h = 0.37$ and 0.52), medium-to-large at 80 min ($h = 0.75$), and very large at 160 min ($h = 1.70$). The 0.1% and 0.01% PBE0 groups showed only small effects at 160 min ($h = 0.37$).

Probit analysis

Maximum-likelihood probit regression of 160-min mortality on $\log_{10}$ concentration yielded the equation $y = 0.255 + 1.692x$ (pseudo- $R^2 = 0.909$), where x represents $\log_{10}$ concentration (% v/v) and y represents the normal equivalent deviate. The estimated LC_{50} was 0.71% (95% confidence interval [CI]: 0.47–1.06%), and the Hill model yielded a concordant LC_{50} of 0.71% (Figure 1).

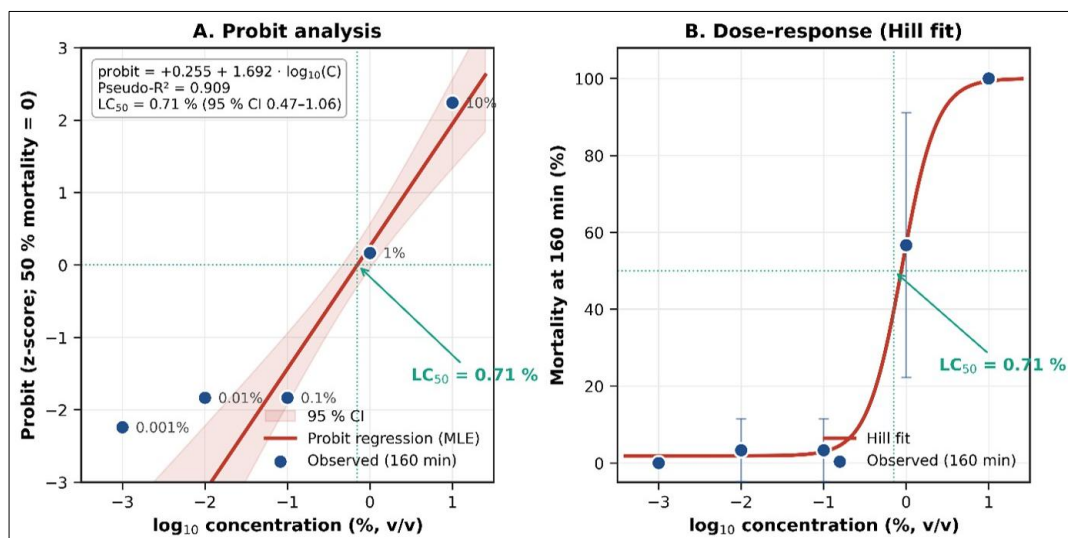


Figure 1: Probit and dose-response modeling of PBE0-induced mortality in adult *Ascaridia galli*. (A) Probit regression of adult-worm mortality at 160 min plotted against $\log_{10}$ PBE0 concentration (% v/v). The fitted model shows a concentration-dependent increase in mortality, with an estimated LC_{50} of 0.71% PBE0 (95% CI: 0.47–1.06%). The dashed horizontal line indicates 50% mortality, and the dashed vertical line indicates the corresponding LC_{50} . (B) Dose-response curve fitted using a Hill model, showing increasing mortality with increasing PBE0 concentrations and 100% mortality at 10% PBE0 after 160 min of exposure. Points represent observed mortality values, vertical bars indicate variation around the observed estimates, and the curve represents the fitted model. PBE0 = *Piper betle* L. essential oil, LC_{50} = Median lethal concentration.

Ultrastructural cuticular damage

SEM examination showed that vehicle-control *A. galli* had an intact, smooth cuticular surface with regular transverse annuli and subannuli along the body (Figures 2A and 2E). In contrast, worms exposed to 10% PBEO exhibited marked topographical alterations. The anterior region showed ventrolateral lip abrasion, anterior cuticle distortion, and prominent longitudinal folding (Figure 2B). At $\times 2,000$ magnification, the abraded lip region showed disorganization of the inner surface layer (Figure 2C). The mid-body region exhibited irregular longitudinal and transverse folds, cuticular shrinkage, and focal peeling at $\times 2,000$ magnification (Figure 2D). At $\times 2,000$ magnification, disruption of annular and subannular boundaries was evident, indicating loss of normal cuticular architecture (Figure 2F).

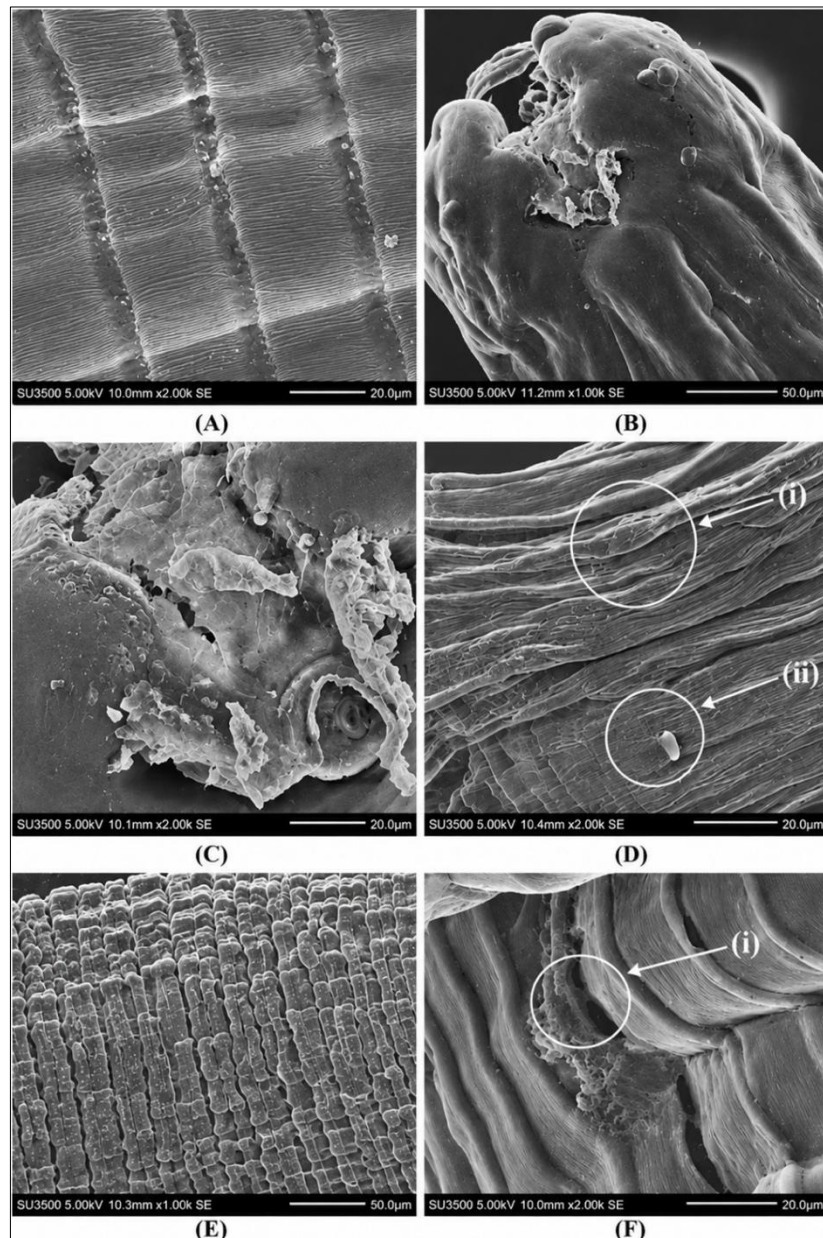


Figure 2: SEM of adult *Ascaridia galli* following exposure to PBEO. (A) Control mid-body cuticular surface showing intact, regularly arranged transverse annuli and subannuli without apparent surface disruption. (B) PBEO-treated worm, anterior cephalic region, showing abrasion and distortion of the cuticular surface around the ventrolateral lip region. (C) PBEO-treated worm, anterior cephalic cuticle, showing extensive surface abrasion, peeling, and loss of normal cuticular integrity. (D) PBEO-treated worm, mid-body cuticular surface, showing irregular longitudinal folding and disruption of the normal transverse cuticular pattern; circled regions indicate longitudinal depression (i) and transverse distortion (ii). (E) Control mid-body cuticular surface showing regular transverse annulation and subannulation for comparison with the treated specimens. (F) PBEO-treated worm, mid-body cuticle at the annulus-subannulus boundary, showing localized cuticular erosion and disruption of the external surface architecture (circle, i). Scale bars: A, C, D, and F = 20 μm ; B and E = 50 μm . SEM = Scanning electron microscopy, PBEO = *Piper betle* L. essential oil.

Histopathological lesions

Histopathological examination demonstrated internal structural injury in PBEO-treated worms. Control worms had intact ovoid intrauterine eggs with smooth, thick, sharply defined shells; a smooth, continuous cuticle; and compact underlying fibrillar muscle layers. In contrast, PBEO-treated worms showed uterine disorganization, egg shell deformation, dissociation of egg contents, and crescent-shaped deformed eggs (Figure 3). The external cuticle appeared rough, discontinuous, and partially detached, while the underlying fibrillar musculature was fragmented (Figure 4). These findings demonstrated structural alterations involving both external and internal tissues.

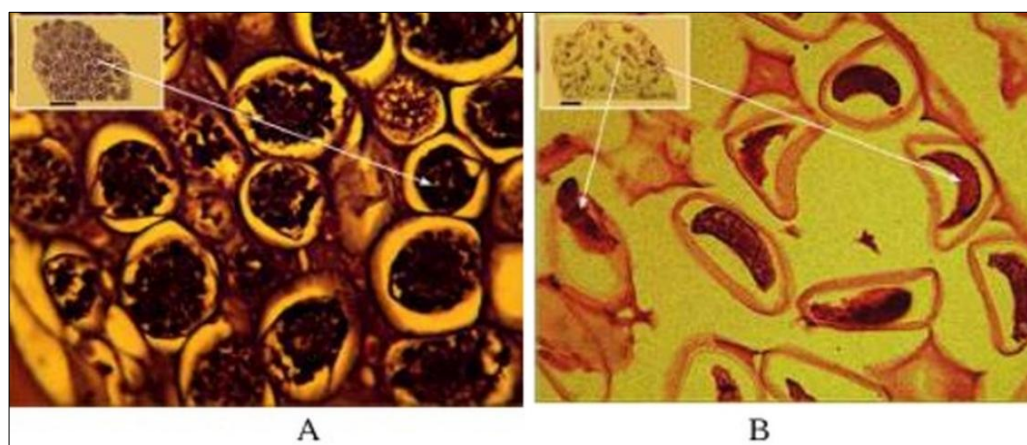


Figure 3: Histopathological changes in intrauterine eggs of adult *Ascaridia galli* following exposure to PBEO. (A) Vehicle control showing numerous compactly arranged intrauterine eggs with predominantly regular ovoid profiles, intact smooth egg shells, and preserved internal contents. The arrow indicates an intact egg with a continuous shell and preserved internal structure. (B) PBEO-treated worm showing marked alterations in intrauterine egg morphology compared with the control, characterized by irregular and deformed egg profiles, distortion or collapse of the egg shell, separation of internal contents from the shell, and formation of crescent-shaped structures. Arrows indicate representative deformed or collapsed eggs and dissociation of internal egg contents from the surrounding shell. H&E staining; ×40 magnification; scale bar = 100 µm. PBEO = *Piper betle* L. essential oil, H&E = Hematoxylin and eosin.

