

In vitro anthelmintic activity of Aetoxylon sympetalum agarwood essential oil against adult *Ascaridia galli* and its putative multi-target mechanism of action against bioenergetic and cytoskeletal proteins

Dita Cahyani

Sebelas Maret University

Penny Hamid

`pennyhumaidahamid@staff.uns.ac.id`

Sebelas Maret University

Syahputra Wibowo

National Research and Innovation Agency (BRIN)

Nur Balqis Maulydia

National Research and Innovation Agency (BRIN)

Dinda Eka Pramesti

Sebelas Maret University

Intan Dwi Pangesti

Sebelas Maret University

Herjuno Ari Nugroho

National Research and Innovation Agency (BRIN)

Dwi Cahyo Budi Setiawan

Universitas Gadjah Mada

Margaretta Christita

National Research and Innovation Agency (BRIN)

Pamungkas Rizki Ferdian

National Research and Innovation Agency (BRIN)

Fabien Schultz

Bernhard Nocht Institute for Tropical Medicine

Research Article

Keywords: agarwood, sesquiterpenoids, anthelmintic, infectious disease

Posted Date: August 25th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10724505/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

In vitro anthelmintic activity of *Aetoxylon sympetalum* agarwood essential oil against adult *Ascaridia galli* and its putative multi-target mechanism of action against bioenergetic and cytoskeletal proteins

Dita Cahyani¹, Penny Humaidah Hamid^{1,2*}, Syahputra Wibowo³, Nur Balqis Maulydia³, Dinda Eka Pramesti¹, Intan Dwi Pangesti¹, Herjuno Ari Nugroho⁴, Margaretta Christita^{4,5}, Pamungkas Rizki Ferdian⁶, Dwi Cahyo Budi Setiawan⁷, Fabien Schultz^{8,9}

¹Faculty of Animal Science, Universitas Sebelas Maret, Indonesia, ²Genomic Department, Tropical OneHealth and EcoHealth Institute, Indonesia, ³Eijkman Research Center for Molecular Biology, National Research and Innovation Agency (BRIN), Indonesia, ⁴Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Indonesia, ⁵Research Collaboration Center for Agarwood, Institut Teknologi Bandung, Bandung, Indonesia, ⁶Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Indonesia, ⁷Department of Pharmacology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Indonesia, ⁸Ethnopharmacology and Zoopharmacognosy, Bernhard Nocht Institute for Tropical Medicine, Germany, ⁹Department of Agriculture and Food Sciences, Neubrandenburg University of Applied Sciences, Germany,

Corresponding author:

Penny Humaidah Hamid : pennyhumaidahhamid@staff.uns.ac.id

Co-author's email addresses:

Dita Cahyani : ditacahyani@student.uns.ac.id

Syahputra Wibowo : syah031@brin.go.id

Nur Balqis Maulydia : nur.balqis.maulydia@brin.go.id

Dinda Eka Pramesti : dindaekapramesti@student.uns.ac.id

Intan Dwi Pangesti : intandwip_28@student.uns.ac.id

- 26 Herjuno Ari Nugroho : herj001@brin.go.id
- 27 Dwi Cahyo Budi Setiawan : dwicahyobs@ugm.ac.id
- 28 Margaretta Christita : margaretta.christita@brin.go.id
- 29 Pamungkas Rizki Ferdian : pamu003@brin.go.id
- 30 Fabien Schultz : Fabien.Schultz@bnitm.de

Abstract

Ascaridia galli is the most prevalent and economically damaging nematode of chickens, and its control rests on a chemical base so narrow that anthelmintic resistance has already been confirmed in the species. Agarwood (*Aetoxylon sympetalum*) is a sesquiterpenoid-rich resinous wood of established pharmacological interest whose anthelmintic potential has never been examined. Herein, adult *A. galli* were immersed in *A. sympetalum* essential oil (ASEO) at 0.001-10% (v/v) and in levamisole, piperazine or ivermectin at five concentrations each, and mortality was scored at 10, 20, 40, 80 and 160 min ($n = 4$ replicates $\times$ 10 worms) and fitted by probit regression. ASEO killed worms in a concentration- and time-dependent manner (concentration $\times$ time interaction, $\chi^2(1) = 17.13$, $P < 0.001$), reaching 100% mortality at 1-10% within 160 min. Its median lethal concentration of 0.037% v/v (95% CI 0.007-0.202, heterogeneity-corrected) was statistically indistinguishable from ivermectin (0.062%, 0.039-0.100) and piperazine (0.024%, 0.016-0.034), though significantly higher than levamisole (0.0009%, 0.0006-0.0013), and median lethal times of 10.5 min at 10% and 34.5 min at 1% matched ivermectin at 1% (29.0 min). GC-MS resolved 100 peaks dominated by oxygenated sesquiterpenes, principally γ -eudesmol (10.93%), β -eudesmol (7.16%), α -eudesmol (5.79%), α -humulene (5.86% across two peaks), elemol (3.25%) and agarospirol (1.78%), and the five most abundant of these were docked against three conserved *A. galli* proteins: NADH-ubiquinone oxidoreductase chain 4 (UniProt S4UCE9), β -tubulin (R4VG92) and ATP synthase subunit 6 (S4UGP6). Affinities were strongest and most uniform at ATP synthase subunit 6, where α -humulene, β -eudesmol and agarospirol each reached -7.3 kcal/mol, approaching ivermectin (-7.5) and exceeding levamisole (-6.3) and piperazine (-3.3); agarospirol bound Complex I most strongly (-6.5) and γ -eudesmol β -tubulin most strongly (-6.6), with alkyl and π -alkyl contacts at Phe, Leu, Val and Pro residues predominating alongside conventional

hydrogen bonds. ASEO is therefore a potent *in vitro* nematicide against adult *A. galli*, matching ivermectin on a concentration basis.

Keywords: agarwood, sesquiterpenoids, anthelmintic, infectious disease

Introduction

Poultry meat and eggs are among the most affordable sources of animal protein worldwide, and demand continues to rise with population growth. Intensification of production has amplified the burden of parasitic disease, and among the gastrointestinal helminths of chickens, *Ascaridia (A.) galli*, a large ascaridid of the small intestine, is the most prevalent and economically important (Höglund *et al.*, 2023; Shifaw *et al.*, 2021). A systematic review of global surveys placed the pooled prevalence of helminth infection in chickens well above 50%, with the highest burdens in non-caged systems (Shifaw *et al.*, 2021). The parasite has a direct life cycle: embryonated eggs are ingested from a contaminated environment, hatch, and undergo a histotrophic phase in the intestinal mucosa before maturing in the lumen (Höglund *et al.*, 2023). Heavy infections cause mucosal damage, impaired nutrient absorption, reduced weight gain and depressed egg output and egg quality, and predispose birds to secondary infection (Sharma *et al.*, 2019). The expansion of cage-free and free-range husbandry, in which birds contact infective eggs in litter and soil, has further heightened the epidemiological relevance of *A. galli*, and reports in European laying hens rose after the 2012 ban on conventional battery cages (Höglund *et al.*, 2023).

Control has long depended on synthetic anthelmintics, most of which act on the parasite's neuromuscular system to induce paralysis and promote expulsion. The principal drug classes engage distinct molecular effectors: benzimidazoles bind β -tubulin and disrupt microtubule-dependent processes; the cholinergic agonists levamisole and pyrantel activate nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction to cause spastic paralysis;

piperazine acts as a GABA-receptor agonist; and macrocyclic lactones such as ivermectin open glutamate-gated chloride channels to produce flaccid paralysis (Zirintunda *et al.*, 2021). Because persistence in the gut depends on coordinated motility, these effectors are well-validated, druggable targets. However, repeated, frequently sub-therapeutic and indiscriminate use of a narrow drug arsenal has driven the emergence of resistance across all major classes (Zirintunda *et al.*, 2021). The problem is now documented in poultry ascarids specifically: fenbendazole, the only approved treatment in many jurisdictions, has lost efficacy against *A. galli* and against the co-endemic *Heterakis gallinarum*, with resistance confirmed in a first *A. galli* isolate and in every *H. gallinarum* isolate examined in a recent survey (Collins *et al.*, 2025). The same trajectory has been traced in other host-parasite systems, most notably triclabendazole resistance in *Fasciola hepatica* (Kelley *et al.*, 2016). Resistance, drug residues in eggs and meat, and tightening regulation together create an urgent need for anthelmintic alternatives that are safe, effective and mechanistically distinct.

Plant-derived compounds, and essential oils in particular, have accordingly attracted attention as complements or replacements for conventional drugs. As chemically complex mixtures of terpenoids, phenylpropanoids and other volatiles, essential oils can engage several parasite targets simultaneously, an attribute that lowers the probability of resistance emerging against any single mechanism (Zirintunda *et al.*, 2021). Several act on the very receptors exploited by synthetic drugs. The monoterpenoid carvacrol interacts directly with the nAChR and GABA receptors of *Ascaris suum*, producing rapid, concentration-dependent paralysis (Trailović *et al.*, 2015), and antagonises both nicotine- and morantel-sensitive receptor subtypes in *Parascaris* sp. (Trailović *et al.*, 2021). Thymol, carvacrol and eugenol act as negative allosteric modulators of the levamisole-sensitive nAChR and the UNC-49 GABA_A receptor of *Caenorhabditis elegans* (Hernando *et al.*, 2019), menthol behaves as a positive allosteric

modulator of levamisole-sensitive nAChRs (Choudhary *et al.*, 2019), and *trans*-cinnamaldehyde engages multiple Cys-loop targets and acts synergistically with levamisole and monepantel (Hernando *et al.*, 2025). Carvacrol and geraniol in combination are synergistically nematocidal, reducing individual LC₅₀ values almost ten-fold (Stojković *et al.*, 2025). Plant preparations also damage the nematode surface directly: ethanolic betel-nut extract produces gross cuticular disruption in adult *A. galli* (Balqis *et al.*, 2017). Comparable ovicidal and larvicidal activity has been reported for *Moringa oleifera* (Hegazi *et al.*, 2018), *Zingiber officinale* (Moazeni and Khademolhoseini, 2016), curcumin-based nanoformulations (Oyeyemi *et al.*, 2018; Alcala-Canto *et al.*, 2023), *Mentha pulegium* oil (Bouajila and Eddouks, 2021) and *Artemisia vulgaris* (Nurlaelasari *et al.*, 2023).

Agarwood is the fragrant, resin-impregnated heartwood that forms in trees of the family Thymelaeaceae following wounding and biotic or abiotic stress, when the activation of host defence responses, frequently but not invariably in association with colonising microbial communities, redirects metabolism towards the deposition of sesquiterpenes and 2-(2-phenylethyl)chromones (Sampasivam *et al.*, 2024; Hermawaty *et al.*, 2025; Herath and Jinendra, 2023). Agarwood-producing trees occur across South and Southeast Asia and parts of southern China, and the resinous wood and the oil distilled from it are among the most highly valued natural products in the world, with a long history of use across Asia and the Middle East as incense, in perfumery, as an essential oil, in traditional medicine, and in cultural and religious practice (Xie *et al.*, 2024; Sampasivam *et al.*, 2024). This sustained demand has driven intensive exploitation of agarwood-producing species, particularly within the principal producing genera *Aquilaria* and *Gyrinops*, because natural resin formation is spatially heterogeneous, sporadic, slow, and limited to a small fraction of wild trees (Tan *et al.*, 2019; Sampasivam *et al.*, 2024).

131

132 Because resin deposition cannot be detected from outside the stem, collectors commonly fell
133 or hack whole trees to locate relatively small quantities of resin-rich wood, a destructive
134 practice that has hampered natural regeneration and, together with habitat loss, has severely
135 depleted wild populations across the agarwood-producing genera (Tan *et al.*, 2019; Akter *et*
136 *al.*, 2013; Sampasivam *et al.*, 2024). Two distinct frameworks govern this situation and
137 should not be conflated. International trade is regulated under CITES Appendix II, which lists
138 all species of *Aquilaria* and *Gyrinops* under annotation #14; *A. malaccensis* was listed in
139 1995 and the remaining *Aquilaria* species together with all *Gyrinops* species in 2005 (CITES,
140 2026). This is a taxon-based trade-control instrument rather than a prohibition, and it does not
141 extend to every tree that yields a commercially traded agarwood: *Aetoxylon sympetalum*, the
142 species examined here, is not listed in the CITES Appendices, so its wood and oil enter
143 international trade outside the CITES permitting system and are subject to national legislation
144 alone (see below). CITES listing is likewise not equivalent to an IUCN Red List assessment,
145 and the two do not coincide across agarwood taxa: eight *Aquilaria* species, including *A.*
146 *sinensis*, are assessed as Endangered on the IUCN Red List and five of the twenty assessed
147 *Aquilaria* species as Critically Endangered (Sampasivam *et al.*, 2024; Liu *et al.*, 2013; Ullah,
148 2020), whereas several other agarwood-producing taxa, *Aetoxylon* among them, remain
149 globally unassessed.

150

151 These conservation concerns have stimulated interest in plantation cultivation and artificial
152 induction of agarwood formation as alternatives to harvesting from natural populations (Tan *et*
153 *al.*, 2019; Sampasivam *et al.*, 2024). Mass cultivation of agarwood-producing trees across
154 Asian countries has become a primary strategy to address supply shortages while reducing
155 pressure on wild populations (Tan *et al.*, 2019). *A. malaccensis* and *G. versteegii* are the most

cultivated agarwood-producing species in Malaysia and Indonesia, respectively, providing income for local populations without harming natural ecosystems (Yunus *et al.*, 2024). By simulating natural wounding and infection, artificial induction technologies expedite agarwood production in plantation trees to a 1-2 year timeframe (Sampasivam *et al.*, 2024; Herath and Jinendra, 2023). Current methods span mechanical (e.g., nailing, drilling, bark removal, trunk breaking, and the whole-tree agarwood-inducing technique Agar-Wit; Herath and Jinendra, 2023; Tan *et al.*, 2019; Liu *et al.*, 2013), biological (fungal inoculant use, e.g., *Fusarium solani* or *Xylaria*; Faizal *et al.*, 2022; Chhipa *et al.*, 2017), chemical (chemical inducers; Ngadiran *et al.*, 2023) and integrated combination approaches (Auri *et al.*, 2025; Chen *et al.*, 2025).