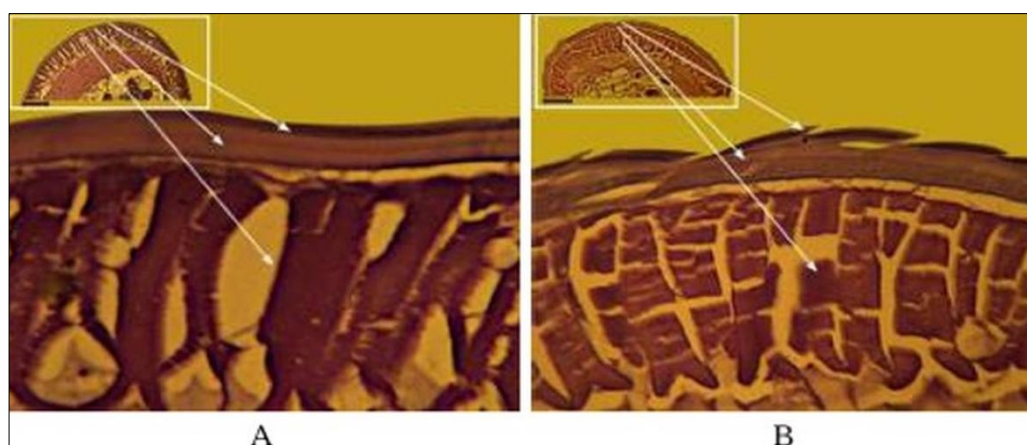


Figure 4: Histopathological changes in the cuticle and underlying somatic musculature of adult *Ascaridia galli* following exposure to PBEO. (A) Vehicle control showing a smooth, continuous external cuticle with preserved thickness and regularly arranged underlying somatic muscle cells. Arrows indicate the intact external cuticle and preserved underlying somatic musculature. (B) PBEO-treated worm showing marked structural alterations compared with the vehicle control, including an irregular and disrupted cuticular surface with focal separation or detachment of the cuticle and disorganization of the underlying somatic musculature. Arrows indicate focal cuticular disruption or detachment and disorganization of the underlying muscle layer. H&E staining; ×40 magnification; scale bar = 100 µm. PBEO = *Piper betle* L. essential oil, H&E = Hematoxylin and eosin.

GC-MS chemical profile

The GC-MS chromatogram of PBEO contained 50 peaks, of which 49 were identified by database comparison and one remained unidentified (Table 1). The chemical profile included hydrocarbons, bicyclic compounds, aromatic compounds, terpenoids, and oxygenated constituents. Identified constituents comprised

sesquiterpene hydrocarbons (including α - and β -selinene, β -caryophyllene, γ -muurolene, α -humulene, (*E*)- β -farnesene, α -santalene, and β -bisabolene), phenylpropanoids (eugenol, chavicol, estragole, chavicol acetate, chavibetol acetate, and 4-allylcatechol diacetate), monoterpenes (including α -pinene, camphene, γ -terpinene, *p*-cymene, linalool, terpinen-4-ol, and α -terpineol), and aliphatic alkanes and aldehydes (Table 1).

The most abundant single chromatographic peak was eugenol (7.96%, peak 27), while a second eugenol-matched peak (0.52%, peak 25) increased the total eugenol contribution to 8.48% of the PBEO profile. Other major constituents included α -selinene (6.47%), β -selinene (6.16%), 4-allylphenyl acetate (5.67%), 3-allyl-6-methoxyphenyl acetate (5.47%), β -caryophyllene (5.21%), γ -muurolene (4.97%), humulene (3.76%), chavicol (3.63%), *n*-heneicosane (3.54%), and (–)-terpinen-4-ol (3.44%).

Table 4: Predicted active-site residues of nematode protein targets used for molecular docking.

Protein (database ID)	Predicted active-site residues
nAChR, non- α subunit (UniProt Q17328)	Ile71(A), Val73(A), Tyr168(A), Asn211(A), Phe212(A), Arg243(A), Tyr247(A), Tyr248(A), Lys298(A), Leu299(A)
Aquaporin (AQP; UniProt Q19949)	Val53(A), Phe56(A), His57(A), Trp66(A), Val69(A), Trp73(A), Ile147(A), Leu148(A), Gly149(A), Gly152(A), Gly155(A), Ala158(A), Ser159(A), Tyr160(A), Ser162(A), Gly216(A), Met217(A), Asn218(A), Ile219(A), Gly220(A), Arg227(A)
Na ⁺ /K ⁺ -ATPase β -1 subunit (UniProt Q93235)	Leu134(A), Asp138(A), Leu169(A), Phe172(A), Asp173(A), Ser177(A), Glu178(A)
Glutamate-gated chloride channel (GluCl; PDB reference crystal structure)	Phe91(C), Pro93(C), Asn94(C), Ser129(C), Pro131(C), Ser145(C), Asp147(C), Ala149(C), Ser150(C), Tyr151(C), Cys191(C), Ser193(C), Thr195(C), Thr197(C), Tyr200(C), Cys202(C), Arg204(C), Leu35(D), Arg37(D), Thr38(D), Thr54(D), Arg56(D), Leu109(D), Ser121(D), Lys171(D), Val172(D), Gly173(D)

AQP = Aquaporin, GluCl = Glutamate-gated chloride channel, nAChR = Nicotinic acetylcholine receptor, PDB = Protein Data Bank, UniProt = Universal Protein Resource. Active-site residues were predicted using P2Rank and refined with CurPocket as implemented in CB-Dock2. Residues are reported with their corresponding protein-chain identifiers in parentheses. These predicted cavities were used to evaluate interactions between the selected nematode protein targets and major *Piper betle* L. essential oil constituents. Because CB-Dock2 docked each ligand into the top five predicted cavities and retained the best-scoring pose, the contact residues of several complexes (see the interaction profiles below) lie in alternative cavities of the same receptor model and therefore differ from the top-ranked cavity residues listed here. GluCl cavity residues are those of the pentameric 3RHW reference structure (chains C and D) used for protocol validation, whereas GluCl complexes were docked into the single-chain predicted GLC-1 model (chain A).

Molecular docking against nematode targets

The predicted binding affinities of the 10 PBEO constituents against AQP, GluCl, nAChR, and Na⁺/K⁺-ATPase are summarized in Table 5. Binding energies ranged from –7.2 to –4.4 kcal/mol. The most favorable predicted interactions were observed for 4-allylphenyl acetate-AQP and β -caryophyllene-AQP, both at –7.2 kcal/mol, followed by *n*-heptadecane-AQP at –7.0 kcal/mol. Humulene showed predicted affinities of –6.9 kcal/mol for both AQP and GluCl. Among the reference compounds, ivermectin showed moderate-to-strong binding, whereas levamisole and pyrantel pamoate, which were docked against nAChR only, showed predicted affinities of –5.1 and –5.4 kcal/mol, respectively. The weakest predicted interactions were observed for *n*-heptadecane-Na⁺/K⁺-ATPase (–4.4 kcal/mol) and chavicol-nAChR (–4.6 kcal/mol).

Table 5: Predicted binding affinities of major PBEO constituents and reference anthelmintics against selected nematode protein targets.

Ligand	AQP	GluCl	nAChR	Na ⁺ /K ⁺ -ATPase
Eugenol	–6.1	–5.4	–5.2	–5.1
α -Selinene	–6.9	–6.4	–6.0	–6.3
β -Selinene	NR	–6.5	–5.8	–6.0
4-Allylphenyl acetate	–7.2	–5.6	–5.3	–5.1
3-Allyl-6-methoxyphenyl acetate	–6.0	–6.1	–5.3	–5.6
β -Caryophyllene	–7.2	–6.6	–5.8	–5.5
<i>n</i> -Heptadecane	–7.0	NR	–5.6	–4.4
Humulene	–6.9	–6.9	–5.7	–5.8
Chavicol	–6.1	–5.4	–4.6	–4.9
(–)-Terpinen-4-ol	–6.1	–5.3	–5.1	–4.8
Ivermectin (control)	–9.3	–9.4	—	–8.4
Levamisole (control)	—	—	–5.1	—
Pyrantel pamoate (control)	—	—	–5.4	—

Values are predicted docking binding free energies expressed as kcal/mol. More negative values indicate more favorable predicted binding. AQP = Aquaporin, GluCl = Glutamate-gated chloride channel, nAChR = Nicotinic acetylcholine receptor, PBEO = *Piper betle* L. essential oil. — = Not evaluated for the corresponding ligand-target combination. NR = Not reported; the β -selinene-AQP and *n*-heptadecane-GluCl runs were excluded from analysis because their docking output could not be verified.

Detailed receptor-ligand interaction profiles are presented in Table 6. Across the complexes, conventional hydrogen bonds, carbon-hydrogen bonds, π -sigma, π - π stacked, π -cation, alkyl, and π -alkyl interactions were identified. For AQP, eugenol, 4-allylphenyl acetate, and 3-allyl-6-methoxyphenyl acetate formed hydrogen bonds involving residues such as Ser159, Asn224, and His200, whereas sesquiterpenes and *n*-heptadecane interacted predominantly through alkyl and π -alkyl contacts involving Val53, Phe56, Trp66, Trp73, Leu181, and Tyr221. For GluCl, notable interactions included a π -cation interaction between eugenol and Arg402, a π - π stacked interaction between 4-allylphenyl acetate and Tyr386, and alkyl/ π -alkyl interactions involving Arg385, Pro390, and Ala395.

For nAChR, 4-allylphenyl acetate and 3-allyl-6-methoxyphenyl acetate formed hydrogen bonds involving Thr238 and Leu239, whereas α -selinene interacted through alkyl/ π -alkyl contacts involving Tyr261, Leu456, and Tyr457. For Na⁺/K⁺-ATPase, several PBE0 constituents formed hydrogen bonds with Tyr254 and alkyl/ π -alkyl interactions with Phe167, Val171, Pro246, and Ile250.

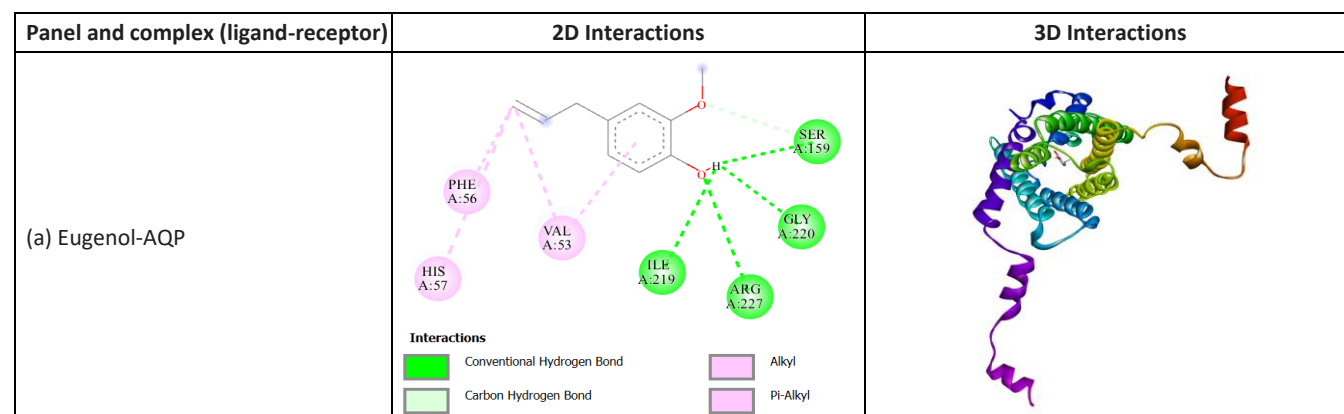
Table 6: Molecular docking interaction profiles of major *PBE0* constituents and reference anthelmintics against selected nematode protein targets.