Natural resin accumulation occurs only in localized regions of the stem where host defense responses have been activated following wounding, animal grazing, insect attack, or microbial invasion, whereas adjacent tissues remain as normal, non-resinous sapwood (Sampasivam *et al.*, 2024). As a result, a single piece of agarwood-bearing wood often exhibits a clear distinction between the dark, resin-rich agarwood and the surrounding pale wood that has not undergone sufficient resin deposition (Figure S1). This localized pattern reflects the progressive spread of infection and host defense responses within the stem. The resin-rich dark portion is the commercially valuable fraction that is selectively harvested and distilled to obtain agarwood essential oil, which contains the majority of the sesquiterpenes and other volatile compounds responsible for the characteristic aroma and diverse biological activities of agarwood. The resin accumulated within these infected tissues is particularly rich in sesquiterpenes and 2-(2-phenylethyl) chromones, compounds responsible for both the characteristic fragrance and the diverse pharmacological activities of agarwood. Its principal bioactive constituents are sesquiterpenes and 2-(2-phenylethyl)chromones, which carry anti-inflammatory, antioxidant, immunomodulatory and neuroactive properties (Xie *et al.*, 2024).

Guaiane- and humulane-type sesquiterpenes isolated from *Aquilaria malaccensis* agarwood inhibit lipopolysaccharide-induced nitric-oxide production in RAW 264.7 macrophages with IC₅₀ values in the low-micromolar range (Ma *et al.*, 2021), and whole agarwood essential oil exerts antidepressant and neuroprotective effects through NF- κ B/I κ B- α downregulation and BDNF/TrkB/CREB upregulation (Zhang *et al.*, 2025). Sesquiterpenes are also predicted, *in silico*, to bind human nAChR subtypes with appreciable affinity (Fakih *et al.*, 2025), which makes a neuroactive contribution plausible in nematodes as well.

Aetoxylon sympetalum (Steenis & Domke) Airy Shaw (Thymelaeaceae), the accepted name and authority followed by Plants of the World Online and World Flora Online (POWO, 2026), is the sole species of the monotypic genus *Aetoxylon* and is endemic to Borneo, where it occurs in lowland mixed dipterocarp and kerangas forest at elevations up to about 100 m in Indonesian Kalimantan and Malaysian Sarawak. It is known locally as *gaharu buaya* (“crocodile agarwood”), and its fragrant resinous heartwood is harvested and hydro- or steam-distilled to yield gaharu buaya oil, traded internationally as “white oud”, alongside incense and perfumery products. The conservation position of the species differs in one important respect from that of the CITES-listed agarwood genera: *Aetoxylon* is not listed in the CITES Appendices, so trade in its wood and oil is not subject to CITES Appendix II permitting, and the species is instead protected at the national level only. In Indonesia it is nationally categorised as Critically Endangered and has been legally protected since 2018 under Minister of Environment and Forestry Decree No. 106/2018, following intensified harvest pressure generated by agarwood demand; a recent survey of remnant forest in Mentebah District, Kapuas Hulu, West Kalimantan, located only nine mature and seven immature individuals within the area surveyed, and conservation responses have accordingly included *ex situ* propagation from seedlings and cuttings for population recovery (Kusumadewi *et al.*, 2024). The global

assessment is less settled: the species is currently flagged as predicted-threatened under the 2024 Angiosperm Extinction Risk Predictions curated by Kew, while a World Flora Online record displays a Vulnerable IUCN category (POWO, 2026), so no definitive Red List category should be asserted for it at present. Species-specific ethnomedicinal documentation for *A. sympetalum* likewise remains limited, and most medicinal claims attached to the collective term “agarwood” derive from other taxa, principally *Aquilaria*; we are not aware of any documented ethnoveterinary use of *A. sympetalum* against helminths of poultry, and the present study is therefore chemistry- and target-driven rather than ethnopharmacology-led. Chemically, *A. sympetalum* essential oil (ASEO) is distinguished by an abundance of eudesmane-, guaiane- and agarospirane-type sesquiterpenes and their oxygenated derivatives (Table 2; section 3.2), which underlie its characteristic aroma and provide a plausible basis for biological activity. Despite this distinctive composition, the biological activities of *A. sympetalum* remain poorly investigated, yet its anthelmintic potential is entirely unexplored.

Two properties make ASEO a rational candidate against *A. galli*. First, oxygenated sesquiterpenes are lipophilic molecules able to partition into and destabilise biological membranes, a mechanism demonstrated for cinnamon bark oil against bacterial membranes (Yang *et al.*, 2019). Second, terpenoid-rich oils are established modulators of nematode neuromuscular and metabolic function. The present study therefore (i) quantified the *in vitro* anthelmintic activity of ASEO against adult *A. galli* against three mechanistically distinct reference anthelmintics, (ii) characterised its volatile composition by GC-MS, and (iii) used molecular docking against three conserved *A. galli* proteins, NADH-ubiquinone oxidoreductase chain 4 (respiratory Complex I), β -tubulin and ATP synthase subunit 6, to generate a testable mechanistic hypothesis for its major constituents. Paralytic Cys-loop receptors were deliberately not modelled, because no experimentally resolved or reliably

modelled *A. galli* structure was available for them; they are discussed as the principal untested alternative.

Materials and methods

Parasite collection

Adult *A. galli* worms were retrieved from the small-intestinal lumen of free-range chickens (*Gallus gallus domesticus*) routinely slaughtered for human consumption at Ngebong Market, Boyolali Regency, Indonesia. Worms were placed immediately in 50 mL sterile conical tubes containing pre-warmed (37 °C) phosphate-buffered saline (PBS, pH 7.4), transported to the laboratory within 2 h, and transferred to fresh 0.9% (w/v) NaCl at 37 °C for a 30 min adaptation period. Species identity was confirmed morphologically on the basis of the three lips, the prominent preanal sucker of males and body dimensions. Only macroscopically intact, actively motile worms were used, and all treatments were initiated within 3 h of collection, in line with published recommendations for *in vitro* efficacy testing against *A. galli* (Feyera *et al.*, 2022, Ristanti *et al.*, 2023).

ASEO source and preparation

Steam-distilled *A. sympetalum* essential oil was obtained from a commercial supplier (PT Darjeeling Sembrani Aroma, Indonesia) as a finished retail product; the batch/lot number, production date and purchase date recorded for the bottle used throughout this study are given in Supplementary Table S2, together with the supplier's stated geographic origin of the raw heartwood (province and district, Kalimantan, Indonesia) and the certificate of analysis supplied on request and stored in amber glass vials at 4 °C in the dark until use. A single batch was used throughout, so that the chemical profile reported in section 2.5 applies to all bioassays. Two limitations of this provenance should be stated explicitly. First, because the material was acquired as a distilled commercial product rather than collected in the field, no herbarium voucher specimen could be prepared or deposited, and botanical identity therefore

rests on the supplier's declaration together with the GC-MS profile determined here (section 3.2), rather than on independent taxonomic authentication of the source tree; molecular authentication such as DNA barcoding is not reliably achievable from a distilled oil. Second, since *Aetoxylon* is not listed in the CITES Appendices, no CITES export or import documentation attaches to the material, and legality of origin rests on compliance with Indonesian protected-species legislation (Minister of Environment and Forestry Decree No. 106/2018); the supplier's documentation to this effect is reproduced in the supplementary material. Results are interpreted throughout as applying to a commercially traded oil declared to be of *A. sympetalum* origin and of the chemical composition reported in section 3.2.

***In vitro* anthelmintic assay**

Anthelmintic activity was assessed by adult-worm immersion, following the design principles established for *A. galli* by Feyera *et al.* (2022). Because ASEO is not miscible with saline, all test articles were dissolved in dimethyl sulfoxide (DMSO) and diluted in 0.9% (w/v) NaCl to a constant final DMSO concentration of 0.9% (v/v) in every treatment well, so that vehicle exposure was identical across treatments and concentrations. Five concentrations were tested per compound: ASEO at 10, 1, 0.1, 0.01 and 0.001% (v/v); levamisole at 0.1, 0.05, 0.01, 0.005 and 0.001% (w/v); piperazine at 1, 0.5, 0.1, 0.05 and 0.01% (w/v); and ivermectin at 1, 0.5, 0.1, 0.05 and 0.01% (w/v). Warmed 0.9% NaCl at 37 °C served as the negative control.

For each treatment, 5 mL of working solution was dispensed into a sterile watch glass and ten adult worms were transferred into it with non-surgical forceps so that they were fully immersed. Preparations were held at 37 °C throughout. Mortality was assessed at 10, 20, 40, 80 and 160 min. A worm was scored dead when it remained motionless for 30 s of continuous observation under a stereomicroscope and failed to respond to gentle mechanical stimulation with a blunt probe applied at three body regions. Provisionally non-motile worms were transferred to fresh

0.9% NaCl at 37 °C and re-examined after 5 min; only those failing to regain motility were recorded as dead. This recovery step distinguishes irreversible death from reversible paralysis and was applied at every time point. A binary lethality endpoint was used in preference to a graded motility score, and all scoring was performed by a single trained observer throughout, in order to remove between-observer variation in the perception of residual worm movement. Each treatment was performed in four independent biological replicates ($n = 4$), each replicate using worms from a different host, giving 40 worms per concentration per time point.

The 160 min end point was selected because (i) worms held in 0.9% NaCl at 37 °C remained $> 95\%$ viable for at least 4 h in preliminary trials, beyond which background mortality became non-negligible; (ii) the approximately logarithmic 10-160 min schedule resolves the rapid onset expected of a membrane-active terpenoid mixture; and (iii) all groups had reached 100% mortality or a clear plateau by 160 min. Levamisole, piperazine and ivermectin were included as reference anthelmintics representing three distinct paralytic modes of action, nAChR agonism, GABA-receptor agonism and glutamate-gated chloride-channel activation, respectively, and all three are in clinical use against *A. galli* (Höglund *et al.*, 2023).

Statistical analysis

Mortality was recorded as the number of dead worms out of the total exposed ($n = 4$ replicates $\times 10$ worms = 40 per concentration per time point) and analysed as bounded binomial proportions. Mortality in the 0.9% NaCl control was 0% at every time point, so Abbott's correction (Abbott, 1925) left all estimates unchanged; uncorrected values are therefore reported. Data are presented as mean percentage mortality $\pm$ SD.

The effects of concentration and exposure time on mortality were evaluated with binomial generalised linear models (logit link) fitted in R (version 4.4.1), with $\log_{10}$ concentration, $\log_{10}$

time and their interaction as predictors. The interaction was tested by likelihood-ratio comparison against the additive model. Replicate-level data were additionally analysed with a generalised linear mixed model (lme4) including replicate as a random effect; because the random-effect variance was negligible and the fixed-effect conclusions were unchanged, the simpler fixed-effect model is reported. Pairwise post-hoc contrasts between concentrations were obtained with the emmeans package using Tukey's adjustment.

Concentration-mortality relationships were quantified by probit regression of mortality on $\log_{10}$ concentration at the 160 min end point, fitted as a generalised linear model with binomial error and probit link (Finney, 1971). For each treatment the median and 90% lethal concentrations (LC_{50} , LC_{90}) were derived from the fitted function, together with the regression slope ($\pm$ SE) and 95% fiducial limits estimated by the delta method. Goodness of fit was assessed by the residual deviance χ^2 statistic. Where significant heterogeneity was detected, the variance-covariance matrix was inflated by the heterogeneity factor $h = \chi^2/df$ before computing confidence limits, following Finney (1971). Differences in potency between ASEO and the reference anthelmintics were considered statistically significant when the 95% confidence intervals of their LC_{50} values did not overlap. Median lethal times (LT_{50}) were estimated analogously by probit regression of mortality on $\log_{10}$ exposure time at each concentration; LT_{50} values falling beyond the 160 min observation window are reported as extrapolations and flagged as such. Statistical significance was set at $P < 0.05$ throughout. The a priori sample size (40 worms per treatment per concentration) was determined for a two-proportion comparison detecting a difference of 50% versus 0% at $\alpha = 0.05$ and power $(1 - \beta) = 0.95$.

Gas chromatography-mass spectrometry (GC-MS) assay of *Aetoxylon sympetalum* essential oil (ASEO)

GC-MS analysis of ASEO was performed at the Advanced Characterization Laboratory, National Research and Innovation Agency (Badan Riset dan Inovasi Nasional, BRIN), Cibinong, Indonesia. ASEO was dissolved in dichloromethane to 1000 µg/mL and filtered through a 0.22 µm Minisart syringe membrane. The filtrate was injected into a Shimadzu GCMS-QP2020NX instrument (Shimadzu, Japan) fitted with an Rtx-5MS column (5% diphenyl : 95% dimethylpolysiloxane; 30 m × 0.25 mm). The carrier gas was ultra-high-purity helium at 30 kPa. The injector was held at 200 °C, the ion source at 230 °C and the interface at 280 °C, with splitless injection. The oven programme began at 60 °C and rose to 150 °C at 10 °C/min, with a 3 min hold. Chromatograms and mass spectra were compared with the National Institute of Standards and Technology library (NIST-17) for putative identification. Relative abundances are expressed as percentage peak area of the total ion chromatogram; no internal standard or response-factor correction was applied, so values are semi-quantitative. The n-hexane peak was attributed to the extraction solvent and excluded from compositional interpretation. Because several eudesmane alcohols are reported by NIST under systematic 2-naphthalenemethanol nomenclature, these entries were mapped to their trivial names before selection of ligands for docking (Table 2).

Molecular docking analysis

CB-Dock2 web server (<https://cadd.labshare.cn/cb-dock2/index.php>), which uses AutoDock Vina for scoring and identifies binding sites automatically by detecting surface cavities (Fitrianita *et al.*, 2026; Liu *et al.*, 2022; Maulydia *et al.*, 2025). Amino-acid sequences of the target proteins were retrieved from UniProt (Ahmad *et al.*, 2025): NADH-ubiquinone oxidoreductase chain 4 (S4UCE9), β-tubulin (R4VG92) and ATP synthase subunit 6 (S4UGP6), all of *A. galli*. Three-dimensional structures were obtained from the AlphaFold

Protein Structure Database (<https://alphafold.ebi.ac.uk>), no experimentally resolved structures being available for these proteins; only models with per-residue pLDDT > 70 across the predicted binding cavities were carried forward. Receptors were prepared by removing water molecules and heteroatoms, adding polar hydrogens and assigning Kollman charges in AutoDock Tools 1.5.7.

Ligands were the five most abundant identified constituents of ASEO (γ -eudesmol, β -eudesmol, α -eudesmol, α -humulene and agarospirol; Table 2) together with the reference anthelmintics ivermectin, levamisole and piperazine. Agarospirol was retained despite ranking sixth by peak area because it is the diagnostic agarospiropane chemomarker of agarwood; elemol, which is marginally more abundant, was not docked and this is acknowledged as a limitation. Structures were downloaded from PubChem and energy-minimised with the Universal Force Field using a conjugate-gradient algorithm (Wibowo *et al.*, 2026; Sailah *et al.*, 2024). For each target the CB-Dock2 algorithm defined the search space from the predicted cavities. Each docking run was performed in triplicate and the pose with the lowest binding free energy (ΔG , kcal/mol) was retained (Wibowo *et al.*, 2026; Abiyyu *et al.*, 2026). Intermolecular interactions, hydrogen bonds, hydrophobic contacts (alkyl, π -alkyl, π -sigma), carbon-hydrogen bonds and unfavourable contacts, were mapped in Discovery Studio 2021 Client 21.1 (Dassault Systèmes, France). More negative ΔG values indicate stronger predicted binding. Docking scores are relative, are not free energies of binding in the thermodynamic sense, and are treated throughout as hypothesis-generating rather than confirmatory.

Results

In vitro anthelmintic activity of *Aetoxylon sympetalum* essential oil (ASEO)

ASEO produced concentration- and time-dependent mortality of adult *A. galli* (Table 1, Fig. 1). Both main effects were highly significant ($\log_{10}$ concentration, $z = 7.19$; $\log_{10}$ time, $z =$

11.40; both $P < 0.001$) and the concentration $\times$ time interaction was significant (likelihood-ratio $\chi^2(1) = 17.13$, $P < 0.001$), indicating that the rate of killing itself increased with concentration. At 10% (v/v), mortality reached $50.0 \pm 25.8\%$ within 10 min and 100% by 80 min. At 1%, mortality rose progressively to 100% by 160 min. Lower concentrations were substantially less active: 0.1% reached $37.5 \pm 45.0\%$ only at 160 min, while 0.01% and 0.001% produced $35.0 \pm 10.0\%$ and $15.0 \pm 19.2\%$, respectively, at the same time point. Mortality at 0.1% and 0.01% was therefore effectively indistinguishable (37.5% versus 35.0%), a departure from monotonicity that reflects high between-replicate variance at 0.1% (SD 45.0%, individual replicates ranging from 0 to 100%) and that is the direct cause of the poor probit fit reported below. The five sub-groups of the NaCl control did not differ from one another and remained at 0% throughout, justifying their pooling. Effect sizes versus the pooled control at 160 min were very large for 10% and 1% (Cohen's $h = 2.92$ each) and large for 0.1%, 0.01% and 0.001% ($h = 1.32, 1.27$ and 0.80).

Probit analysis at 160 min (Table 3) gave an ASEO LC_{50} of 0.037% v/v. The fit showed significant heterogeneity ($\chi^2 = 24.53$, $df = 3$, $P < 0.001$; $h = 8.18$), so fiducial limits were inflated accordingly, giving a wide 95% CI of 0.0068-0.2018%. On this basis the potency of ASEO was statistically indistinguishable from ivermectin (LC_{50} 0.062%, 95% CI 0.0387-0.0997%) and from piperazine (0.024%, 95% CI 0.0165-0.0341%), because their confidence intervals overlap, but significantly lower than levamisole (0.00090%, 95% CI 0.00060-0.00134%), whose interval does not overlap that of ASEO. The ASEO probit slope was shallow (0.858 ± 0.288) relative to levamisole (2.372 ± 0.486) and piperazine (1.688 ± 0.241), consistent with a mixture of constituents of differing potency rather than a single high-affinity ligand. The ASEO LC_{90} (1.15%) carried a fiducial interval spanning more than two orders of magnitude (0.078-16.9%) and is not considered reliably estimable from the present design.

403

404 Time-mortality analysis (Table 4) showed rapid onset at the higher concentrations. The LT₅₀
405 of ASEO was 10.5 min (95% CI 7.7-14.2) at 10% and 34.5 min (29.2-40.8) at 1%; at 0.1% the
406 LT₅₀ extrapolated to 191.6 min, beyond the observation window, and at 0.01% and 0.001% it
407 was not estimable. For comparison, ivermectin at 1% gave an LT₅₀ of 29.0 min (21.9-38.5),
408 piperazine at 1% 8.0 min (5.4-11.8) and levamisole at 0.01% 12.6 min (10.2-15.6). ASEO at
409 1% therefore killed adult *A. galli* at a rate statistically indistinguishable from ivermectin at the
410 same nominal concentration.

411 **Chemical composition of *Aetoxylon sympetalum* essential oil (ASEO)**

412 The GC-MS chromatogram resolved 100 peaks, all putatively identified by NIST library
413 comparison (Table S1). The profile was chemically diverse and dominated by sesquiterpenoids,
414 with smaller fractions of monoterpenes, aliphatic hydrocarbons and other oxygenated
415 constituents. Aliphatic hydrocarbons included 3-methylpentane, n-hexane, 2,4-
416 dimethylheptane, 4,6-dimethyldodecane, 2,6,11-trimethyldodecane and 5-methyl-5-
417 propylnonane, together with methylcyclopentane. Monoterpenes included the hydrocarbons α -
418 pinene, sabinene, β -pinene, 3-carene, (+)-4-carene, o-cymene, limonene and γ -terpinene, and
419 the oxygenated monoterpenes linalool and (R)-terpinen-4-ol.

420

421 The sesquiterpene fraction was the most prominent and structurally varied, comprising the
422 hydrocarbons caryophyllene, α -humulene, α -muurolene, β -bisabolene, α -copaene,
423 alloaromadendrene, α -guaiene, selina-3,7(11)-diene, (E)-longipinane, longifolene and
424 isolongifolene, alongside numerous octahydronaphthalene- and cyclopropazulene-type
425 structures. Oxygenated sesquiterpenes were especially abundant and included γ -eudesmol, 8-
426 epi- γ -eudesmol, agarospirol, elemol, selina-6-en-4-ol, isoshyobunone, 6-epi-shyobunol, *trans*-
427 longipinocarveol, thunbergol, caryophyllene oxide and eudesmane- and agarofuran-type 2-

naphthalenemethanol derivatives. Minor higher-molecular-weight constituents comprised fatty-acid methyl esters (e.g. methyl hexadecanoate), androstanone derivatives and the triterpene squalene.

The most abundant single peak was γ -eudesmol (10.93%, peak 61); two additional γ -eudesmol-matched peaks (0.21% each, peaks 45 and 59) and 8-epi- γ -eudesmol (0.16%, peak 50) raised the total eudesmol contribution to approximately 11.5%. Two further major peaks were reported by NIST under systematic nomenclature and correspond to the same chemical family: 2-naphthalenemethanol, decahydro- $\alpha,\alpha,4a$ -trimethyl-8-methylene- (7.16%, peak 64) is β -eudesmol, and 2-naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro- $\alpha,\alpha,4a,8$ -tetramethyl- (5.79%, peak 65) is α -eudesmol. This mapping (Table 2) reconciles the chromatographic output with the ligand set used for docking. Other dominant constituents were 2-isopropenyl-4a,8-dimethyloctahydronaphthalene (4.53%, peak 27), α -humulene (4.03%, peak 55; a second humulene-matched peak of 1.83% at peak 22 brought the total to approximately 5.86%), the guaiane sesquiterpene bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl- (3.90%, peak 29), a selina-diene octahydronaphthalene (3.67%, peak 31), elemol (3.25%, peak 44), agarospirol (1.78%, peak 62), (E)-longipinane (2.34%, peak 69) and selina-3,7(11)-diene (2.31%, peak 42). The n-hexane peak (3.25%, peak 2) was attributed to the extraction solvent and excluded.

This oxygenated-sesquiterpene-dominated profile, in which eudesmane-, agarospiro- and agarofuran-type constituents prevail, is characteristic of genuine agarwood essential oil (Xie *et al.*, 2024) and indicates that ASEO bioactivity is likely to be driven principally by eudesmol-type sesquiterpene alcohols. The contribution of co-occurring sesquiterpene hydrocarbons and minor terpenoids cannot be excluded, however, because essential oils act as complex mixtures.

Molecular docking against bioenergetic and cytoskeletal targets

Docking evaluated the binding affinities of the five selected ASEO constituents against three conserved *A. galli* proteins: NADH-ubiquinone oxidoreductase chain 4 (S4UCE9), β -tubulin (R4VG92) and ATP synthase subunit 6 (S4UGP6). The compounds showed target-specific binding, and the most favourable and most consistent affinities were obtained at ATP synthase subunit 6.

Against NADH-ubiquinone oxidoreductase (Table 5), agarospirol gave the strongest affinity at -6.5 kcal/mol, forming conventional hydrogen bonds with Tyr A:49 and Leu A:210 alongside hydrophobic contacts with Tyr A:152, Lys A:272 and Phe A:276. This exceeded levamisole (-4.8) and piperazine (-3.2) but was weaker than ivermectin (-7.6). α -Humulene, γ -eudesmol, α -eudesmol and β -eudesmol gave affinities of -6.2 to -6.1 kcal/mol through hydrophobic and hydrogen-bond interactions, principally with Val, Tyr and Phe residues. One unfavourable acceptor-acceptor contact was recorded for β -eudesmol at Leu A:210, indicating that this pose is less well accommodated than the corresponding agarospirol pose.

Against β -tubulin (Table 6), γ -eudesmol bound most strongly (-6.6 kcal/mol), followed by α -eudesmol (-6.3) and β -eudesmol (-6.1); α -humulene (-5.8) and agarospirol (-6.0) were weaker. Ivermectin outperformed all ASEO constituents (-8.8), while levamisole (-5.7) and piperazine (-3.3) were weaker. Interactions were dominated by alkyl and π -alkyl bonds with Leu A:215, His A:227, Leu A:228, Val A:231 and Phe A:270, residues lying within the tubulin dimer interface. An unfavourable donor-donor contact was noted for β -eudesmol at Arg A:276. Affinities at β -tubulin were consistently lower than at ATP synthase, so on the present evidence β -tubulin is a secondary rather than a primary candidate target.

The strongest and most uniform binding was obtained against ATP synthase subunit 6 (Table 7). α -Humulene, β -eudesmol and agarospirol each reached -7.3 kcal/mol and γ -eudesmol and α -eudesmol -7.1 kcal/mol. These values approach ivermectin (-7.5) and substantially exceed levamisole (-6.3) and piperazine (-3.3). Interactions were dominated by hydrophobic contacts, including alkyl and π -alkyl bonds with Phe A:4, Leu A:11, Phe A:12, Leu A:15, Pro A:77 and Phe A:83, supplemented by hydrogen bonds with Gly A:68, Ser A:74, Cys A:75 and Tyr A:99. Notably, agarospirol occupied a distinct cavity (Val A:96, Tyr A:99, Phe A:100, Tyr A:105, Leu A:123, Ile A:127) from the eudesmols and α -humulene, so the two chemotypes are predicted to engage non-identical sites on the same subunit. Representative poses are shown in Fig. 2.

Taken together, the docking results indicate that all five constituents bind the membrane-embedded, highly hydrophobic subunit 6 of ATP synthase more favourably than either of the other targets, and that they do so with affinities comparable to a clinically used anthelmintic. These are computational predictions only; they identify ATP synthase as the priority target for experimental validation rather than establishing it as the mechanism

Discussion

The essential oil of *A. sympetalum* killed adult *A. galli* rapidly and in a concentration-dependent manner, with an LC_{50} of 0.037% (v/v) at 160 min and an LT_{50} of 10.5 min at 10%. Its potency was statistically indistinguishable from that of ivermectin and of piperazine, and it was surpassed only by levamisole. To our knowledge this is the first report of anthelmintic activity for *A. sympetalum*, and one of very few studies to benchmark a plant essential oil directly against three mechanistically distinct reference anthelmintics under identical conditions. The closest published precedents are the comparison of *trans*-cinnamaldehyde with levamisole and monepantel at *C. elegans* Cys-loop receptors (Hernando *et al.*, 2025), the benchmarking of

carvacrol-geraniol combinations as nematocides against their individual reference potencies (Stojković *et al.*, 2025), and the standardised adult-immersion protocol for *A. galli* validated against levamisole and piperazine by Feyera *et al.* (2022); to our knowledge no agarwood-derived oil has previously been examined in such a design.

The finding matters because the chemical base for controlling poultry ascarids is exceptionally narrow. Fenbendazole is the only approved treatment in many jurisdictions, and reduced efficacy consistent with benzimidazole resistance has now been confirmed in a first *A. galli* isolate and in every *Heterakis gallinarum* isolate tested in a recent survey (Collins *et al.*, 2025). Against a background in which helminth infection affects the majority of chicken flocks worldwide (Shifaw *et al.*, 2021) and *A. galli* measurably depresses feed conversion, egg output and egg quality (Sharma *et al.*, 2019), the loss of the sole approved drug class would be difficult to mitigate. The trajectory is familiar from other systems: triclabendazole resistance has similarly narrowed the options against *Fasciola hepatica* (Kelley *et al.*, 2016). A botanically derived agent that matches ivermectin on a concentration basis is therefore worth pursuing, provided its claims are stated at the strength the data support.