Complex (ligand-receptor)	Binding affinity (kcal/mol)	Interaction type and residues
Eugenol-AQP	-6.1	Conventional H-bond: Ser A:159, Ile A:219, Gly A:220, Arg A:227. Carbon-H bond: Ser A:159. Alkyl/ π -alkyl: Val A:53, Phe A:56, His A:57.
Eugenol-GluCl	-5.4	Conventional H-bond: Ala A:395. π -Cation: Arg A:402. Alkyl/ π -alkyl: Arg A:385, Tyr A:386, Pro A:390.
Eugenol-nAChR	-5.2	Conventional H-bond: Arg A:57, Glu A:61, Phe A:98. Carbon-H bond: Val A:56. Alkyl/ π -alkyl: Met A:64, Ile A:65.
Eugenol-Na ⁺ /K ⁺ -ATPase	-5.1	Conventional H-bond: Tyr A:254. Alkyl/ π -alkyl: Val A:171, Lys A:220, Pro A:246.
α -Selinene-AQP	-6.9	Alkyl/ π -alkyl: Val A:53, Phe A:56, His A:57, Trp A:66, Val A:69, Trp A:73, Tyr A:221.
α -Selinene-GluCl	-6.4	Alkyl/ π -alkyl: Arg A:385, Tyr A:386, Pro A:390, Ala A:395.
α -Selinene-nAChR	-6.0	π -Sigma: Phe A:460. Alkyl/ π -alkyl: Tyr A:261, Leu A:456, Tyr A:457.
α -Selinene-Na ⁺ /K ⁺ -ATPase	-6.3	Alkyl/ π -alkyl: Phe A:167, Val A:171, Pro A:246, Tyr A:254.
β -Selinene-GluCl	-6.5	π -Sigma: Tyr A:386. Alkyl/ π -alkyl: Arg A:385, Pro A:390, Ala A:395, Phe A:399.
β -Selinene-nAChR	-5.8	π -Sigma: Phe A:460. Alkyl/ π -alkyl: Tyr A:261, Leu A:456, Tyr A:457.
β -Selinene-Na ⁺ /K ⁺ -ATPase	-6.0	Alkyl: Val A:15, Arg A:22.
4-Allylphenyl acetate-AQP	-7.2	Conventional H-bond: Asn A:224. π -Sigma: Leu A:181. Alkyl/ π -alkyl: Ile A:77, Val A:96, Leu A:185, Phe A:204.
4-Allylphenyl acetate-GluCl	-5.6	Conventional H-bond: Arg A:402. π - π stacked: Tyr A:386. Alkyl/ π -alkyl: Lys A:382, Arg A:385.
4-Allylphenyl acetate-nAChR	-5.3	Conventional H-bond: Thr A:238, Leu A:239. π -Donor H-bond: Thr A:238. Alkyl: Arg A:235.
4-Allylphenyl acetate-Na ⁺ /K ⁺ -ATPase	-5.1	Conventional H-bond: Tyr A:254. Carbon-H bond: Arg A:253. π -Donor H-bond: Phe A:167. Alkyl/ π -alkyl: Val A:171, Pro A:246, Ile A:250.
3-Allyl-6-methoxyphenyl acetate-AQP	-6.0	Conventional H-bond: His A:200. π -Sigma: Ile A:189. Alkyl/ π -alkyl: Val A:96, Leu A:100, Leu A:185, Phe A:204.
3-Allyl-6-methoxyphenyl acetate-GluCl	-6.1	Conventional H-bond: Arg A:402. Carbon-H bond: Asp A:270. π - π stacked: Tyr A:386. Alkyl/ π -alkyl: Arg A:385.
3-Allyl-6-methoxyphenyl acetate-nAChR	-5.3	Conventional H-bond: Thr A:238, Leu A:239. Carbon-H bond: Thr A:238. Alkyl: Arg A:235.
3-Allyl-6-methoxyphenyl acetate-Na ⁺ /K ⁺ -ATPase	-5.6	Conventional H-bond: Arg A:253, Tyr A:254. Carbon-H bond: Arg A:253. Alkyl/ π -alkyl: Phe A:167, Pro A:246, Ile A:250.
β -Caryophyllene-AQP	-7.2	Alkyl/ π -alkyl: Val A:53, Phe A:56, His A:57, Trp A:66, Val A:69, Trp A:73, Tyr A:160, Tyr A:221.
β -Caryophyllene-GluCl	-6.6	Alkyl/ π -alkyl: Leu A:271, Tyr A:386, Pro A:390, Ala A:395.
β -Caryophyllene-nAChR	-5.8	Alkyl/ π -alkyl: Leu A:122, Val A:137, Met A:140, Trp A:153.
β -Caryophyllene-Na ⁺ /K ⁺ -ATPase	-5.5	Alkyl/ π -alkyl: Lys A:81, Val A:82, Ile A:199, Val A:258.
<i>n</i> -Heptadecane-AQP	-7.0	Alkyl/ π -alkyl: Leu A:45, Trp A:73, Ile A:77, Val A:96, Leu A:100, Leu A:181, Leu A:185, Ile A:189, Phe A:204, Val A:208, Ile A:223, Arg A:227.
<i>n</i> -Heptadecane-nAChR	-5.6	Alkyl/ π -alkyl: Pro A:171, Phe A:172, Ile A:246, Val A:250, Leu A:300, Val A:301, Tyr A:304, Ile A:470, Ile A:471, Val A:477.

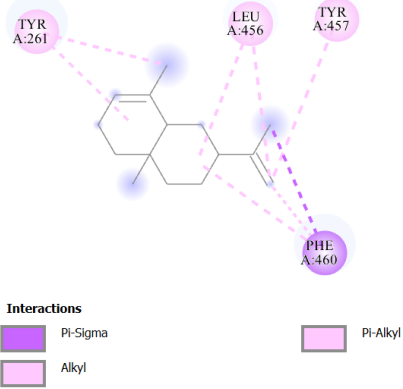
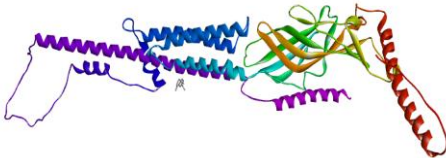
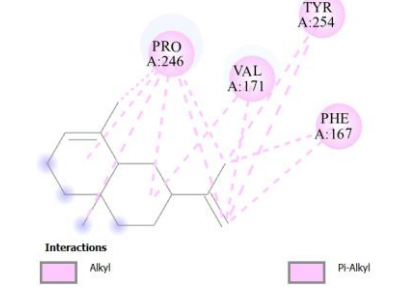
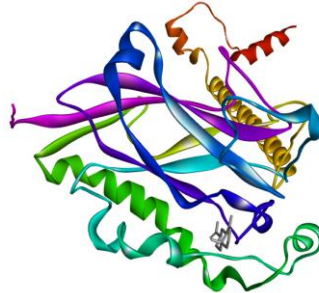
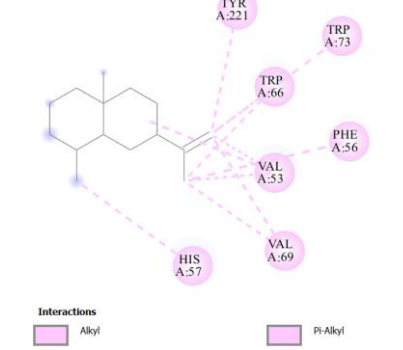
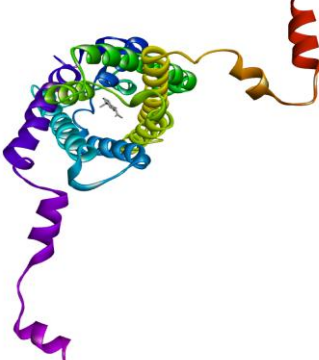
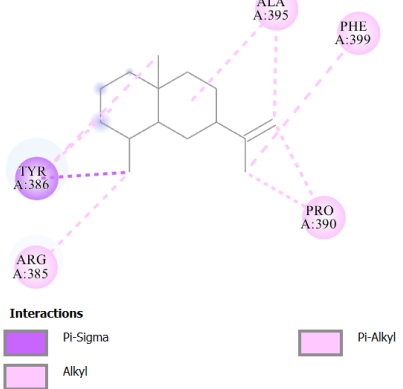
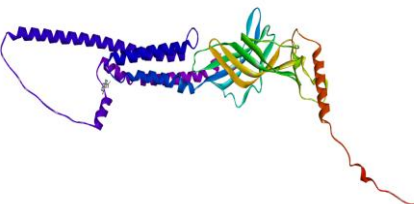
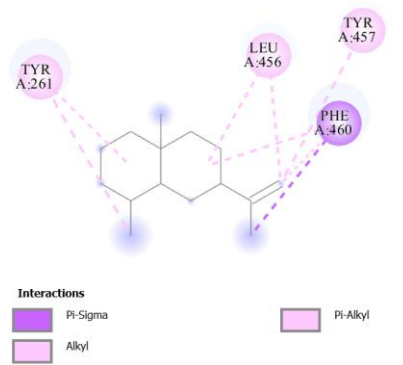
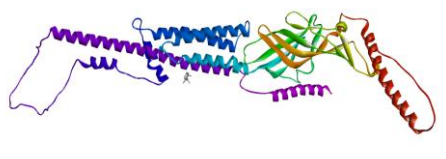
Complex (ligand-receptor)	Binding affinity (kcal/mol)	Interaction type and residues
<i>n</i> -Heptadecane- Na^+/K^+ -ATPase	-4.4	Alkyl: Lys A:131, Leu A:134, Leu A:169.
Humulene-AQP	-6.9	Alkyl/ π -alkyl: Val A:53, Phe A:56, Trp A:66, Val A:69, Trp A:73, Tyr A:160, Tyr A:221.
Humulene-GluCl	-6.9	Alkyl: Arg A:164, Leu A:167, Ala A:427.
Humulene-nAChR	-5.7	Alkyl/ π -alkyl: Phe A:172, Lys A:237.
Humulene- Na^+/K^+ -ATPase	-5.8	Alkyl/ π -alkyl: Tyr A:100, Trp A:103, Val A:303.
Chavicol-AQP	-6.1	π -Sigma: Leu A:181. Alkyl/ π -alkyl: Leu A:45, Ile A:77, Val A:96, Val A:208.
Chavicol-GluCl	-5.4	Conventional H-bond: Gln A:166. Alkyl/ π -alkyl: Arg A:164, Leu A:167, Ala A:427.
Chavicol-nAChR	-4.6	Conventional H-bond: Asn A:130, Lys A:160. Carbon-H bond: Tyr A:159. Alkyl/ π -alkyl: Ile A:158.
Chavicol- Na^+/K^+ -ATPase	-4.9	Conventional H-bond: Tyr A:254. π -Donor H-bond: Phe A:167. Alkyl/ π -alkyl: Val A:171, Lys A:220, Pro A:246.
(-)-Terpinen-4-ol-AQP	-6.1	Alkyl: Leu A:45, Ile A:77, Val A:96, Leu A:181, Leu A:185.
(-)-Terpinen-4-ol-GluCl	-5.3	Alkyl: Leu A:271, Pro A:390, Ala A:395. Unfavorable donor-donor: Arg A:402.
(-)-Terpinen-4-ol-nAChR	-5.1	Conventional H-bond: Pro A:55. Alkyl: Val A:56. Unfavorable acceptor-acceptor: Asp A:96.
(-)-Terpinen-4-ol- Na^+/K^+ -ATPase	-4.8	Conventional H-bond: Asp A:251. Alkyl/ π -alkyl: Phe A:167, Val A:171, Pro A:246, Tyr A:254.
Ivermectin-AQP (control)	-9.3	Conventional H-bond: His A:57, Ser A:159. Carbon-H bond: Gly A:155, Gly A:220. π -Sigma: Trp A:66. Alkyl/ π -alkyl: Val A:53, Phe A:56. Unfavorable acceptor-acceptor: Ile A:219.
Ivermectin-GluCl (control)	-9.4	Conventional H-bond: Arg A:131. Carbon-H bond: Glu A:126, Ala A:129. Alkyl/ π -alkyl: Ile A:118, Phe A:123, Tyr A:151.
Ivermectin- Na^+/K^+ -ATPase (control)	-8.4	Conventional H-bond: Tyr A:133, Ser A:194, Gln A:265. Carbon-H bond: Arg A:197. Alkyl: Pro A:83, Ile A:92, Ile A:199, Leu A:300.
Levamisole-nAChR (control)	-5.1	Carbon-H bond: Thr A:264, Lys A:445. Van der Waals: Ser A:449. π -Sulfur: Phe A:446. Amide- π stacked: Ala A:448.
Pyrantel pamoate-nAChR (control)	-5.4	Conventional H-bond: Thr A:238. Carbon-H bond: Asp A:213, Glu A:479. π -Alkyl: Lys A:237.