Two aspects of the concentration-response deserve emphasis rather than concealment. First, the ASEO probit fit was significantly heterogeneous ($h = 8.18$) because mortality stepped sharply between 1% and 0.1% while remaining at 15-37.5% across the three lowest concentrations, leaving no observation near the inflection point. The 95% confidence interval on the LC_{50} is consequently wide (0.0068-0.2018%), and the claim of equivalence with ivermectin rests on that inflated interval. It is a defensible statement of statistical indistinguishability, not a demonstration of equal potency. Second, the shallow probit slope (0.858) is itself informative. Steep slopes are characteristic of single-target agents acting

through one high-affinity site; shallow slopes are typical of mixtures in which several constituents of differing potency contribute additively or sub-additively. The slope therefore independently supports a multi-constituent, and probably multi-target, mode of action, the same conclusion the docking data suggest by a different route.

The chemical profile provides a rational basis for the activity. ASEO was dominated by oxygenated sesquiterpenes: γ -eudesmol (10.93%), β -eudesmol (7.16%), α -eudesmol (5.79%), α -humulene (5.86%), elemol (3.25%) and agarospirol (1.78%). This composition is characteristic of genuine, resin-derived agarwood oil, in which sesquiterpenoids frequently constitute more than half of the total volatiles and carry the bulk of the reported bioactivity (Xie *et al.*, 2024). Agarwood sesquiterpenes are established bioactive principles: guaiane- and humulane-type constituents of *Aquilaria malaccensis* inhibit nitric-oxide production in LPS-stimulated macrophages at low-micromolar IC₅₀ (Ma *et al.*, 2021), and whole agarwood essential oil produces antidepressant and neuroprotective effects mediated through NF- κ B/I κ B- α and BDNF/TrkB/CREB signalling (Zhang *et al.*, 2025). These oils are therefore demonstrably able to reach and modulate membrane-associated and intracellular targets in animal tissue, which is a prerequisite for the mechanism proposed here.

Comparison with the reference drugs frames the mechanistic question. Levamisole is an nAChR agonist causing spastic paralysis, ivermectin gates glutamate-activated chloride channels to produce flaccid paralysis, and piperazine is a GABA-receptor agonist; each paralyzes the worm by a distinct route (Zirintunda *et al.*, 2021). A substantial body of electrophysiological work shows that plant terpenes act on these same Cys-loop receptors. Carvacrol interacts directly with the nAChR and GABA receptors of *Ascaris suum*, producing rapid concentration-dependent paralysis (Trailović *et al.*, 2015), and antagonises both nicotine-

and morantel-sensitive nAChR subtypes in *Parascaris* sp. (Trailović *et al.*, 2021). Single-channel recordings show that thymol, carvacrol and eugenol reduce channel opening frequency at the *C. elegans* levamisole-sensitive nAChR and UNC-49 GABA_A receptor, that is, they act as negative allosteric modulators (Hernando *et al.*, 2019). Menthol conversely potentiates levamisole-sensitive nAChRs as a positive allosteric modulator (Choudhary *et al.*, 2019), *trans*-cinnamaldehyde engages several Cys-loop targets and synergises with levamisole and monepantel (Hernando *et al.*, 2025), and carvacrol combined with geraniol is synergistically nematocidal, lowering individual LC₅₀ values by almost an order of magnitude (Stojković *et al.*, 2025). Structure-based virtual screening against the *A. suum* nAChR has already been used successfully to prioritise novel modulators for electrophysiological testing (Stevanovic *et al.*, 2021), and sesquiterpenes specifically are predicted to bind nAChR subtypes with appreciable affinity (Fakih *et al.*, 2025).

This literature is important precisely because it identifies the principal alternative hypothesis that the present study did not test. Almost all the electrophysiological evidence concerns monoterpenoids, carvacrol, thymol, eugenol, menthol, which are small, phenolic or alcoholic C₁₀ molecules. ASEO contains very little of these; it is dominated by C₁₅ sesquiterpene alcohols. Whether the receptor pharmacology established for monoterpenoids transfers to bulkier sesquiterpenoids is an open question, and the present data cannot answer it because no paralytic receptor was modelled. The reason was practical: no experimentally resolved *A. galli* Cys-loop receptor structure exists, and homology models of pentameric ligand-gated ion channels built without a close template are unreliable for docking. Docking sesquiterpenes against the *A. suum* or *Parascaris* nAChR, for which better structural and functional data exist, is the obvious next computational step.

What the present docking does show is a consistent preference for bioenergetic targets. All five constituents bound ATP synthase subunit 6 more favourably than either Complex I or β -tubulin, with α -humulene, β -eudesmol and agarospirol reaching -7.3 kcal/mol, close to ivermectin (-7.5) and well beyond levamisole (-6.3) and piperazine (-3.3). Three features of this pattern are worth noting. First, subunit 6 (ATP6) is the membrane-embedded proton-translocating component of F_0 , so its binding cavities are strongly hydrophobic, precisely the environment into which lipophilic sesquiterpene alcohols partition preferentially. The interaction profile bears this out: contacts were dominated by alkyl and π -alkyl bonds with Phe, Leu, Val and Pro. Second, agarospirol occupied a different cavity from the eudesmols, so the mixture is predicted to engage more than one site on a single subunit, which is consistent with additive or synergistic behaviour of the kind demonstrated for carvacrol and geraniol (Stojković *et al.*, 2025). Third, no single constituent dominated: affinities spanned only 0.2 kcal/mol across five chemically distinct ligands. Multi-target engagement of this kind, rather than exclusive binding to one high-affinity site, is widely regarded as protective against the evolution of resistance (Zirintunda *et al.*, 2021), and would be a substantial advantage over the single-target benzimidazoles whose efficacy is already eroding (Collins *et al.*, 2025).

A bioenergetic mechanism is also physiologically coherent for this parasite. Adult *A. galli* inhabits the small-intestinal lumen, an environment of low oxygen tension in which the worm relies on anaerobic and microaerophilic pathways that nonetheless converge on a functional mitochondrial ATP synthase. Rapid ATP depletion would impair the sustained muscular activity by which the worm maintains position against peristalsis, causing loss of motility and expulsion, a phenotype indistinguishable, on a motility assay, from receptor-mediated paralysis. This is the key interpretive caution: the immersion assay used here scores irreversible loss of motility and cannot discriminate between neuromuscular blockade, metabolic arrest and

cuticular damage. Notably, all three remain plausible for ASEO. Lipophilic terpenoids destabilise biological membranes directly, as demonstrated for cinnamon bark essential oil against bacterial membranes, where oxidative stress and loss of membrane integrity were the primary lesions (Yang *et al.*, 2019), and botanical extracts produce gross cuticular disruption in adult *A. galli* specifically (Balqis *et al.*, 2017). Scanning electron microscopy of ASEO-treated worms would discriminate this pathway from the intracellular mechanisms proposed here and should accompany any follow-up study.

Set against comparable botanical work, the potency observed here is encouraging. Most published plant anthelmintics against nematode and trematode targets act on eggs or larvae rather than adults, and typically require milligram-per-millilitre extract concentrations: examples include *Moringa oleifera* leaf extracts (Hegazi *et al.*, 2018), *Zingiber officinale* methanolic extract (Moazeni and Khademolhoseini, 2016), curcumin-nisin and curcumin nanoparticle formulations (Oyeyemi *et al.*, 2018; Alcala-Canto *et al.*, 2023), *Mentha pulegium* oil (Bouajila and Eddouks, 2021) and *Artemisia vulgaris* (Nurlaelasari *et al.*, 2023). An LC₅₀ of 0.037% v/v against the adult stage, achieved within a 160 min exposure, compares favourably with that body of work. Whether the activity is superior to that of other agarwood or Indonesian aromatic species, *Curcuma xanthorrhiza*, for instance, whose leaf oil and xanthorrhizol content are well characterised (Devkota, 2021; Oon *et al.*, 2015; Rahmat *et al.*, 2021), has not been tested and would require head-to-head assays.

Several limitations temper these conclusions and define the next experiments. The assay measured the response of adult worms only; efficacy against embryonated eggs and tissue-phase larvae, which govern transmission, remains unknown, and adult-only activity would not by itself interrupt the life cycle. The probit fit for ASEO was significantly heterogeneous, and

additional intermediate concentrations in the 0.03-0.3% range would tighten the LC₅₀ and permit a defensible LC₉₀. A DMSO-only vehicle control was not run in parallel; although the final DMSO concentration was constant at 0.9% (v/v) across all treatments and the saline control showed no mortality, a vehicle arm should be included in any replication. Mortality was scored as a binary, irreversible endpoint rather than as a graded motility score; ordinal motility scales resolve sub-lethal impairment more sensitively but are laborious and are subject to between-observer variation in the perception of movement, and automated video- or image-based motility detection would improve both throughput and reproducibility in future work. Agarospirol was docked in preference to the marginally more abundant elemol on chemotaxonomic grounds, and elemol should be included in future ligand sets. The docking models were AlphaFold predictions rather than experimental structures, blind docking scores are relative and are not thermodynamic binding free energies, and no molecular-dynamics refinement or MM-GBSA rescoring was performed; the affinity rankings reported here should therefore be treated as a prioritisation, not a measurement. As an *in vitro* immersion study the results cannot be extrapolated to *in vivo* efficacy, where bioavailability, the gut milieu, host toxicity and residue behaviour intervene; the safety margin of ASEO and the pharmacokinetics of its sesquiterpenes require dedicated evaluation before any field use is contemplated.

Most importantly, the proposed mechanism remains a hypothesis. Confirming it requires direct measurement rather than further computation: oxygen-consumption and ATP-content assays on treated worms to test bioenergetic inhibition, isolated-mitochondria or reconstituted F₁F₀ preparations to test direct enzyme inhibition, electrophysiological recording from *A. galli* or *A. suum* muscle to test the alternative receptor-mediated route and scanning electron microscopy to quantify cuticular damage. Fractionation of ASEO followed by testing of purified γ -, α - and β -eudesmol, α -humulene and agarospirol, alone and in combination, would establish which

constituents drive the activity and whether they interact synergistically, as carvacrol and geraniol do (Stojković *et al.*, 2025). Until those experiments are done, the defensible conclusion is narrower than the docking scores alone might suggest: ASEO is a potent *in vitro* nematocide against adult *A. galli* whose chemistry, concentration-response slope and predicted binding profile all point towards a multi-constituent, multi-target mechanism in which bioenergetic proteins are the leading candidate, and which warrants prioritised experimental validation.

Finally, the translational potential of these findings must be set against the conservation status of the source species, and the two stand in direct tension. *A. sympetalum* is the sole species of a monotypic genus, endemic to Borneo, nationally categorised as Critically Endangered in Indonesia and legally protected since 2018; a recent survey of remnant forest in Kapuas Hulu located only sixteen individuals in total, nine of them mature (Kusumadewi *et al.*, 2024). Unlike *Aquilaria* and *Gyrinops*, it is not listed in CITES Appendix II, so any demand generated by a positive pharmacological result would not encounter that regulatory brake on international trade. Recommending wild-harvested ASEO as an ethnoveterinary anthelmintic on the strength of an *in vitro* assay would therefore be irresponsible, and we do not do so. Three routes could make the present results usable without adding pressure to wild populations. First, cultivation: *ex situ* propagation from seedlings and cuttings has already been demonstrated for this species as a conservation measure (Kusumadewi *et al.*, 2024), and the plantation and artificial-induction technologies developed for *Aquilaria* and *Gyrinops* (Tan *et al.*, 2019; Ngadiran *et al.*, 2023; Auri *et al.*, 2025) provide a template, although the silvicultural tolerances of *A. sympetalum*, particularly outside Borneo, are essentially undocumented and would have to be established experimentally. Second, constituent-level substitution: the activity reported here is attributed to eudesmane- and agarospirane-type sesquiterpenes that are not unique to *A. sympetalum*, and γ -, α - and β -eudesmol and α -humulene are obtainable from more abundant,

cultivated aromatic plants; establishing which constituents drive the activity would decouple any application from the species itself. Third, biotechnological supply: chemical or chemoenzymatic synthesis, and microbial terpene-synthase platforms of the kind proposed for agarwood metabolites (Sampasivam *et al.*, 2024), would allow production independent of the tree. Until one of these routes is in place, the defensible position is that *A. sympetalum* is a source of chemical leads whose documentation is itself an argument for its conservation, rather than a harvestable remedy.

Declarations

Funding

This research was funded by the Tropical Onehealth and EcoHealth Institute (TROPI) under grant number (grant no. 02/TROPI-GRANT/IV/2023), and by Universitas Sebelas Maret (UNS), Indonesia by its research support programme. Additional in-kind support was provided by the National Research and Innovation Agency (BRIN), Indonesia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgement

We thank the Zoology Laboratory at the National Research and Innovation Agency (BRIN), Cibinong, Indonesia, for technical assistance with GC-MS. We further acknowledge PT. Darjeeling Sembrani Aroma, Indonesia, for providing the essential oil used in this study.

Conflict of interest

The authors declare that they have no conflicts of interest, financial or otherwise, that could have influenced the outcomes, interpretation or reporting of this research.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary materials.

Ethical statement

Adult *A. galli* specimens were collected from chickens routinely slaughtered for human consumption at a local market; no live animal experimentation was conducted. No animals were handled, treated, anaesthetised or sacrificed for the purpose of this study, and therefore the ethical clearance was waived. Field access and sample-collection activities were authorised by the National and Political Unity Agency (Kesbangpol, Central Java) under permit no. 070/642/XII/5.5/2021 (for 5-years research period).

Author s contribution

DC: investigation, formal analysis, visualization. PHH: conceptual framework, supervised the study, performed funding acquisition, edited the manuscript. DEP: investigation, methodology. IDP: investigation, methodology. SW: investigation, formal analysis. NBM: investigation. HAN: investigation, edited the manuscript. PRF: investigation, methodology. MC: investigation, edited the manuscript. DCBS: investigation, edited the manuscript. FS: provided the ethnopharmacological, conservation framing, and contributed to manuscript writing and editing. All authors read and approved the final version of the manuscript.