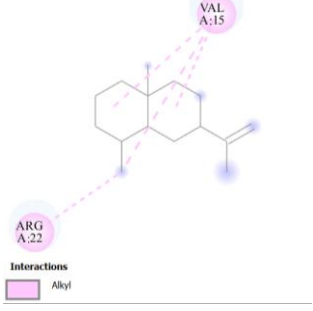
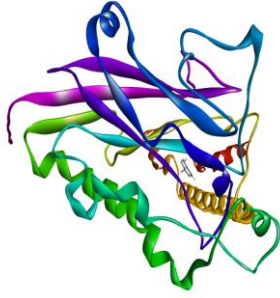
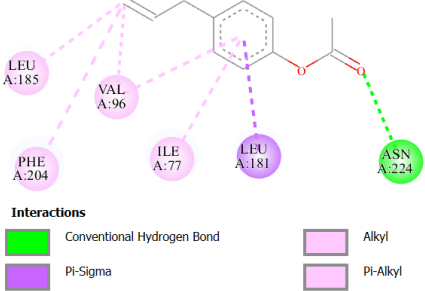
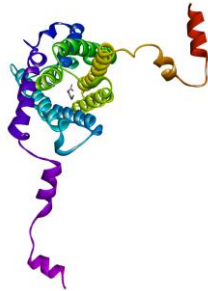
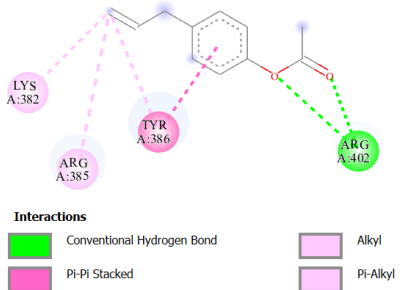
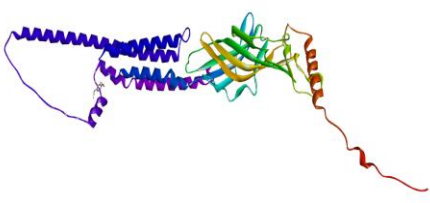
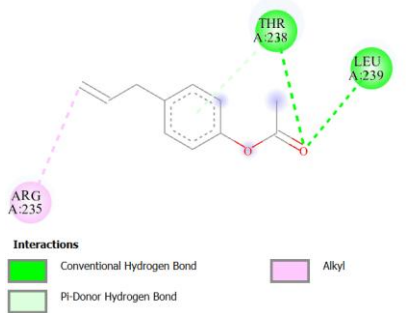
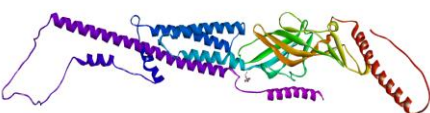
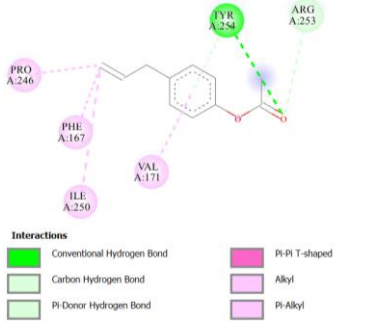
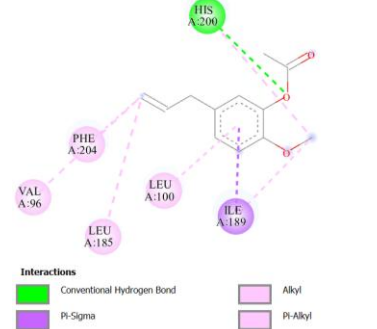
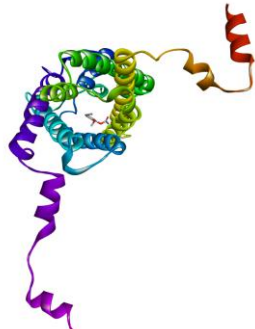
AQP = Aquaporin, GluCl = Glutamate-gated chloride channel, nAChR = Nicotinic acetylcholine receptor, PBEO = *Piper betle* L. essential oil. Binding affinities are predicted docking scores expressed as kcal/mol; more negative values indicate more favorable predicted binding. Interaction residues are reported with the corresponding protein-chain identifier. Reference anthelmintics were included for comparative docking.

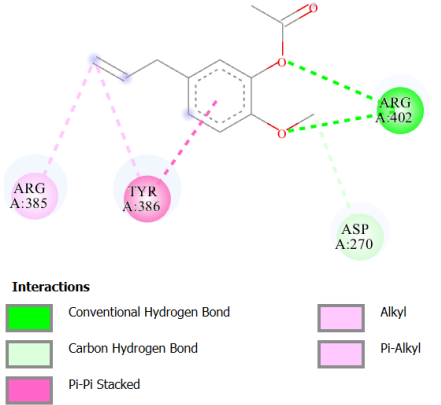
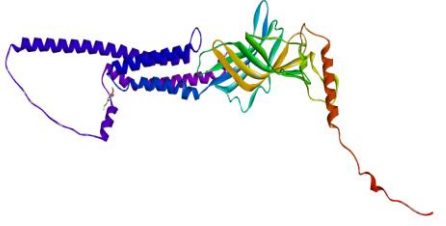
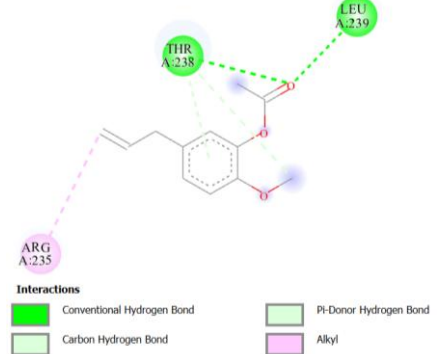
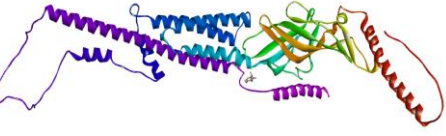
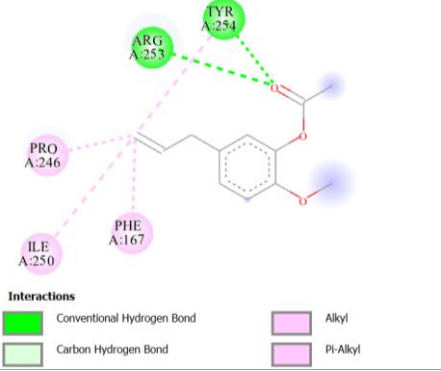
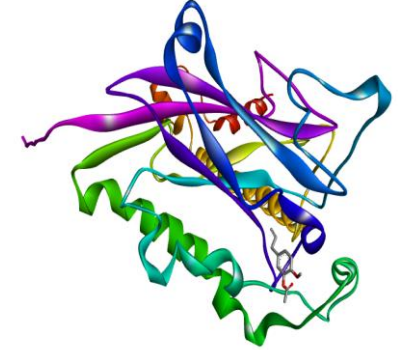
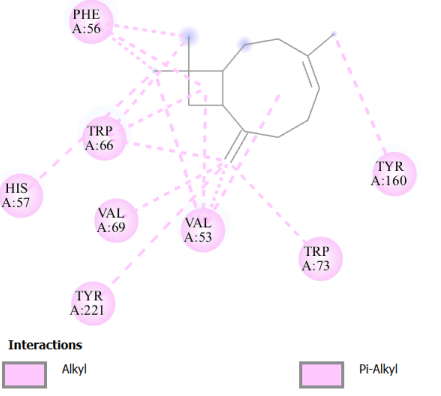
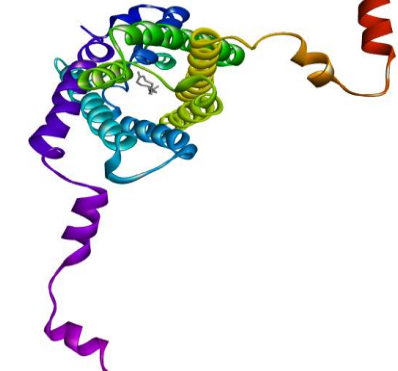
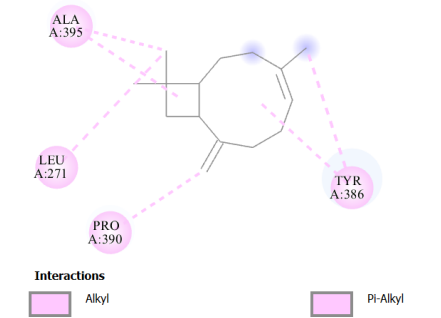
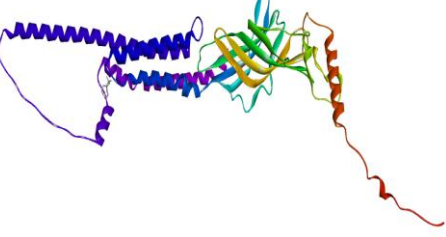
The reference compounds showed distinct residue-interaction patterns. Ivermectin interacted with AQP through His57, Ser159, and Trp66, with an unfavorable acceptor-acceptor interaction at Ile219; with GluCl through Arg131, Glu126, and Ala129; and with Na^+/K^+ -ATPase through Tyr133, Ser194, and Gln265. Levamisole interacted with nAChR through Thr264, Lys445, and Phe446, whereas pyrantel pamoate interacted with Thr238, Asp213, and Glu479. The predicted affinities of PBEO constituents for AQP and GluCl (up to -7.2 kcal/mol) were weaker than those of ivermectin (-9.3 and -9.4 kcal/mol, respectively), whereas several constituents showed predicted nAChR affinities (-6.0 to -5.6 kcal/mol) comparable to or more favorable than those of levamisole and pyrantel pamoate (-5.1 and -5.4 kcal/mol), while engaging different residue-interaction patterns (Figure 5).

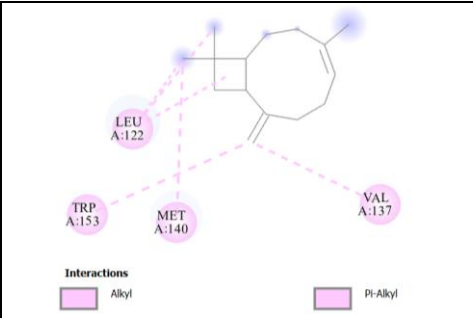
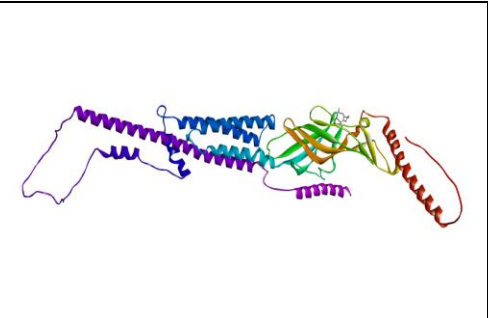
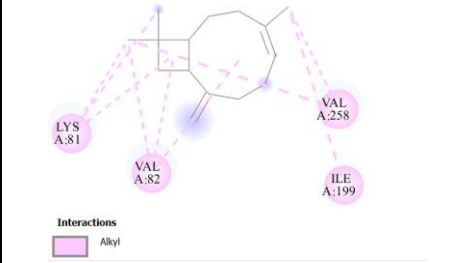
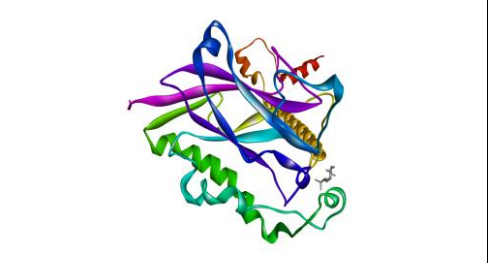
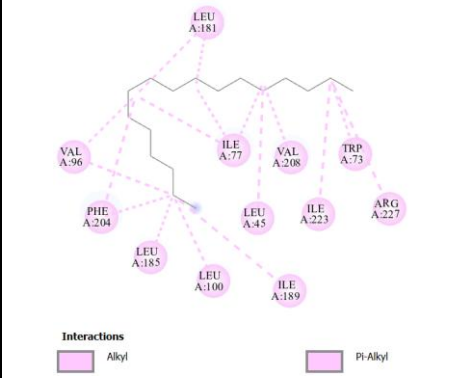
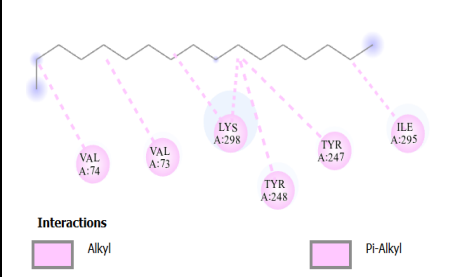
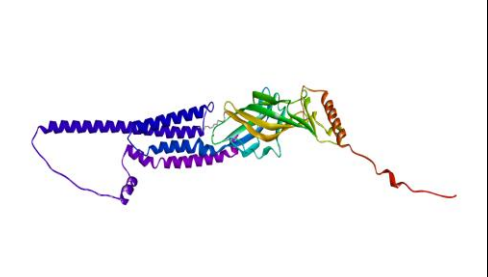
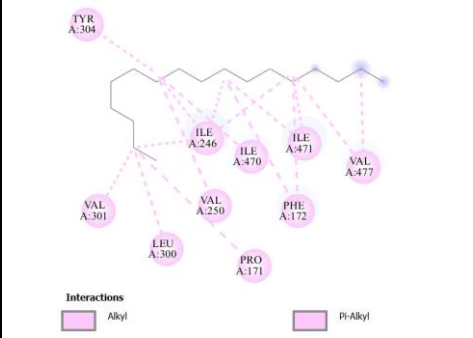
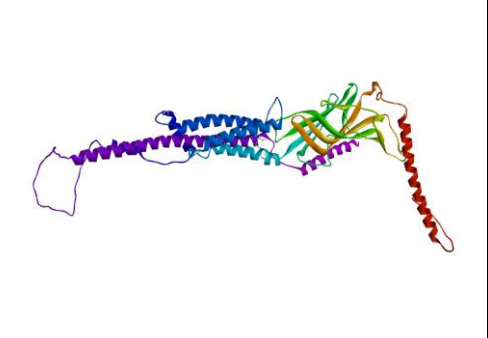
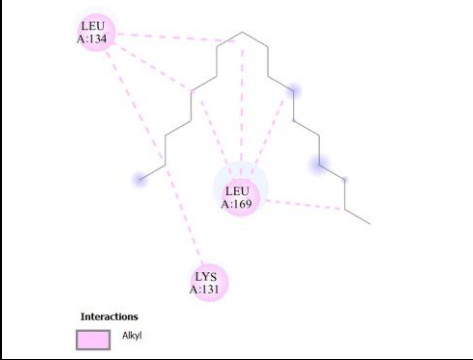
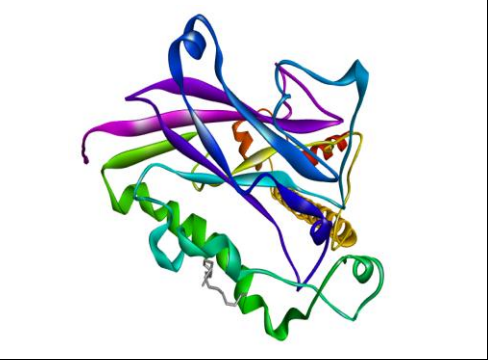