Generative AI statement

During the preparation of this work the authors used ChatGPT for sentence-level language polishing only. After using this tool the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Abbreviations

ASEO	<i>Aetoxylon sympetalum</i> Essential Oil
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
BRIN	Badan Riset dan Inovasi Nasional (National Research and Innovation Agency of Indonesia)

CB-Dock2	Cavity Detection-guided Blind Docking 2
CI	Confidence Interval
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CREB	cAMP Response Element-Binding Protein
df	Degrees of Freedom
DMSO	Dimethyl Sulfoxide
F ₁ F ₀	ATP Synthase Complex (membrane-bound F ₀ and catalytic F ₁ subunits)
GABA	Gamma-Aminobutyric Acid
GC-MS	Gas Chromatography-Mass Spectrometry
h	Heterogeneity Factor (probit analysis)
IUCN	International Union for Conservation of Nature
LC ₅₀	Median Lethal Concentration
LPS	Lipopolysaccharide
LT ₅₀	Median Lethal Time
MM-GBSA	Molecular Mechanics with Generalized Born and Surface Area Solvation
nAChR	Nicotinic Acetylcholine Receptor
NaCl	Sodium Chloride (physiological saline)
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NIST	National Institute of Standards and Technology
PBS	Phosphate-Buffered Saline
POWO	Plants of the World Online (Royal Botanic Gardens, Kew)
RT	Retention Time (GC-MS)
SD	Standard Deviation

SE	Standard Error
TrkB	Tropomyosin Receptor Kinase B
TROPI	Tropical Onehealth and EcoHealth Institute
UniProt	Universal Protein Resource
UNS	Universitas Sebelas Maret (Indonesia)
v/v	Volume per Volume (concentration)
w/v	Weight per Volume (concentration)
χ^2	Chi-Square (statistical test)

722

723 **References**

- 724 Abbott, W.S. (1925) A method of computing the effectiveness of an insecticide, *Journal of*
725 *Economic Entomology*, 18(2), pp. 265-267. doi: 10.1093/jee/18.2.265a.
- 726 Abiyyu, N., Fitrianita, A., Artika, I.M., Siregar, J.E., Wibowo, S., Wardhani, B.W.K., Budi,
727 C.S. and Nuringtyas, T.R. (2026) The role of astaxanthin–Cu²⁺ in stabilizing glycated human
728 serum albumin for type 2 diabetes mellitus management: a computational approach,
729 *Indonesian Journal of Biotechnology*, 31(1), pp. 49–61. doi: 10.22146/ijbiotech.109830.
- 730 Ahmad, S., Jose da Costa Gonzales, L., Bowler-Barnett, E.H., Rice, D.L., Kim, M., Wijerathne,
731 S., Luciani, A., Kandasamy, S., Luo, J., Watkins, X., Turner, E. and Martin, M.J. (2025)
732 The UniProt website API: facilitating programmatic access to protein knowledge, *Nucleic*
733 *Acids Research*, 53(W1), pp. W547-W553. doi: 10.1093/nar/gkaf394.
- 734 Akter, S., Islam, M.T., Zulkefeli, M. and Khan, S.I. (2013) Agarwood production - a
735 multidisciplinary field to be explored in Bangladesh, *International Journal of*
736 *Pharmaceutical and Life Sciences*, 2(1), pp. 22-32. doi: 10.3329/ijpls.v2i1.15132.
- 737 Alcala-Canto, Y., Alberti-Navarro, A., Ibarra-Velarde, F., Vera-Montenegro, Y., Cervantes-
738 Valencia, M.E., Murcia-Mejia, C. and Olmedo-Juárez, A. (2023) Curcumin-loaded

739 nanoparticles potentiate the *in vitro* ovicidal and larvicidal activity against gastrointestinal
 740 nematodes of small ruminants, *Veterinary Parasitology*, 318, 109925. doi:
 741 10.1016/j.vetpar.2023.109925.

742 Auri, A., Dimara, P.A. and Runtuboi, Y. (2025) Integrated resin induction enhances agarwood
 743 formation in *Gyrinops caudata*, *Biodiversitas Journal of Biological Diversity*, 26(8). doi:
 744 10.13057/biodiv/d260809.

745 Balqis, U., Hambal, M., Rinidar, Athaillah, F., Ismail, Azhar, Vanda, H. and Darmawi (2017)
 746 Cuticular surface damage of *Ascaridia galli* adult worms treated with *Veitchia merrillii* betel
 747 nuts extract *in vitro*, *Veterinary World*, 10(7), pp. 732-737. doi:
 748 10.14202/vetworld.2017.732-737.

749 Bouajila, J. and Eddouks, M. (2021) Essential oil of *Mentha pulegium* induces anthelmintic
 750 effects and reduces parasite-associated oxidative stress in rodent model, *Veterinary*
 751 *Parasitology*, 292, 109399. doi: 10.1016/j.vetpar.2021.109399.

752 Chen, J., Gao, T., Ge, Y., Chen, X.-D., Feng, M., Chen, X., Zhang, W. and Gao, X. (2025) An
 753 efficient harvesting strategy for agarwood based on the correlation analysis of resin
 754 formation and leaves dynamic changes induced by integrated induction method, *PLOS*
 755 *ONE*, 20(8), e0327516. doi: 10.1371/journal.pone.0327516.

756 Chhipa, H., Chowdhary, K. and Kaushik, N. (2017) Artificial production of agarwood oil in
 757 *Aquilaria* sp. by fungi: a review, *Phytochemistry Reviews*, 16, pp. 835-860. doi:
 758 10.1007/s11101-017-9492-6.

759 Choudhary, S., Marjanović, D.S., Wong, C.R., Zhang, X., Abongwa, M., Coats, J.R., Trailović,
 760 S.M., Martin, R.J. and Robertson, A.P. (2019) Menthol acts as a positive allosteric
 761 modulator on nematode levamisole sensitive nicotinic acetylcholine receptors, *International*
 762 *Journal for Parasitology: Drugs and Drug Resistance*, 9, pp. 44-53. doi:
 763 10.1016/j.ijpddr.2018.12.005.

764 CITES (2026) *Appendices I, II and III*. Convention on International Trade in Endangered
 765 Species of Wild Fauna and Flora. Available at: <https://cites.org/eng/app/appendices.php>
 766 (Accessed: 15 August 2026).

767 Collins, J.B., Choo, R., Shaver, A.O., Schaye, E.S., Volpe, T., Nunn, L., Lighty, M.E., Niel,
 768 K.R., Frye, E.M., Zamanian, M. and Andersen, E.C. (2025) A small-scale survey of
 769 fenbendazole resistance in *Ascaridia galli* and *Heterakis gallinarum*, two common ascarid
 770 parasites of poultry, *Poultry Science*, 104(11), 105808. doi: 10.1016/j.psj.2025.105808.

771 Devkota, H.P. (2021) Chemical constituent analysis and antioxidant activity of leaf essential
 772 oil of *Curcuma xanthorrhiza*, *Journal of Essential Oil Bearing Plants*, 24(4), pp. 776-787.
 773 doi: 10.1080/0972060X.2021.1955750.

774 Faizal, A., Hermawaty, D., Junita, E., Rahmawati, A., Azar, A.W.P., Makajanma, M.M. and
 775 Turjaman, M. (2022) Evaluation of biotic and abiotic stressors to artificially induce
 776 agarwood production in *Gyrinops versteegii* (Gilg.) Domke seedlings, *Symbiosis*, 86, pp.
 777 229-239. doi: 10.1007/s13199-022-00835-2.

778 Fakih, T.M., Rizkita, A.D., Dewi, S.A. and Muchtaridi, M. (2025) In silico approaches for
 779 developing sesquiterpene derivatives as antagonists of human nicotinic acetylcholine
 780 receptors (nAChRs) for nicotine addiction treatment, *Current Research in Structural*
 781 *Biology*, 9, 100162. doi: 10.1016/j.crstbi.2024.100162.

782 Feyera, T., Elliott, T., Sharpe, B., Ruhnke, I., Shifaw, A. and Walkden-Brown, S.W. (2022)
 783 Evaluation of *in vitro* methods of anthelmintic efficacy testing against *Ascaridia galli*,
 784 *Journal of Helminthology*, 96, e29. doi: 10.1017/S0022149X22000177.

785 Finney, D.J. (1971) *Probit analysis*. 3rd edn. Cambridge: Cambridge University Press.

786 Fitrianita, A., Abiyyu, N., Juliandi, B., Sabarudin, A., Nuringtyas, T.R., Gunosewoyo, H.,
 787 Wibowo, S. and Syahputra, R.A. (2026) Astaxanthin–Zn²⁺ complexes and glycated human

788 serum albumin: a molecular mechanism study for protein integrity in diabetes mellitus,
789 *Results in Chemistry*, 22, 103124. doi: 10.1016/j.rechem.2026.103124.

790 Hegazi, A.G., El-Megeed, K.N.A., Hassan, S.E., Abdelaziz, M.M., Toaleb, N.I., El
791 Shanawany, E.E. and Aboelsoued, D. (2018) Comparative ovicidal activity of *Moringa*
792 *oleifera* leaf extracts on *Fasciola gigantica* eggs, *Veterinary World*, 11(2), pp. 215-220. doi:
793 10.14202/vetworld.2018.215-220.

794 Herath, H.J.B. and Jinendra, B. (2023) Recent advancement in agarwood induction technology:
795 a comprehensive review for the transformation of artificial agar resin induction methods,
796 *Journal of Agro-Technology and Rural Sciences*, 3(1). doi: 10.4038/atrsj.v3i1.46.

797 Hermawaty, D., Setyobudi, T., Nugrahapraja, H., Turjaman, M. and Faizal, A. (2025) *De novo*
798 transcriptome assembly and analysis during agarwood induction in *Gyrinops versteegii*
799 Gilg. seedling, *Scientific Reports*, 15(1), 2977. doi: 10.1038/s41598-025-87486-7.

800 Hernando, G., Turani, O. and Bouzat, C. (2019) *Caenorhabditis elegans* muscle Cys-loop
801 receptors as novel targets of terpenoids with potential anthelmintic activity, *PLOS Neglected*
802 *Tropical Diseases*, 13(11), e0007895. doi: 10.1371/journal.pntd.0007895.

803 Hernando, G., Turani, O., Rodriguez Araujo, N., Pulido Carrasquero, A. and Bouzat, C. (2025)
804 Unraveling anthelmintic targets and mechanisms of action of *trans*-cinnamaldehyde from
805 cinnamon essential oil, *Scientific Reports*, 15(1), 5422. doi: 10.1038/s41598-025-89883-4.

806 Höglund, J., Daş, G., Tarbiat, B., Geldhof, P., Jansson, D.S. and Gauly, M. (2023) *Ascaridia*
807 *galli* - an old problem that requires new solutions, *International Journal for Parasitology:*
808 *Drugs and Drug Resistance*, 23, pp. 1-9. doi: 10.1016/j.ijpddr.2023.07.003.

809 Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P. and Spithill, T.W. (2016)
810 Current threat of triclabendazole resistance in *Fasciola hepatica*, *Trends in Parasitology*,
811 32(6), pp. 458-469. doi: 10.1016/j.pt.2016.03.002.

812 Kusumadewi, Y., Widodo, R.M.W., Rahmawati, K., Pratama, B.A., Syah, G., Suriyanto, I.,
813 Randi, A., Petrus, S. and Rachmat, H.H. (2024) Rescuing the rare monotypic *Aetoxylon*
814 *sympetalum* from remnant forests in Kapuas Hulu, West Kalimantan, *Oryx*, 58(1), p. 13.
815 doi: 10.1017/S0030605323001357.

816 Liu, Y., Chen, H., Yang, Y., Zhang, Z., Wei, J., Meng, H., Chen, W., Feng, J., Gan, B., Chen,
817 X., Gao, Z., Huang, J.-Q. and Chen, H. (2013) Whole-tree agarwood-inducing technique:
818 an efficient novel technique for producing high-quality agarwood in cultivated *Aquilaria*
819 *sinensis* trees, *Molecules*, 18(3), pp. 3086-3106. doi: 10.3390/molecules18033086.

820 Liu, Y., Yang, X., Gan, J., Chen, S., Xiao, Z.X. and Cao, Y. (2022) CB-Dock2: improved
821 protein-ligand blind docking by integrating cavity detection, docking and homologous
822 template fitting, *Nucleic Acids Research*, 50(W1), pp. W159-W164. doi:
823 10.1093/nar/gkac394.

824 Ma, C.T., Ly, T.L., Le, T.H.V., Tran, T.V.A., Kwon, S.W. and Park, J.H. (2021) Sesquiterpene
825 derivatives from the agarwood of *Aquilaria malaccensis* and their anti-inflammatory effects
826 on NO production of macrophage RAW 264.7 cells, *Phytochemistry*, 183, 112630. doi:
827 10.1016/j.phytochem.2020.112630.

828 Maulydia, N.B., Khairan, K., Tallei, T.E. and Idroes, R. (2025) Metabolomic profiling and
829 anti-inflammatory activity of *Blumea balsamifera*: insights from *in silico* and *in vitro*
830 studies, *Pharmacia*, 72, pp. 1-15. doi: 10.3897/pharmacia.72.e146995.

831 Moazeni, M. and Khademolhoseini, A.A. (2016) Ovicidal effect of the methanolic extract of
832 ginger (*Zingiber officinale*) on *Fasciola hepatica* eggs: an *in vitro* study, *Journal of*
833 *Parasitic Diseases*, 40(3), pp. 662-666. doi: 10.1007/s12639-014-0550-3.

834 Ngadiran, S., Baba, S., Nor, N.F.A., Yahayu, M., Muhamad, M.F., Kushairi, A.K.A., Zaidel,
835 D.N.A. and Muhamad, I.I. (2023) The induction techniques of resinous agarwood

836 formation: a review, *Bioresource Technology Reports*, 21, 101337. doi:
837 10.1016/j.biteb.2023.101337.

838 Nurlaelasari, A., Wulandari, A.R., Muñoz Caro, T., Nugroho, H.A., Sukaryo, S., Cahyadi, M.,
839 Kurniawan, W. and Hamid, P.H. (2023) *Artemisia vulgaris* anthelmintic activities to eggs
840 and adult stages of *Fasciola gigantica* *in vitro*, *Veterinary World*, 16(5), pp. 1141-1153. doi:
841 10.14202/vetworld.2023.1141-1153.