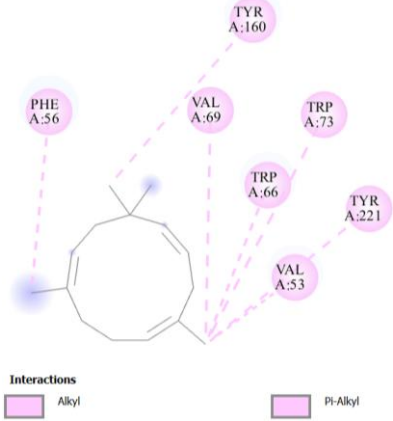
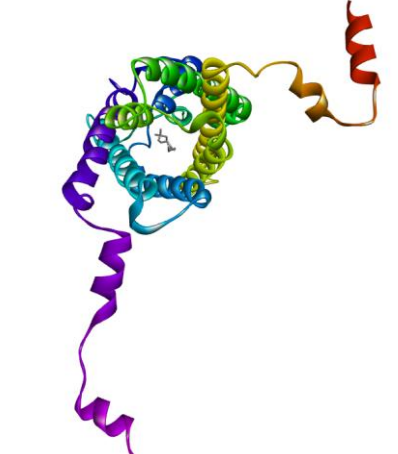
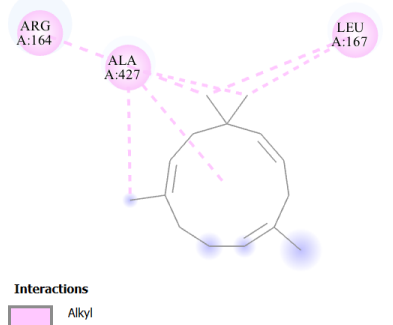
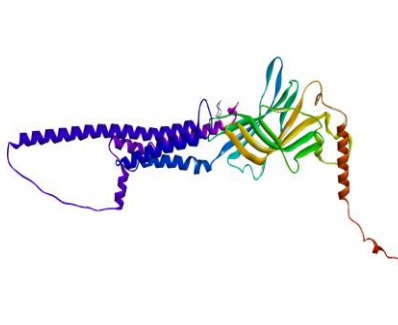
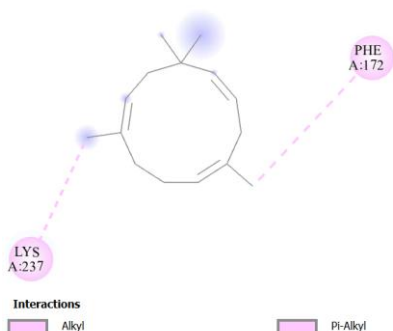
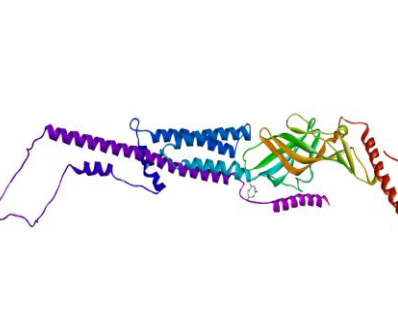
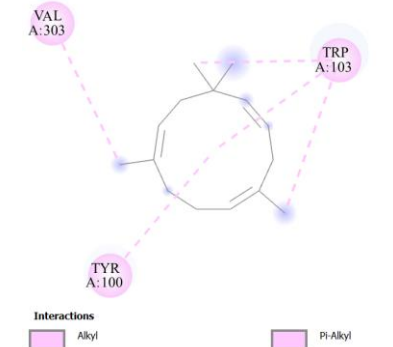
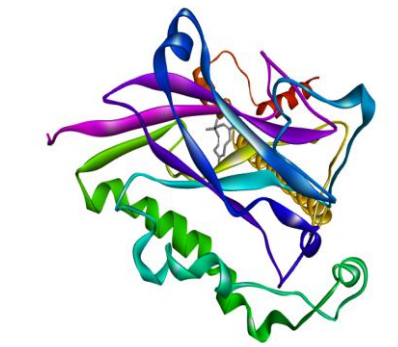
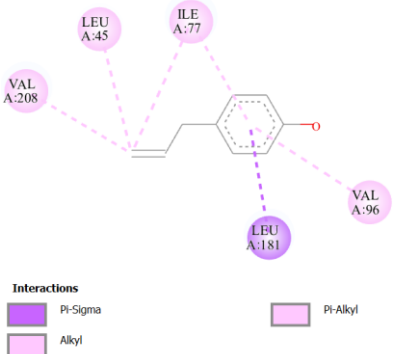
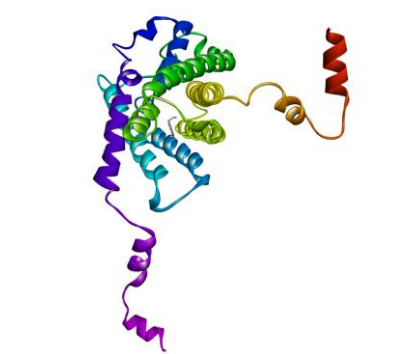
(b) Eugenol-GluCl	Interactions - Conventional Hydrogen Bond - Pi-Cation - Alkyl - Pi-Alkyl	
(c) Eugenol-nAChR	Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Alkyl - Pi-Alkyl	
(d) Eugenol-Na ⁺ /K ⁺ -ATPase	Interactions - Conventional Hydrogen Bond - Alkyl - Pi-Alkyl	
(e) α-Selinene-AQP	Interactions - Alkyl - Pi-Alkyl	
(f) α-Selinene-GluCl	Interactions - Alkyl - Pi-Alkyl	

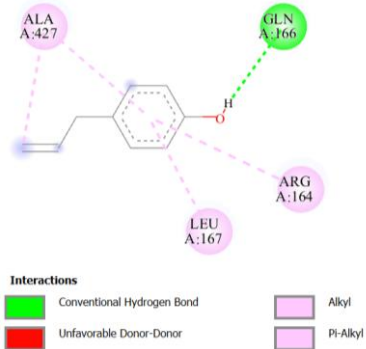
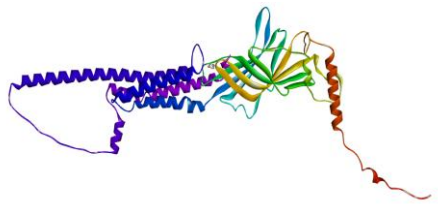
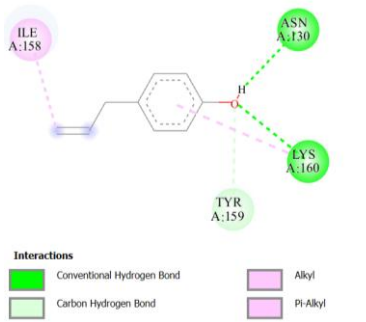
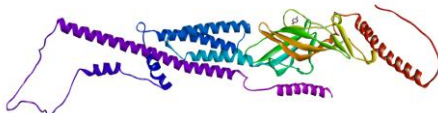
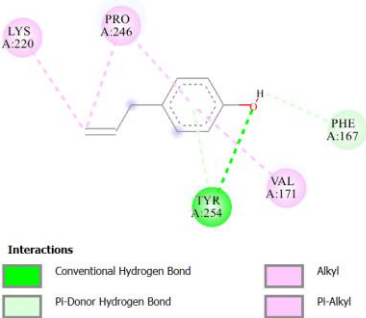
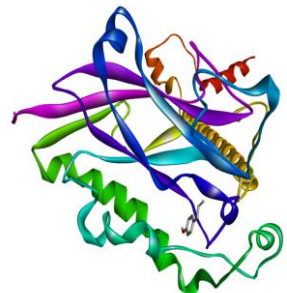
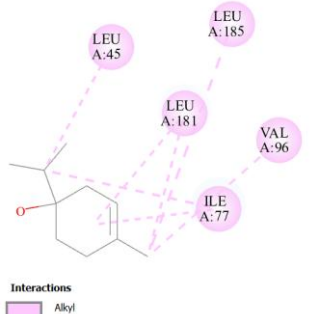
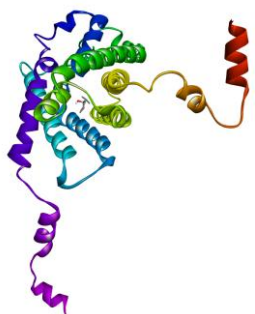
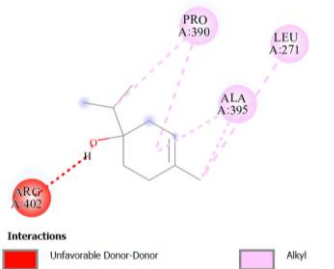
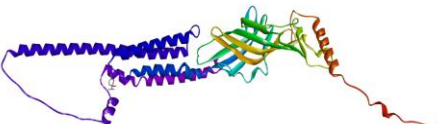
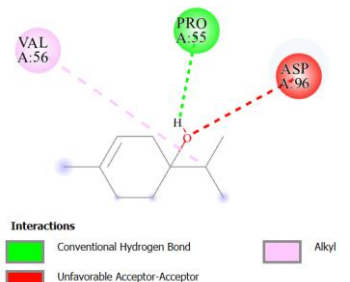
(g) α -Selinene-nAChR	 Interactions - Pi-Sigma - Alkyl - Pi-Alkyl	
(h) α -Selinene- Na^+/K^+ -ATPase	 Interactions - Alkyl - Pi-Alkyl	
	 Interactions - Alkyl - Pi-Alkyl	
(i) β -Selinene-GluCl	 Interactions - Pi-Sigma - Alkyl - Pi-Alkyl	
(j) β -Selinene-nAChR	 Interactions - Pi-Sigma - Alkyl - Pi-Alkyl	

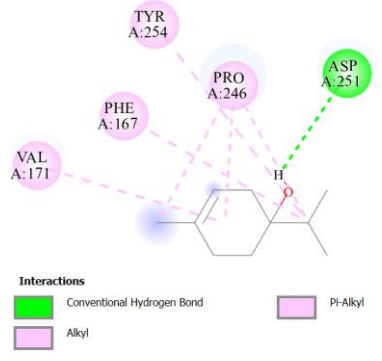
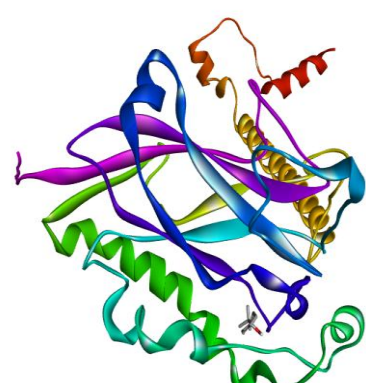
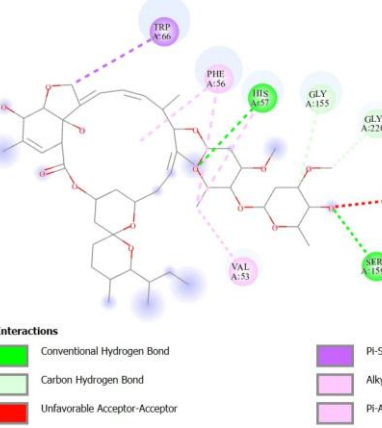
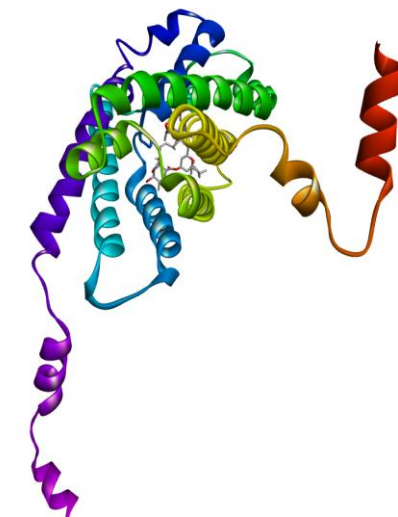
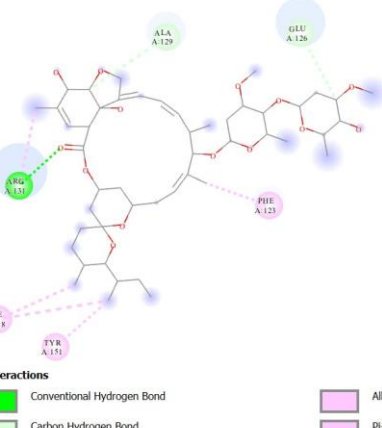
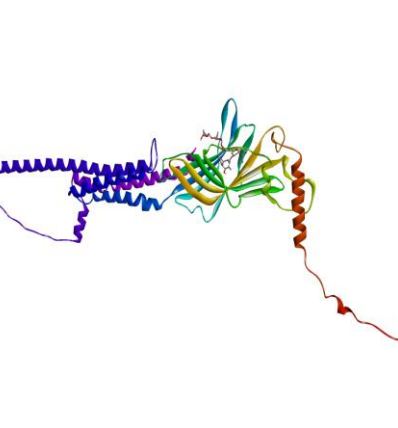
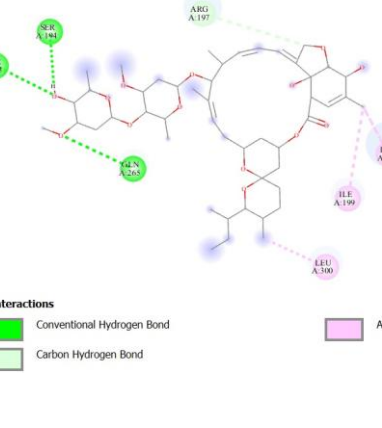
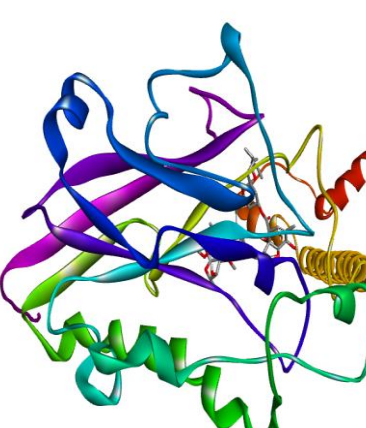
(k) β -Selinene- Na^+/K^+ -ATPase	 Interactions Alkyl	
(l) 4-Allylphenyl acetate-AQP	 Interactions - Conventional Hydrogen Bond - Pi-Sigma - Alkyl - Pi-Alkyl	
(m) 4-Allylphenyl acetate-GluCl	 Interactions - Conventional Hydrogen Bond - Pi-Pi Stacked - Alkyl - Pi-Alkyl	
(n) 4-Allylphenyl acetate-nAChR	 Interactions - Conventional Hydrogen Bond - Pi-Donor Hydrogen Bond - Alkyl	
(o) 4-Allylphenyl acetate- Na^+/K^+ -ATPase	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Donor Hydrogen Bond - Pi-Pi T-shaped - Alkyl - Pi-Alkyl	
(p) 3-Allyl-6-methoxyphenyl acetate-AQP	 Interactions - Conventional Hydrogen Bond - Pi-Sigma - Alkyl - Pi-Alkyl	

(q) 3-Allyl-6-methoxyphenyl acetate-GluCl	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Pi Stacked - Alkyl - Pi-Alkyl	
(r) 3-Allyl-6-methoxyphenyl acetate-nAChR	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Pi-Donor Hydrogen Bond - Alkyl - Pi-Alkyl	
(s) 3-Allyl-6-methoxyphenyl acetate-Na⁺/K⁺-ATPase	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Alkyl - Pi-Alkyl	
(t) β-Caryophyllene-AQP	 Interactions - Alkyl - Pi-Alkyl	
(u) β-Caryophyllene-GluCl	 Interactions - Alkyl - Pi-Alkyl	

(v) β -Caryophyllene-nAChR		
(w) β -Caryophyllene- Na^+/K^+ -ATPase		
(x) n-Heptadecane-AQP		
		
(y) n-Heptadecane-nAChR		
(z) n-Heptadecane- Na^+/K^+ -ATPase		

(aa) Humulene-AQP		
(ab) Humulene-GluCl		
(ac) Humulene-nAChR		
(ad) Humulene-Na⁺/K⁺-ATPase		
(ae) Chavicol-AQP		

(af) Chavicol-GluCl	 Interactions - Conventional Hydrogen Bond - Unfavorable Donor-Donor - Alkyl - Pi-Alkyl	
(ag) Chavicol-nAChR	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Alkyl - Pi-Alkyl	
(ah) Chavicol-Na ⁺ /K ⁺ -ATPase	 Interactions - Conventional Hydrogen Bond - Pi-Donor Hydrogen Bond - Alkyl - Pi-Alkyl	
(ai) (-)-Terpinen-4-ol-AQP	 Interactions Alkyl	
(aj) (-)-Terpinen-4-ol-GluCl	 Interactions - Unfavorable Donor-Donor - Alkyl	
(ak) (-)-Terpinen-4-ol-nAChR	 Interactions - Conventional Hydrogen Bond - Unfavorable Acceptor-Acceptor - Alkyl	

(al) (-)-Terpinen-4-ol- Na^+/K^+ -ATPase	 Interactions - Conventional Hydrogen Bond - Alkyl - Pi-Alkyl	
(am) Ivermectin-AQP	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Unfavorable Acceptor-Acceptor - Pi-Sigma - Alkyl - Pi-Alkyl	
(an) Ivermectin-GluCl	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Alkyl - Pi-Alkyl	
(ao) Ivermectin- Na^+/K^+ -ATPase	 Interactions - Conventional Hydrogen Bond - Carbon Hydrogen Bond - Alkyl	

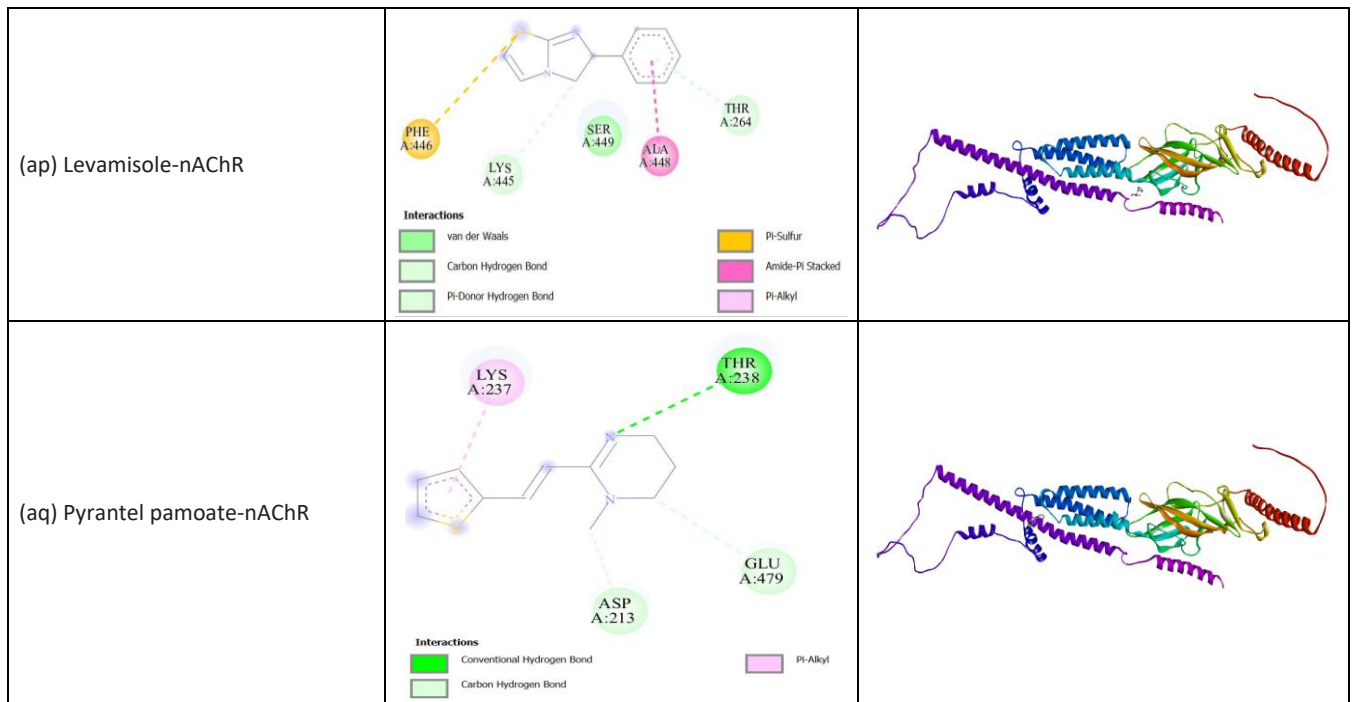


Figure 5: Representative molecular docking poses of major *PBEO* constituents and reference anthelmintics against selected nematode protein targets, shown as two-dimensional and three-dimensional interaction views. Panels show, for each complex, the two-dimensional interaction diagram (left) and the three-dimensional binding pose (right) against AQP, GluCl, nAChR, and Na⁺/K⁺-ATPase, respectively: (a–d) eugenol; (e–h) α-selinene; (i–k) β-selinene against GluCl, nAChR, and Na⁺/K⁺-ATPase; (l–o) 4-allylphenyl acetate; (p–s) 3-allyl-6-methoxyphenyl acetate; (t–w) β-caryophyllene; (x–z) *n*-heptadecane against AQP, nAChR, and Na⁺/K⁺-ATPase; (aa–ad) humulene; (ae–ah) chavicol; (ai–al) (–)-terpinen-4-ol; (am–ao) ivermectin against AQP, GluCl, and Na⁺/K⁺-ATPase; (ap) levamisole-nAChR; and (aq) pyrantel pamoate-nAChR. The poses illustrate predicted ligand orientations within the binding cavities of the nAChR, aquaporin, glutamate-gated chloride channel, and Na⁺/K⁺-ATPase. Representative predicted noncovalent interactions include hydrogen bonds, hydrophobic contacts, alkyl/π-alkyl interactions, and other stabilizing contacts. These docking poses illustrate potential interactions of *PBEO* constituents with multiple nematode targets but do not establish functional target modulation. *PBEO* = *Piper betle* L. essential oil, nAChR = Nicotinic acetylcholine receptor, AQP = Aquaporin, GluCl = Glutamate-gated chloride channel.

DISCUSSION

Novelty and integrated characterization of *PBEO* activity

To our knowledge, this is the first study to characterize the activity of *PBEO* against adult *A. galli* and to integrate mortality kinetics, external and internal structural alterations, chemical composition, and predicted molecular-target interactions within a single experimental framework. Previous studies have examined these components individually, but no study has combined them to evaluate *PBEO* against *A. galli*.

Previous investigations of *PBEO* have primarily focused on insecticidal and anticoccidial activities. Acetylcholinesterase inhibition has been proposed as a plausible neuromuscular mechanism underlying its insecticidal activity [13]. *PBEO* has also demonstrated anticoccidial activity against *E. tenella*, including disruption of the oocyst wall and inhibition of sporogony, with an IC₅₀ of 1.31% at 72 h [10]. However, neither application involved a helminth. The only previous report evaluating *P. betle* against nematodes used a methanolic leaf extract combined with *A. catechu* seed extract against adult *Haemonchus* spp. [15]. That preparation inhibited worm motility and caused cuticular fissuring and leakage of internal contents, as demonstrated by SEM [15]. However, the study differed substantially from the present investigation because it evaluated a combined solvent extract rather than *PBEO*, involved a ruminant trichostrongylid rather than an avian ascaridid, and did not integrate histopathology, chemical characterization, and molecular docking.