842 Oon, S.F., Nallappan, M., Tee, T.T., Shohaimi, S., Kassim, N.K., Sa ariwijaya, M.S.F. and
843 Cheah, Y.H. (2015) Xanthorrhizol: a review of its pharmacological activities and anticancer
844 properties, *Cancer Cell International*, 15, 100. doi: 10.1186/s12935-015-0255-4.

845 Oyeyemi, O.T., Adegbeyeni, O.O., Oyeyemi, I.T., Meena, J. and Panda, A.K. (2018) *In vitro*
846 ovicidal activity of poly lactic acid curcumin-nisin co-entrapped nanoparticle against
847 *Fasciola* spp. eggs and its reproductive toxicity, *Journal of Basic and Clinical Physiology*
848 *and Pharmacology*, 29(1), pp. 73-79. doi: 10.1515/jbcpp-2017-0157.

849 POWO (2026) *Plants of the World Online*. Facilitated by the Royal Botanic Gardens, Kew.
850 Available at: <https://powo.science.kew.org> (Accessed: 15 August 2026).

851 Rahmat, E., Lee, J. and Kang, Y. (2021) Javanese turmeric (*Curcuma xanthorrhiza* Roxb.):
852 ethnobotany, phytochemistry, biotechnology, and pharmacological activities, *Evidence-*
853 *Based Complementary and Alternative Medicine*, 2021, 9960813. doi:
854 10.1155/2021/9960813.

855 Ristanti, R., Iqbal, M., Attahirah, C.W., Curin'in, N.M., Pawestri, W. and Hamid, P.H. (2023)
856 Major intestinal parasites of domestic chicken in Boyolali, Central Java, Indonesia, *Revista*
857 *Electrónica de Veterinaria*, 24(1): 32-41.

858 Sailah, I., Tallei, T. E., Safitri, L., Tamala, Y., Halimatushadyah, E., Ekatanti, D., Maulydia,
859 N. B., & Celik, I. (2024). A network pharmacology approach to elucidate the anti-

860 inflammatory and antioxidant effects of bitter leaf (*Vernonia amygdalina* Del.). Narra J,
861 4(3), Article e1016. <https://doi.org/10.52225/narra.v4i3.1016>

862 Sampasivam, Y., Razman, K.K., Mazlan, N.S.M., Azizan, K.A., Ahlawat, Y.K. and Othman,
863 R. (2024) Towards sustainable agarwood production: integrating microbial interactions,
864 anatomical changes, and metabolite biosynthesis, *Journal of Industrial Microbiology &*
865 *Biotechnology*, 52, kuaf025. doi: 10.1093/jimb/kuaf025.

866 Sharma, N., Hunt, P.W., Hine, B.C. and Ruhnke, I. (2019) The impacts of *Ascaridia galli* on
867 performance, health, and immune responses of laying hens: new insights into an old
868 problem, *Poultry Science*, 98(12), pp. 6517-6526. doi: 10.3382/ps/pez422.

869 Shifaw, A., Feyera, T., Walkden-Brown, S.W., Sharpe, B., Elliott, T. and Ruhnke, I. (2021)
870 Global and regional prevalence of helminth infection in chickens over time: a systematic
871 review and meta-analysis, *Poultry Science*, 100(5), 101082. doi: 10.1016/j.psj.2021.101082.

872 Stevanovic, S., Marjanović, D.S., Trailović, S.M., Zdravković, N., Perdih, A. and Nikolic, K.
873 (2021) Potential modulating effect of the *Ascaris suum* nicotinic acetylcholine receptor
874 (nAChR) by compounds GSK575594A, diazepam and flumazenil discovered by structure-
875 based virtual screening approach, *Molecular and Biochemical Parasitology*, 242, 111350.
876 doi: 10.1016/j.molbiopara.2021.111350.

877 Stojković, M., Marjanović, D.S., Medić, D., Charvet, C.L. and Trailović, S.M. (2025)
878 Neuromuscular system of nematodes is a target of synergistic pharmacological effects of
879 carvacrol and geraniol, *Pharmaceuticals*, 18(8), 1232. doi: 10.3390/ph18081232.

880 Tan, C.S., Isa, N.M., Ismail, I. and Zainal, Z. (2019) Agarwood induction: current
881 developments and future perspectives, *Frontiers in Plant Science*, 10, 122. doi:
882 10.3389/fpls.2019.00122.

883 Trailović, S.M., Marjanović, D.S., Nedeljković Trailović, J., Robertson, A.P. and Martin, R.J.
884 (2015) Interaction of carvacrol with the *Ascaris suum* nicotinic acetylcholine receptors and

gamma-aminobutyric acid receptors, potential mechanism of antinematodal action,
Parasitology Research, 114(8), pp. 3059-3068. doi: 10.1007/s00436-015-4508-x.

Trailović, S.M., Rajković, M., Marjanović, D.S., Neveu, C. and Charvet, C.L. (2021) Action
of carvacrol on *Parascaris* sp. and antagonistic effect on nicotinic acetylcholine receptors,
Pharmaceuticals, 14(6), 505. doi: 10.3390/ph14060505.

Ullah, M.A. (2020) Boosting up the economy of Pakistan through agarwood production,
International Journal of Horticulture and Food Science, 2(2), pp. 49-51. doi:
10.33545/26631067.2020.v2.i2a.49.

Wibowo, S., Hamid, P.H., Ristanti, R., Nugroho, H.A., Nashrulloh, M.M., Nuringtyas, T.R.,
Ferdian, P.R., Amalia, R.L.R., Caro, T.M., Maulydia, N.B., Kusumarini, S., Wardhana,
A.H., Cahyadi, M. and Matsubayashi, M. (2026) Putative binding of *Piper betle* L.
phytochemicals to mannitol pathway enzymes during sporulation of *Eimeria tenella*,
Veterinary and Animal Science, 34, 100763. doi: 10.1016/j.vas.2026.100763.

Xie, C., Dong, J.-Z., Lu, B.-S., Yan, P.-Y., Zhao, Y.-S., Ding, X.-Y., Lv, C.-E. and Zheng, X.
(2024) Pharmacology and therapeutic potential of agarwood and agarwood tree leaves in
periodontitis, *Frontiers in Pharmacology*, 15, 1468393. doi: 10.3389/fphar.2024.1468393.

Yang, S.K., Yusoff, K., Ajat, M., Thomas, W., Abushelaibi, A., Akseer, R., Lim, S.H.E. and
Lai, K.S. (2019) Disruption of KPC-producing *Klebsiella pneumoniae* membrane via
induction of oxidative stress by cinnamon bark essential oil, *PLOS ONE*, 14(4), e0214326.
doi: 10.1371/journal.pone.0214326.

Yunus, M.F., Salleh, M.S., Ponari, M.H.A., Kuswandi, P.C. and Hamdan, M.S.M. (2024)
Nutrient requirement and fertilizer management for agarwood plantations: a review,
AGRIVITA Journal of Agricultural Science, 46(2). doi: 10.17503/agrivita.v46i2.4437.

Zhang, S., Chen, X., Wang, C., Sun, Y., Gong, B., Li, D., Wu, Y., Liu, Y. and Wei, J. (2025)
Antidepressant activity of agarwood essential oil: a mechanistic study on inflammatory and

910 neuroprotective signaling pathways, *Pharmaceuticals*, 18(2), 255. doi:
911 10.3390/ph18020255.

912 Zirintunda, G., Biryomumaisho, S., Kasozi, K.I., Batiha, G.E.-S., Kateregga, J., Vudriko, P.,
913 Nalule, S., Olila, D., Kajoba, M., Matama, K., Kwizera, M.R., Ghoneim, M.M.,
914 Abdelhamid, M., Zaghlool, S.S., Alshehri, S., Abdelgawad, M.A. and Acai-Okwee, J.
915 (2021) Emerging anthelmintic resistance in poultry: can ethnopharmacological approaches
916 offer a solution?, *Frontiers in Pharmacology*, 12, 774896. doi: 10.3389/fphar.2021.774896.

917

918 **Tables and legend to tables**

919 **Table 1.** Mean percentage mortality of adult *Ascaridia galli* following exposure to different
 920 concentrations of *Aetoxylon sympetalum* essential oil (ASEO) and to levamisole, piperazine
 921 and ivermectin as reference anthelmintics, at five observation times.

Treatment	Concentration	10 min	20 min	40 min	80 min	160 min
ASEO	10%	50.0 ±	75.0 ±	90.0 ±	100.0 ±	100.0 ±
		25.8	19.2	11.6	0.0	0.0
	1%	5.0 ± 10.0	25.0 ±	55.0 ±	85.0 ±	100.0 ±
			10.0	30.0	19.2	0.0
	0.1%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.0 ±	37.5 ±
					11.6	45.0
Levamisole	0.01%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	35.0 ±
						10.0
	0.001%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	15.0 ±
						19.2
	0.1%	100.0 ±	100.0 ±	100.0 ±	100.0 ±	100.0 ±
		0.0	0.0	0.0	0.0	0.0
Piperazine	0.05%	80.0 ±	100.0 ±	100.0 ±	100.0 ±	100.0 ±
		10.0	0.0	0.0	0.0	0.0
	0.01%	40.0 ±	70.0 ±	95.0 ± 5.0	100.0 ±	100.0 ±
		15.0	12.0		0.0	0.0
	0.005%	10.0 ± 8.0	30.0 ±	65.0 ±	85.0 ±	95.0 ± 5.0
			10.0	15.0	10.0	
Ivermectin	0.001%	0.0 ± 0.0	5.0 ± 5.0	15.0 ± 8.0	35.0 ±	55.0 ±
					12.0	15.0
	1%	60.0 ±	85.0 ±	95.0 ± 5.0	100.0 ±	100.0 ±
		15.0	10.0		0.0	0.0
	0.5%	30.0 ±	55.0 ±	85.0 ±	95.0 ± 5.0	100.0 ±
		12.0	15.0	10.0		0.0
Ivermectin	0.1%	10.0 ± 8.0	25.0 ±	45.0 ±	70.0 ±	85.0 ±
			10.0	12.0	15.0	10.0
	0.05%	0.0 ± 0.0	5.0 ± 5.0	20.0 ± 8.0	40.0 ±	65.0 ±
					10.0	12.0
	0.01%	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 5.0	15.0 ± 8.0	30.0 ±
						10.0
Ivermectin	1%	25.0 ±	40.0 ±	55.0 ±	75.0 ±	90.0 ± 8.0
		10.0	12.0	15.0	10.0	
	0.5%	15.0 ± 8.0	25.0 ±	40.0 ±	60.0 ±	80.0 ±
			10.0	12.0	15.0	10.0
	0.1%	5.0 ± 5.0	10.0 ± 8.0	25.0 ±	40.0 ±	60.0 ±
				10.0	12.0	12.0
Ivermectin	0.05%	0.0 ± 0.0	5.0 ± 5.0	15.0 ± 8.0	25.0 ±	40.0 ±
					10.0	10.0
	0.01%	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 5.0	10.0 ± 8.0	25.0 ±
						10.0

NaCl 0.9%	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
-----------	---	-----------	-----------	-----------	-----------	-----------

922 Values are mean percentage mortality ± SD (n = 4 replicates × 10 worms = 40 worms per

923 treatment per concentration per time point). ASE0 concentrations are % v/v; reference-drug

924 concentrations are % w/v. All treatments contained 0.9% (v/v) DMSO as co-solvent.

925

926 **Table 2.** Ten most abundant constituents of *Aetoxylon sympetalum* essential oil (ASEO),
 927 ranked by relative peak area, with NIST systematic names mapped to trivial names. The n-
 928 hexane solvent peak is excluded. Compounds marked † were selected as ligands for molecular
 929 docking.

Peak No.	RT (min)	Relative peak area (%)	NIST systematic name	Trivial name	Chemical class
61	37.040	10.93	γ -Eudesmol	γ -Eudesmol †	Oxygenated sesquiterpene (eudesmane alcohol)
64	37.776	7.16	2-Naphthalenemethanol, decahydro- $\alpha,\alpha,4a$ -trimethyl-8-methylene-	β -Eudesmol †	Oxygenated sesquiterpene (eudesmane alcohol)
65	37.925	5.79	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro- $\alpha,\alpha,4a,8$ -tetramethyl-	α -Eudesmol †	Oxygenated sesquiterpene (eudesmane alcohol)
55 + 22	35.768 / 29.912	4.03 + 1.83 = 5.86	Humulene	α -Humulene †	Sesquiterpene hydrocarbon (humulene)
27	30.872	4.53	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	Selinene-type hydrocarbon	Sesquiterpene hydrocarbon (eudesmane)
29	31.293	3.90	Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl-	Guaiene-type hydrocarbon	Sesquiterpene hydrocarbon (guaiane)
31	31.663	3.67	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-	Selina-diene	Sesquiterpene hydrocarbon (eudesmane)
44	33.843	3.25	Cyclohexanemethanol, 4-ethenyl- $\alpha,\alpha,4$ -trimethyl-3-(1-methylethenyl)-	Elemol	Oxygenated sesquiterpene (elemane alcohol)
69	38.441	2.34	Longipinane, (E)-	(E)-Longipinane	Sesquiterpene hydrocarbon (longipinane)
42	33.490	2.31	Selina-3,7(11)-diene	Selina-3,7(11)-diene	Sesquiterpene hydrocarbon (eudesmane)
62	37.343	1.78	Agarospirol	Agarospirol †	Oxygenated sesquiterpene (agarospirane alcohol)

930 Agarospirol is listed although it ranks eleventh by peak area, because it is the diagnostic
931 agarospirane chemomarker of agarwood and was included in the docking ligand set on that
932 basis. Relative abundances are semi-quantitative percentage peak areas of the total ion
933 chromatogram; no response-factor correction was applied.
934

Table 3. Probit analysis of the concentration-mortality response of adult *Ascaridia galli* at the 160 min end point.

Treatment	Slope $\pm$ SE	LC ₅₀ (%)	95% CI	LC ₉₀ (%)	95% CI	χ^2 (df = 3)	P	h
ASEO (v/v)	0.858 $\pm$ 0.288	0.0370	0.0068-0.2018	1.151	0.078-16.94	24.53	< 0.001	8.18
Levamisole (w/v)	2.372 $\pm$ 0.486	0.00090	0.00060-0.00134	0.00311	0.00205-0.00471	0.66	0.882	1.00
Piperazine (w/v)	1.688 $\pm$ 0.241	0.0237	0.0165-0.0341	0.1362	0.0853-0.2175	2.17	0.539	1.00
Ivermectin (w/v)	0.976 $\pm$ 0.146	0.0621	0.0387-0.0997	1.277	0.536-3.044	1.12	0.773	1.00

Probit regression of mortality on log₁₀ concentration, binomial error, probit link. h = heterogeneity factor (χ^2/df); where h > 1 the variance-covariance matrix was inflated by h before computing fiducial limits (Finney, 1971). Slope $\pm$ SE for ASEO is heterogeneity-corrected. Two treatments are considered to differ significantly in potency when their LC₅₀ confidence intervals do not overlap. The ASEO LC₉₀ is not considered reliably estimable.

Table 4. Median lethal time (LT₅₀) of adult *Ascaridia galli* by treatment and concentration, estimated by probit regression of mortality on log₁₀ exposure time.

Treatment	Concentration	LT ₅₀ (min)	95% CI (min)
ASEO	10%	10.5	7.7-14.2
	1%	34.5	29.2-40.8
	0.1%	191.6 ‡	142.3-258.1
	0.01%	not estimable	-
	0.001%	not estimable	-
Levamisole	0.01%	12.6	10.2-15.6
	0.005%	31.1	25.6-37.7
	0.001%	130.5	94.9-179.4
Piperazine	1%	8.0	5.4-11.8
	0.5%	16.7	13.4-20.9
	0.1%	45.2	35.8-57.0
	0.05%	103.7	80.3-133.9
Ivermectin	1%	29.0	21.9-38.5
	0.5%	52.3	39.4-69.5
	0.1%	112.0	77.3-162.3
	0.05%	209.5 ‡	121.7-360.6

‡ Extrapolated beyond the 160 min observation window; interpret with caution. LT₅₀ was not estimable where fewer than two time points showed non-zero mortality.

Table 5. Molecular docking binding free energies (kcal/mol) of the five major constituents of *Aetoxylon sympetalum* essential oil (ASEO) against *Ascaridia galli* NADH-ubiquinone oxidoreductase chain 4 (UniProt S4UCE9), compared with levamisole, ivermectin and piperazine.

Ligand-receptor complex	Binding affinity (kcal/mol)	Type of interaction	Amino acids involved
α -Humulene	-6.2	Alkyl and π -alkyl	Tyr A:47, Met A:51, Phe A:276, Leu A:411
γ -Eudesmol	-6.2	Conventional hydrogen bond	Val A:151
		Alkyl and π -alkyl	Val A:43, Tyr A:152
α -Eudesmol	-6.1	Conventional hydrogen bond	Val A:151
		Alkyl and π -alkyl	Phe A:35, Val A:43, Tyr A:152
β -Eudesmol	-6.1	Alkyl and π -alkyl	Tyr A:49, Tyr A:152, Lys A:214, Lys A:272
		Unfavourable acceptor-acceptor	Leu A:210
Agarospirol	-6.5	Conventional hydrogen bond	Tyr A:49, Leu A:210
		Alkyl and π -alkyl	Tyr A:152, Lys A:272, Phe A:276
Ivermectin	-7.6	Conventional hydrogen bond	Asp A:8
		Carbon-hydrogen bond	Thr A:65
		π -sigma	Phe A:12
		Alkyl and π -alkyl	Phe A:4, Phe A:9, Val A:13, Phe A:57
Levamisole	-4.8	π -alkyl	Lys A:272
Piperazine	-3.2	Conventional hydrogen bond	Tyr A:307, Asn A:309, Ser A:382

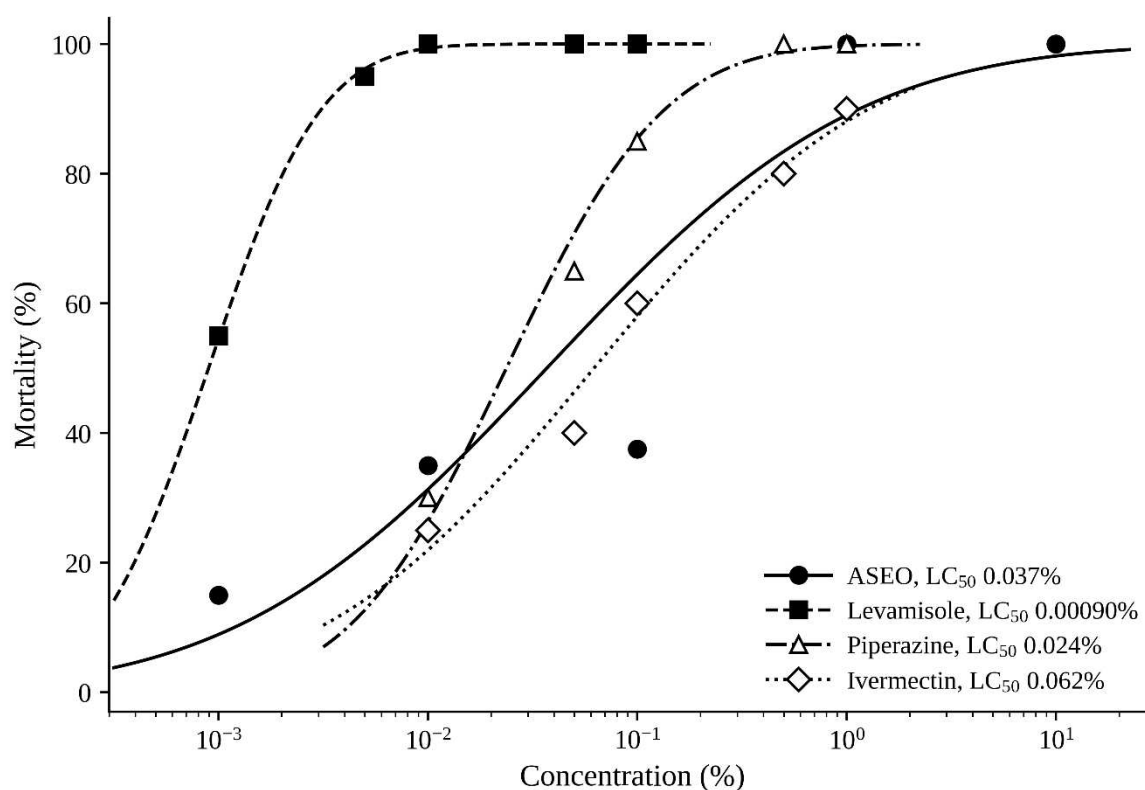
Table 6. Molecular docking binding free energies (kcal/mol) of the five major constituents of ASEO against *Ascaridia galli* β -tubulin (UniProt R4VG92), compared with levamisole, ivermectin and piperazine.

Ligand-receptor complex	Binding affinity (kcal/mol)	Type of interaction	Amino acids involved
α -Humulene	-5.8	Alkyl and π -alkyl	His A:227, Leu A:228, Val A:231, Phe A:270, Leu A:273, Arg A:276, Leu A:361
γ -Eudesmol	-6.6	Alkyl and π -alkyl	Leu A:215, His A:227, Leu A:228, Val A:231, Phe A:270, Leu A:273, Pro A:358, Leu A:361
α -Eudesmol	-6.3	Conventional hydrogen bond	Glu A:181, Asn A:204
		Alkyl and π -alkyl	Cys A:12, Val A:169, Tyr A:222
β -Eudesmol	-6.1	Alkyl and π -alkyl	Leu A:215, Leu A:217, His A:227, Leu A:273, Leu A:361
		Unfavourable donor-donor	Arg A:276
Agarospinol	-6.0	Carbon-hydrogen bond	Gly A:141
		Alkyl	Cys A:12, Val A:178
Ivermectin	-8.8	Conventional hydrogen bond	Ser A:274
		Carbon-hydrogen bond	His A:227, Gly A:360
		Alkyl and π -alkyl	Lys A:19, Val A:23, Phe A:270, Leu A:273, Arg A:359
Levamisole	-5.7	Conventional hydrogen bond	Ser A:138
		Carbon-hydrogen bond	Gln A:11, Glu A:181
Piperazine	-3.3	Conventional hydrogen bond	Asp A:67, Thr A:143
		Carbon-hydrogen bond	Glu A:69

957 **Table 7.** Molecular docking binding free energies (kcal/mol) of the five major constituents of
958 ASEO against *Ascaridia galli* ATP synthase subunit 6 (UniProt S4UGP6), compared with
959 levamisole, ivermectin and piperazine.

Ligand-receptor complex	Binding affinity (kcal/mol)	Type of interaction	Amino acids involved
α -Humulene	-7.3	Alkyl and π -alkyl	Phe A:4, Phe A:12, Leu A:15, Pro A:77
γ -Eudesmol	-7.1	Alkyl and π -alkyl	Phe A:4, Leu A:11, Phe A:12, Leu A:15, Phe A:71, Pro A:77
α -Eudesmol	-7.1	Conventional hydrogen bond	Gly A:68
		Alkyl and π -alkyl	Phe A:4, Leu A:11, Phe A:12, Leu A:15, Phe A:71, Pro A:77
β -Eudesmol	-7.3	Conventional hydrogen bond	Cys A:75
		Carbon-hydrogen bond	Ser A:74
		π -sigma	Phe A:12
		Alkyl and π -alkyl	Phe A:4, Pro A:77, Phe A:83
Agarospirol	-7.3	Conventional hydrogen bond	Tyr A:99
		Alkyl and π -alkyl	Val A:96, Phe A:100, Tyr A:105, Leu A:123, Ile A:127
Ivermectin	-7.5	Conventional hydrogen bond	Met A:1, Ser A:2, Ser A:79
		Carbon-hydrogen bond	Gly A:153
		Alkyl and π -alkyl	Tyr A:70, Ile A:174
Levamisole	-6.3	π -cation and π -anion	Glu A:130, Arg A:133
		π - π stacked	Tyr A:99
Piperazine	-3.3	π -sigma	Tyr A:99

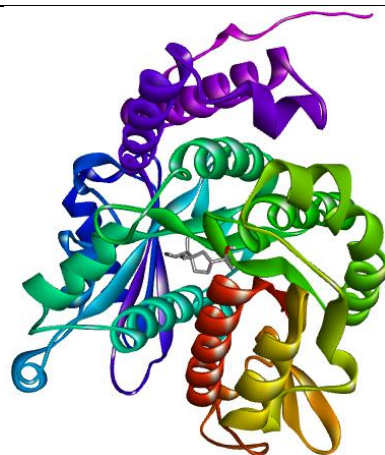
961 **Figures and legend to figures**



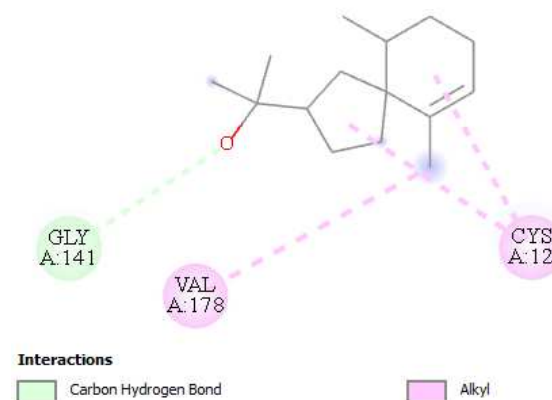
962
 963 **Figure 1.** Concentration-mortality (probit) response of adult *Ascaridia galli* exposed for 160
 964 min to *Aetoxylon sympetalum* essential oil (ASEO) and to the reference anthelmintics
 965 levamisole, piperazine and ivermectin. Points are observed mean mortality ($n = 4$ replicates $\times$
 966 10 worms per concentration); curves are probit fits. LC_{50} values are indicated. Mortality in the
 967 0.9% NaCl control was 0% throughout.

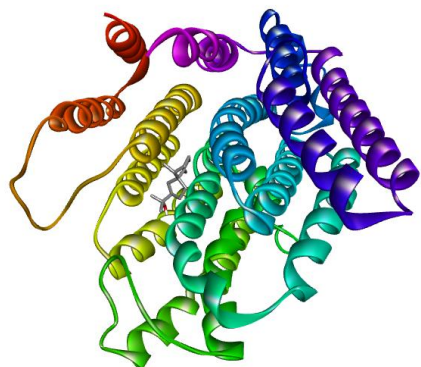
968 .