Essential oils and related plant preparations previously evaluated against *A. galli* have also focused on a narrower range of biological endpoints. Star anise oil reduced fecal egg counts, egg hatchability, and worm motility [11], whereas clove leaf extract caused concentration- and time-dependent mortality accompanied by cuticular damage [12]. Ultrastructural alterations in *A. galli* have been demonstrated following exposure to synthetic anthelmintics [16] and crude plant extracts [17–19], but these investigations did not combine surface ultrastructure with histopathological examination of internal tissues, GC–MS characterization of the tested

preparation, and prediction of molecular-target interactions. The present findings therefore extend the existing literature by linking adulticidal activity with external and internal structural alterations and the chemical composition of PBEO.

Concentration-dependent anthelmintic activity

PBEO demonstrated measurable *in vitro* anthelmintic activity against adult *A. galli*. The effect was strongest at 10% PBEO, which caused complete mortality by 160 min, whereas 1% PBEO produced partial but statistically significant mortality. In contrast, the lower concentrations produced minimal or no mortality, demonstrating a clear concentration-dependent response under the experimental conditions.

The estimated LC₅₀ of 0.71% further characterized the concentration–response relationship. The difference between the LC₅₀ and the 10% concentration required to achieve complete mortality within 160 min reflects the relatively shallow slope of the concentration–response curve. Based on the fitted probit model ($y = 0.255 + 1.692x$, where x represents log₁₀ concentration [% v/v] and y represents the normal equivalent deviate), the estimated LC₉₀ was 4.04%; higher lethal concentrations were not estimated because they would require extrapolation beyond the tested concentration range. The model predicted 97.42% mortality at 10% PBEO and 60.06% at 1%, closely corresponding to the observed values of 100% and $56.67 \pm 34.44\%$, respectively. Given the considerable between-replicate variability (Table 2), the confidence interval of the LC₅₀ should be interpreted with caution.

The relatively broad concentration-response range may reflect the chemical complexity of PBEO. However, the present experimental design does not establish whether this response arises from cumulative membrane partitioning, interactions among multiple constituents, or simultaneous engagement of several biological targets. These possibilities therefore require direct experimental investigation.

Cuticular damage and structural alterations

The biological activity of PBEO is consistent with previous evidence that essential oils can exert antiparasitic effects through interactions with biological membranes [9]. Essential oils are predominantly lipophilic and can partition into membranes, thereby altering membrane fluidity, permeability, and membrane-associated functions [30]. In helminths, the cuticle is a highly organized extracellular structure composed of cross-linked collagens, cuticlins, glycoproteins, and associated lipids. Beyond providing environmental protection, it supports locomotion through its association with underlying musculature and participates in osmoregulation, nutrient exchange, and sensory functions [31]. Damage to this interface may therefore adversely affect structural integrity and physiological homeostasis [8, 32].

SEM revealed prominent alterations in PBEO-treated *A. galli*, including lip abrasion, cuticular folding, disruption of annular architecture, and surface disintegration. These findings support the cuticle as an important structural site affected following PBEO exposure. Comparable damage to the cuticle and buccal capsule has been reported after *Haemonchus contortus* exposure to eugenol-rich *Ocimum micranthum* essential oil [33].

Histopathological examination further demonstrated that structural alterations were not restricted to the external surface. PBEO-treated worms exhibited deformation of intrauterine eggs, uterine disorganization, cuticular detachment, and fragmentation of underlying fibrillar musculature. These observations are compatible with effects extending beyond the external cuticle to reproductive and somatic tissues. Similar cuticular wrinkling, folding, and internal structural alterations have been reported after exposure of *A. galli* to plant-derived preparations from *Veitchia merrillii*, *Millettia pachycarpa*, *Acacia oxyphylla*, *Imperata cylindrica*, and *A. catechu* [17–19]. Nevertheless, the present findings demonstrate structural association rather than the sequence or molecular mechanism by which these lesions developed.

Chemical composition and potential contribution of major constituents

GC-MS profiling showed that PBEO was chemically diverse, with 50 chromatographic peaks, and that eugenol accounted for 8.48% of the total profile across two identified peaks. Eugenol is a phenylpropanoid commonly reported in aromatic plants, including clove and betel-related essential oils, and has been associated with antimicrobial, insecticidal, and anthelmintic activities. Eugenol-containing preparations have been linked to acetylcholinesterase inhibition, neuromuscular disruption, and metabolic impairment in helminths [34, 35].

The present PBEO profile also contained α -selinene, humulene, chavicol, 4-allylphenyl acetate, 3-allyl-6-methoxyphenyl acetate, (–)-terpinen-4-ol, and other terpenoid and phenylpropanoid constituents. Essential oils can exhibit biological activity through combined contributions from multiple chemically distinct components. Terpenoids have been associated with alterations in membrane properties and protein function, whereas

phenylpropanoids may influence neuromuscular or enzymatic pathways. Therefore, based on the present findings, the observed activity cannot be attributed to eugenol alone. Bio-guided fractionation and testing of individual compounds and defined combinations will be required to determine which constituents contribute most strongly to the anthelmintic activity of PBEO.

Predicted interactions with nematode molecular targets

Molecular docking was used to investigate potential interactions between 10 selected PBEO constituents and four nematode targets: AQP, GluCl, nAChR, and Na⁺/K⁺-ATPase. Predicted interactions were compared with those of ivermectin, levamisole, and pyrantel pamoate. These reference compounds represent established anthelmintic pharmacological classes and provided a framework for comparing predicted binding energies and residue-interaction patterns.

For AQP, ivermectin interacted through residues including His57, Ser159, Gly155, Gly220, Trp66, Val53, and Phe56. Several PBEO constituents, notably β -caryophyllene (−7.2 kcal/mol), also interacted with residues within this region, whereas 4-allylphenyl acetate (−7.2 kcal/mol) occupied an adjacent hydrophobic cavity lined by Ile77, Val96, Leu181, Leu185, and Phe204; both predicted affinities were weaker than that of ivermectin (−9.3 kcal/mol). PBEO constituents additionally interacted with residues including Val69, Trp73, and Tyr221. These findings indicate that several PBEO components can occupy predicted AQP binding cavities, although docking alone cannot determine whether these interactions alter AQP function or water transport.

For GluCl, ivermectin interacted with Arg131, Glu126, Ala129, Ile118, Phe123, and Tyr151. PBEO constituents exhibited different predicted interaction patterns involving residues such as Arg385, Tyr386, Pro390, Ala395, Phe399, and Arg402. Humulene showed a predicted affinity of −6.9 kcal/mol, while eugenol and 4-allylphenyl acetate displayed additional interactions involving Arg402, Ala395, and Tyr386. The distinct predicted residue contacts suggest that PBEO constituents may occupy binding regions that differ from those predicted for ivermectin. However, whether these compounds function as agonists, antagonists, or allosteric modulators cannot be determined from molecular docking alone. In addition, because GluCl was docked as a single subunit, the inter-subunit transmembrane site occupied by ivermectin in the pentameric 3RHW structure was not available; the ivermectin–GluCl pose therefore reflects an alternative cavity of the monomer and serves only as a within-model reference.

For Na⁺/K⁺-ATPase, ivermectin interacted with Tyr133, Ser194, Gln265, Arg197, Pro83, Ile92, Ile199, and Leu300. In contrast, PBEO constituents, particularly α -selinene, eugenol, and 4-allylphenyl acetate, showed predicted interactions involving Tyr254, Phe167, Pro246, and Ile250, with affinities ranging from −6.3 to −5.1 kcal/mol. These results indicate potential binding to Na⁺/K⁺-ATPase; however, functional inhibition of the pump and subsequent disruption of ion gradients were not evaluated experimentally and therefore remain hypotheses requiring validation.

For nAChR, levamisole interacted with Thr264, Lys445, Ser449, Phe446, and Ala448, whereas pyrantel pamoate interacted with Thr238, Asp213, Glu479, and Lys237. Several PBEO constituents also interacted with residues within the nAChR structure. For example, 4-allylphenyl acetate and 3-allyl-6-methoxyphenyl acetate interacted with Thr238 and Arg235, while α -selinene showed a predicted affinity of approximately −6.0 kcal/mol. Eugenol and chavicol also formed predicted hydrogen bonds with selected receptor residues. These findings indicate potential receptor engagement, but the pharmacological nature of these interactions cannot be inferred without functional receptor assays.

Overall, docking predicted interactions of PBEO constituents with several pharmacologically relevant nematode proteins rather than with a single target. This multi-target pattern provides testable hypotheses regarding the molecular basis of PBEO activity. However, binding affinity values generated by docking represent computational predictions and should not be interpreted as evidence of receptor activation, inhibition, therapeutic potency, or resistance-circumvention without experimental validation.

Relationship between docking predictions and experimental evidence

Predicted binding affinity alone is insufficient for prioritizing active PBEO constituents. Eugenol showed only moderate predicted affinities in this docking analysis (−6.1 to −5.1 kcal/mol), yet it has strong independent experimental support for nematode activity. Eugenol inhibited *C. elegans* at a median effective dose (ED₅₀) of 62.1 μ g/mL [36] and showed high larvicidal activity at 1% and 0.1% [37]. Electrophysiological evidence also shows that eugenol can modulate levamisole-sensitive nicotinic and UNC-49 GABA-A receptors [32], findings consistent with the predicted nAChR interaction observed in the present study. Similarly, eugenol-rich *O. micranthum* essential oil inhibited

adult *H. contortus* motility with a half-maximal effective concentration (EC₅₀) of 0.046 mg/mL [33], while PBEO itself has demonstrated acetylcholinesterase-inhibitory activity [13].

In contrast, several hydrocarbon terpenoids produced comparatively favorable docking scores despite weaker independent evidence of nematicidal activity. β -Caryophyllene, for example, was not nematicidal against *Meloidogyne incognita* at concentrations up to 5 mg/mL [38], and hydrocarbon terpenoids have generally shown lower activity than oxygenated and phenolic terpenoids in comparative assessments [39]. These observations highlight a key limitation of docking: favorable predicted binding does not necessarily reflect biological potency because it does not adequately account for factors such as solubility, membrane partitioning, target accessibility, metabolism, and compound concentration.

The phenylpropanoid fraction of PBEO, including eugenol, chavicol, chavicol acetate, and chavibetol acetate, therefore represents a logical group for further experimental investigation because both the present chemical profile and independent biological evidence support their potential relevance. The sesquiterpene fraction should also be investigated experimentally, particularly for possible effects on cuticular or membrane properties.

Strengths and limitations

A major strength of this study was the integration of mortality measurements with SEM, histopathology, GC–MS profiling, and molecular docking. Mortality assays establish biological activity but cannot independently identify associated structural alterations. The combination of external surface damage and internal tissue lesions provides complementary morphological evidence of the effects of PBEO exposure. GC–MS characterization further defines the chemical preparation evaluated and provides a foundation for subsequent standardization and bio-guided fractionation. Such chemical characterization is particularly important for essential oils because their composition may vary according to geographic origin, plant age, extraction procedure, storage conditions, and supplier.

Several limitations should also be considered. The *in vitro* immersion model exposes adult worms directly to PBEO and therefore does not reproduce the pharmacokinetic, physiological, and environmental conditions encountered within a living chicken. Structural observations were obtained from worms exposed to the highest PBEO concentration and do not establish whether similar lesions occur across the full concentration range. The molecular docking component used *C. elegans* proteins as structural surrogates, docked as single-chain predicted models rather than as assembled receptor complexes, and provides predicted rather than experimentally confirmed target interactions. Furthermore, the study performed no molecular dynamics simulations or functional target-based assays. These limitations limit conclusions about the precise mechanism of action and underscore the need for experimental validation.

Safety and translational considerations

The effective concentration of 10% used in this study represents an *in vitro* immersion concentration applied directly to adult worms and should not be interpreted as an equivalent oral or systemic dose in chickens. Luminal exposure, systemic absorption, metabolism, and tissue distribution are distinct pharmacological parameters; therefore, an *in vitro* concentration cannot be directly converted into an administered dose.

The principal constituent identified in PBEO was eugenol (8.48%), for which previous toxicological assessments have reported acute oral median lethal dose (LD₅₀) values of 1,930–2,680 mg/kg body weight in rats and approximately 3,000 mg/kg body weight in mice [40]. An acceptable daily intake of 0–2.5 mg/kg body weight has also been established for eugenol by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives [40], while oral administration of *P. betle* leaf extract has reportedly yielded an LD₅₀ exceeding 5,000 mg/kg body weight in mice [14]. However, these data relate to eugenol or *P. betle* preparations tested under different experimental conditions and do not establish the safety of the PBEO preparation evaluated in the present study.

The applied relevance of PBEO should also be considered in relation to the parasitological context of the study area. Smallholder livestock in Boyolali Regency, from which the present *A. galli* specimens were obtained, carry a substantial and taxonomically diverse parasite burden. Intestinal helminths, including *A. galli*, are frequently recorded in domestic chickens in this district [20]; *Fasciola gigantica* was detected in 25.12% (147/585) of cattle slaughtered at a local abattoir [41]; ectoparasites affected 97.85% (637/651) of the sheep and goats examined, and farmer surveys indicated limited knowledge of parasite life cycles and inconsistent acaricide use [42]; and *Babesia naoakii* was detected by polymerase chain reaction in 34.39% (87/253) of apparently healthy cattle [43]. In such systems, parasite control depends predominantly on synthetic compounds, a practice that favors suboptimal dosing and the selection of drug resistance [6, 7, 42]. Locally available plant-derived

preparations therefore constitute a pragmatic area of investigation. Using parasites from the same district, an *Artemisia vulgaris* extract inhibited *F. gigantica* egg hatching and induced tegumental and internal damage in adult flukes [44], and *C. nardus* essential oil produced complete *in vitro* mortality of adult *A. galli* accompanied by cuticular and reproductive lesions [23]. The present findings extend this regional evidence base to PBEO. PBEO prepared from *P. betle* leaves collected in the same area has also exhibited larvicidal, adulticidal, and oviposition-deterrent activity against *Aedes aegypti* [21]. This activity is of additional relevance because Indonesian *Ae. aegypti* populations have developed resistance to routinely applied pyrethroid and carbamate insecticides, with mortality below 50% following exposure to 0.75% permethrin and 0.1% bendiocarb and a significant association between the V1016G voltage-gated sodium channel mutation and the permethrin-resistant phenotype [45]. Resistance to synthetic chemical agents therefore represents a shared constraint on veterinary parasite control and public-health vector control in Indonesia, reinforcing the rationale for investigating chemically complex plant-derived preparations such as PBEO.

From an applied perspective, PBEO may warrant further investigation as a complementary candidate for controlling *A. galli* rather than as a direct replacement for licensed anthelmintics. Its rapid adulticidal activity at the highest tested concentration, along with the associated structural alterations, supports further evaluation. However, formulation characteristics, palatability, host safety, pharmacokinetics, residue profiles, effective dosing, and efficacy against naturally acquired infections must be established before practical application. Standardized PBEO preparations or defined combinations of selected phenylpropanoid and terpenoid constituents could subsequently be evaluated through controlled *in vivo* studies to determine their potential role in integrated parasite control strategies.

Future directions

Several priorities should guide future investigation of PBEO. First, bio-guided fractionation and testing of purified compounds are required to determine which constituents, or combinations of constituents, contribute most strongly to the observed anthelmintic activity and whether interactions among these compounds enhance efficacy. Second, the molecular docking predictions should be validated experimentally using *in vitro* biochemical, receptor-based, and electrophysiological assays targeting AQP, GluCl, nAChR, and Na⁺/K⁺-ATPase to determine whether the predicted interactions result in functional modulation. Third, the activity of PBEO should be evaluated against other developmental stages of *A. galli*, including eggs and larvae, as well as against other economically important poultry nematodes, to define its spectrum of activity. Fourth, because resistance to synthetic compounds has been documented in livestock helminths [6, 7] and in Indonesian *Ae. aegypti* populations [45], the activity of PBEO should be compared directly with that of conventional anthelmintics against susceptible and, where available, anthelmintic-resistant *A. galli* isolates.

Standardization of PBEO composition will also be essential before translational or field evaluation because essential oil composition can vary substantially according to geographic origin, plant chemotype, harvest season, extraction method, and storage conditions. Quality-control procedures should therefore include chromatographic fingerprinting and quantification of representative marker compounds, particularly eugenol, chavicol, and major sesquiterpenes. Such standardization is necessary to ensure reproducible chemical composition and biological activity across PBEO preparations. Subsequent investigations should evaluate formulation characteristics, stability, palatability, host safety, pharmacokinetics, residue profiles, effective dosing, and efficacy under controlled *in vivo* conditions in naturally infected poultry before field application can be considered.

CONCLUSION

PBEO demonstrated concentration- and time-dependent *in vitro* anthelmintic activity against adult *A. galli*, achieving 100% mortality at 10% after 160 min, with an estimated LC₅₀ of 0.71%. SEM and histopathological examinations revealed substantial alterations in the cuticle, reproductive structures, intrauterine eggs, and underlying musculature, while GC-MS profiling identified a chemically diverse mixture dominated by phenylpropanoid and terpenoid constituents, with eugenol as the most abundant identified compound. Molecular docking further predicted interactions of major PBEO constituents with AQP, GluCl, nAChR, and Na⁺/K⁺-ATPase.

These findings indicate that the adulticidal activity of PBEO is associated with extensive external and internal structural alterations and may involve contributions from multiple chemically diverse constituents. The predicted interactions with several nematode molecular targets provide hypotheses for potential target engagement but do not establish the mechanisms responsible for the observed anthelmintic activity.

From a practical perspective, PBEO represents a plant-derived candidate for further development as a

complementary approach to *A. galli* control in poultry. However, its potential application depends on establishing a standardized chemical composition and determining an effective and safe formulation, dose, administration route, residue profile, and efficacy under conditions relevant to poultry production.

A major strength of this study was the integrated evaluation of adult-worm mortality, SEM, histopathology, GC–MS chemical characterization, and molecular docking within a single experimental framework, enabling the biological effects of PBEO to be considered alongside its structural, chemical, and predicted molecular characteristics.

The study was limited by its *in vitro* adult-worm model, evaluation of structural alterations primarily at the highest PBEO concentration, and reliance on *C. elegans* proteins as structural surrogates for molecular docking. Furthermore, docking provides computational predictions and does not confirm receptor binding, activation, inhibition, or the functional mechanisms responsible for parasite mortality.

Future studies should prioritize bio-guided fractionation and testing of individual constituents and defined combinations, experimental validation of the predicted molecular targets, and evaluation of activity against eggs and larval stages of *A. galli*. Standardized PBEO preparations should subsequently be investigated through controlled *in vivo* studies in poultry to establish efficacy, safety, dosage, formulation requirements, pharmacokinetics, and residue profiles.

Overall, the integrated biological, structural, chemical, and computational findings provide a foundation for further investigation of PBEO as a plant-derived complementary candidate for controlling *A. galli*. Experimental target validation and controlled *in vivo* evaluation are essential before its practical use in poultry parasite management can be established.

DATA AVAILABILITY

Supplementary Figure S1 is provided in the manuscript. The datasets generated during the current study are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

The authors used ChatGPT (OpenAI) for sentence-level language polishing, formatting, and internal consistency checking only. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

AUTHORS' CONTRIBUTIONS

NMC: Conducted the investigation, including parasite collection and *in vitro* assays, performed formal analysis and visualization, and drafted the manuscript. PHH: Conceptualized and designed the study, supervised the study, acquired funding, and reviewed and edited the manuscript. HAN: Performed GC-MS and SEM analyses and reviewed the manuscript. PRF: Contributed to the investigation and data interpretation and reviewed the manuscript. SW: Performed molecular docking and formal analysis and reviewed the manuscript. NBM: Performed molecular docking and docking visualization and reviewed the manuscript. All authors have read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This research was funded by the Tropical OneHealth and EcoHealth Institute (TROPI) (grant no. 02/TROPI-GRANT/IV/2023) and by Universitas Sebelas Maret, Indonesia, through its research support program. Additional in-kind support was provided by the National Research and Innovation Agency (BRIN), Indonesia. The authors acknowledge the facilities and scientific and technical support of the Advanced Characterization Laboratory and the Genomic Laboratory, BRIN, Cibinong, Indonesia, for GC-MS and SEM analyses, and the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia, for histological processing.

COMPETING INTERESTS

The authors declare that they have no competing interests.

PUBLISHER'S NOTE

Veterinary World remains neutral with regard to jurisdictional claims in the published institutional affiliations.

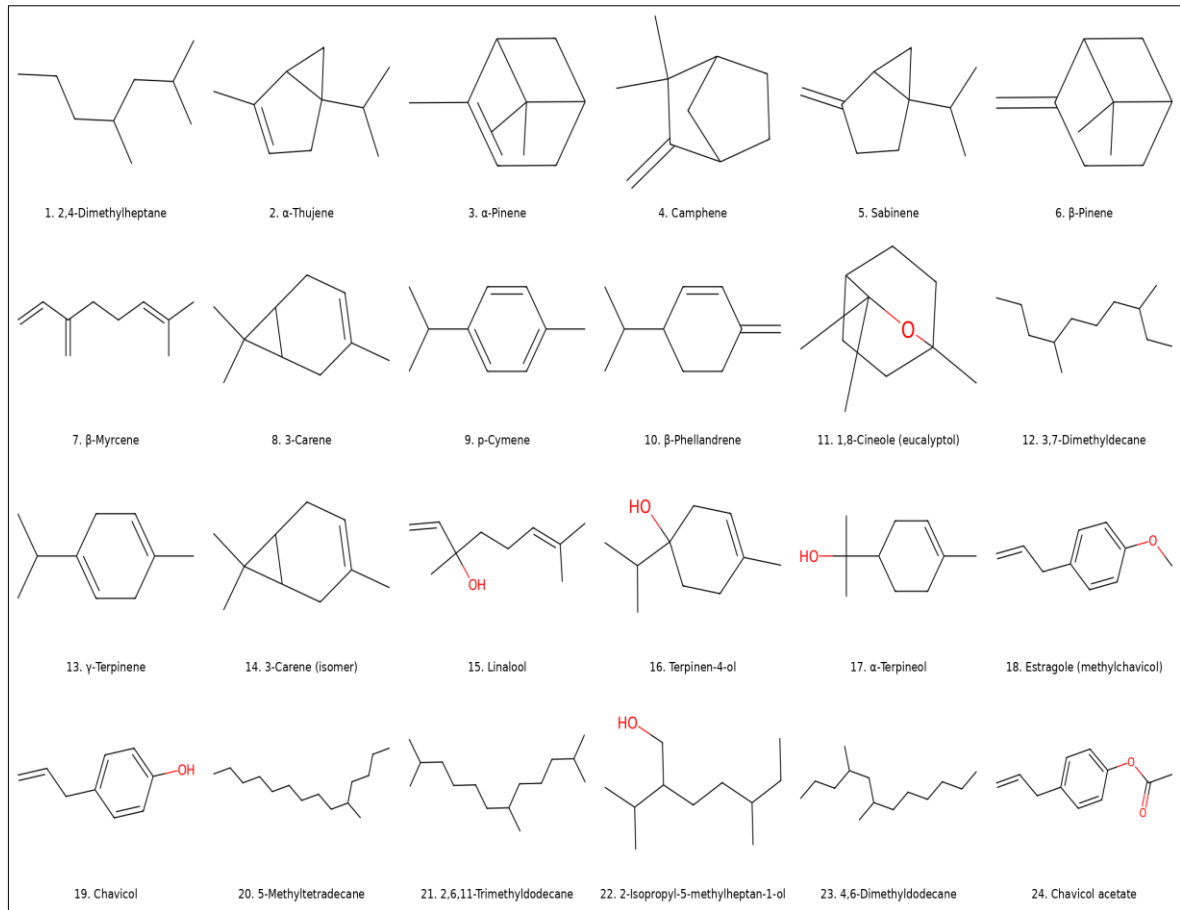
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Supplementary Figure S1. Two-dimensional chemical structures of the constituents of *Piper betle* L. essential oil identified by GC-MS (Table 1). Structures are numbered according to the peak numbering of Table 1. Panel A, peaks 1-24; panel B, peaks 25-50. Peak 43 is omitted because it remained unidentified. Structures were generated from the canonical SMILES in Table 1 (RDKit); structure 35 (α -humulene) was regenerated from the corrected SMILES.

Panel A - peaks 1-24



Panel B - peaks 25-50