969 **Figure 2.** Representative molecular docking poses and two-dimensional interaction diagrams of the major *Aetoxylon sympetalum* essential oil
970 constituents (α -humulene, γ -eudesmol, α -eudesmol, β -eudesmol, agarospirol) and the reference anthelmintics (ivermectin, levamisole, piperazine)
971 with the three *Ascaridia galli* targets: NADH-ubiquinone oxidoreductase chain 4 (S4UCE9), β -tubulin (R4VG92) and ATP synthase subunit 6
972 (S4UGP6). Left panels show the ligand within the predicted binding cavity; right panels show the corresponding residue-level interaction map. #
973 Supplementary material

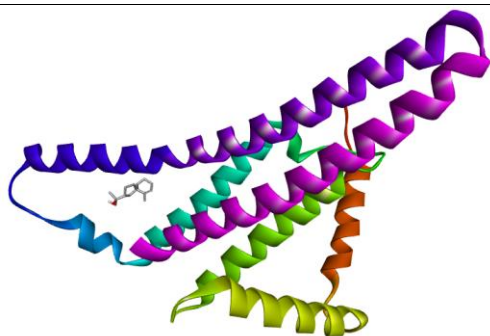
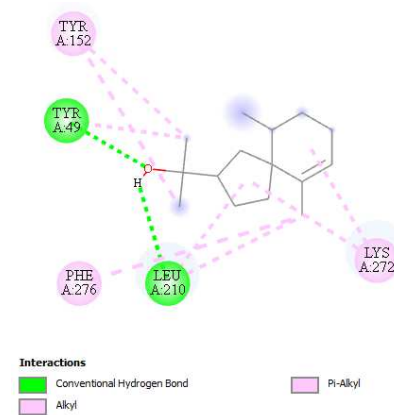


β -tubulin with agarospirol

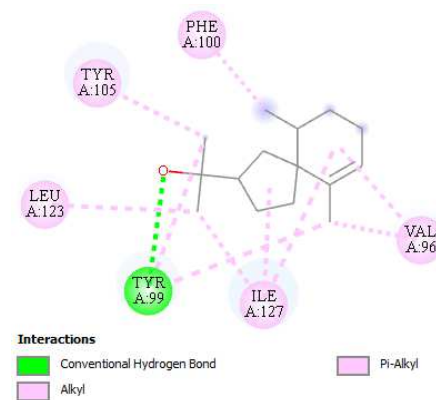


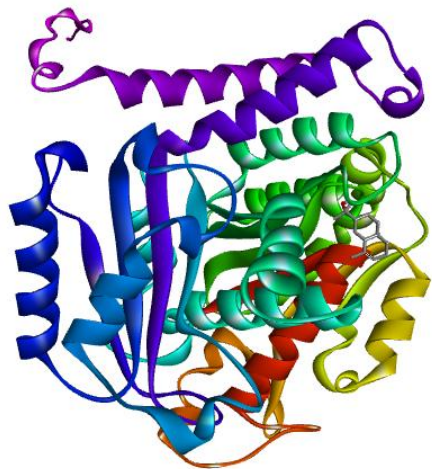


NADH-ubiquinone oxidoreductase chain 4 with agarospirol

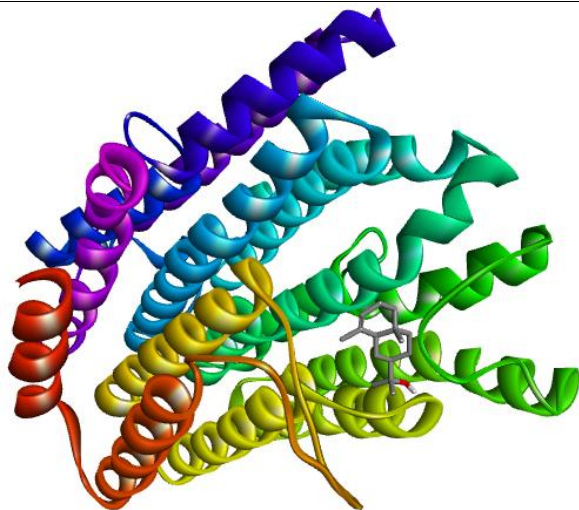
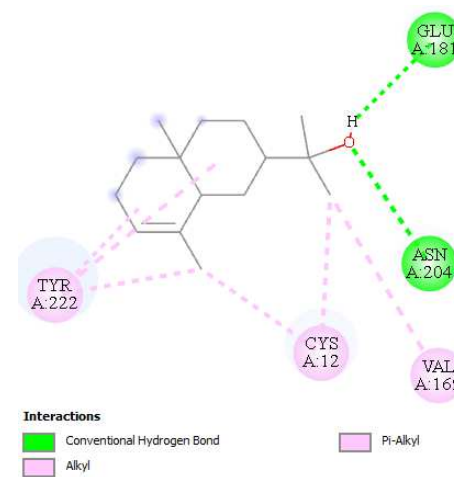


ATP synthase subunit 6 with agarospirol

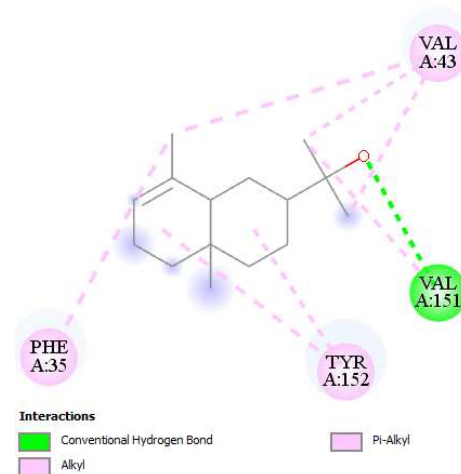


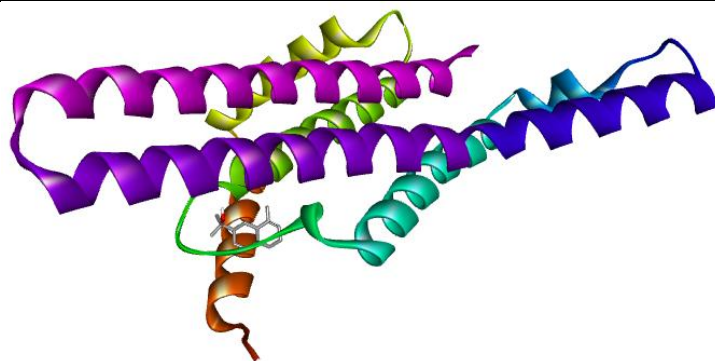


β -tubulin with alpha-eudesmol

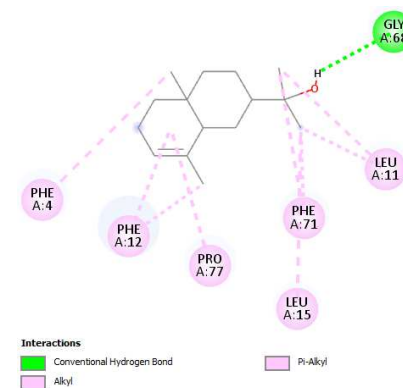


NADH-ubiquinone oxidoreductase chain 4 with alpha-eudesmol

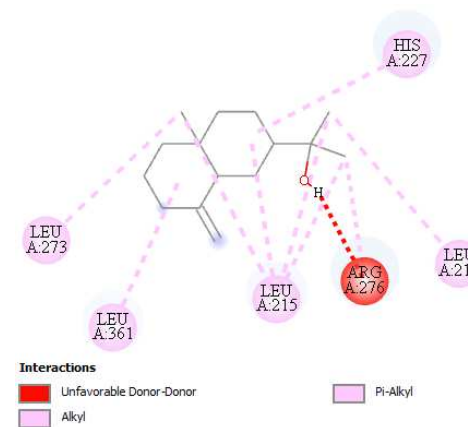


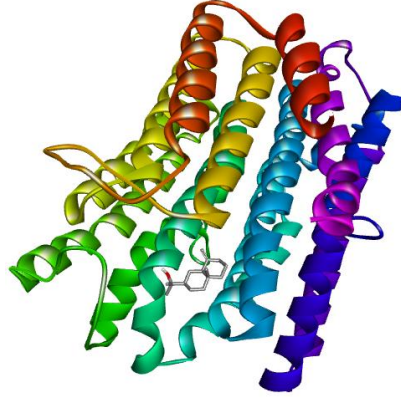


ATP synthase subunit 6 with alpha-eudesmol

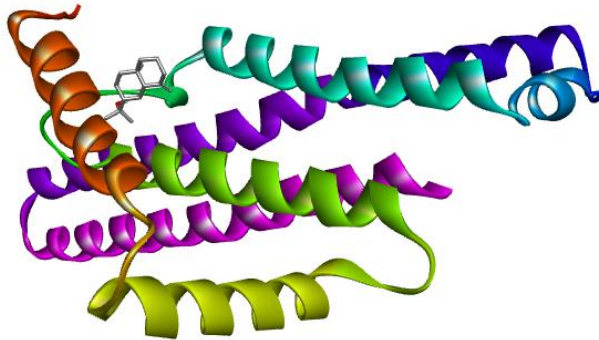
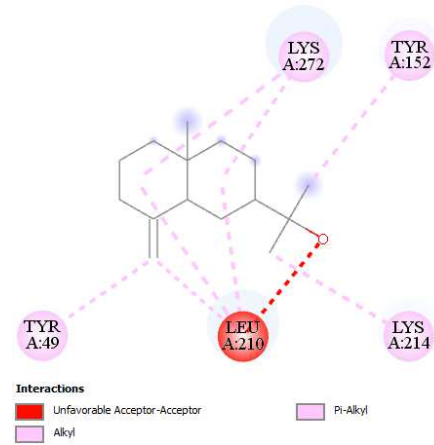


β -tubulin with beta-eudesmol

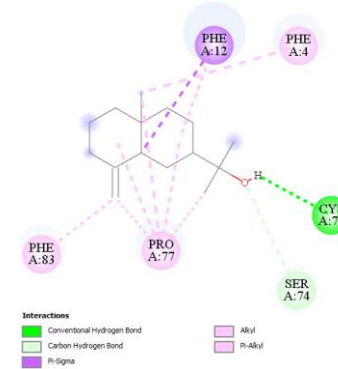


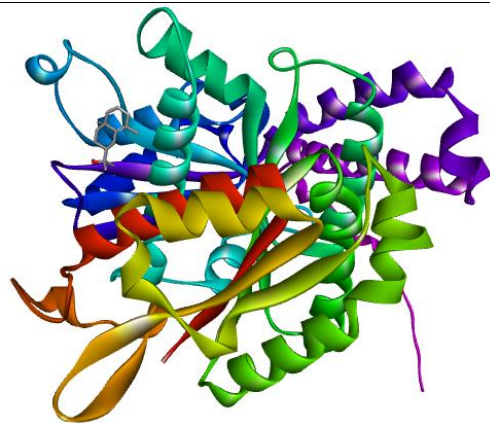


NADH-ubiquinone oxidoreductase chain 4 with beta-eudesmol

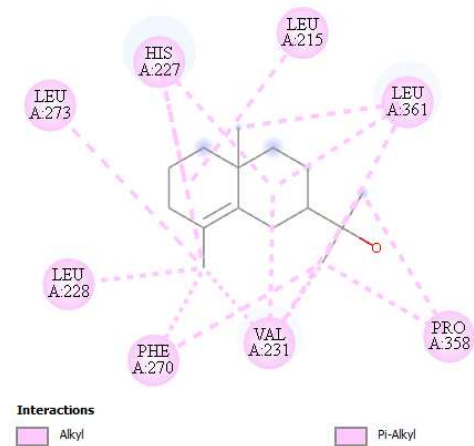


ATP synthase subunit 6 with beta-eudesmol

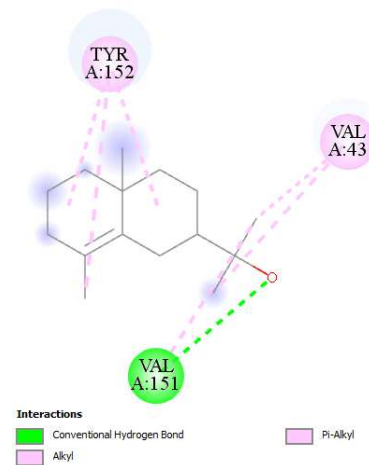


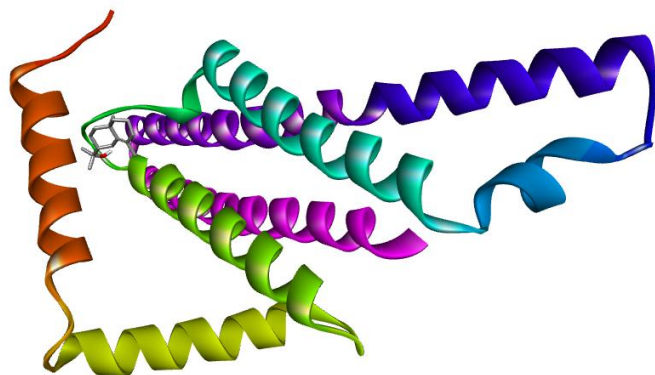


β -tubulin with gamma-eudesmol

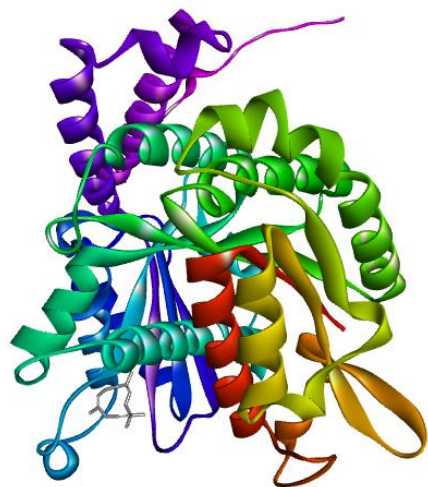
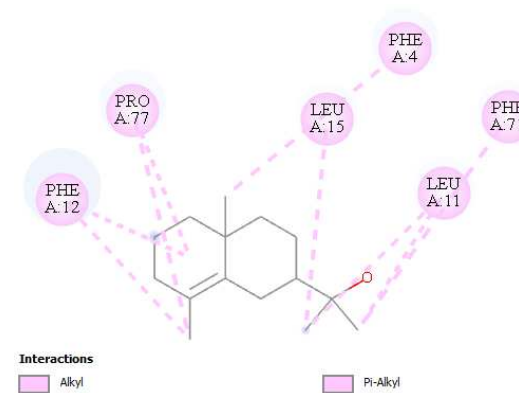


NADH-ubiquinone oxidoreductase chain 4 with gamma-eudesmol

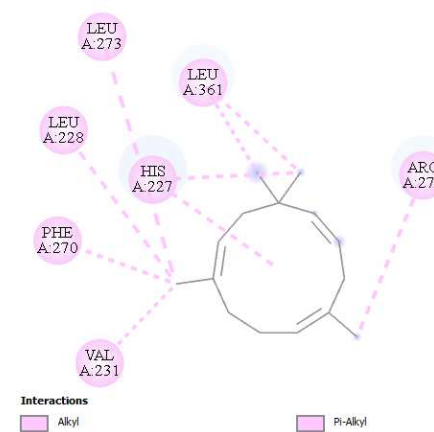


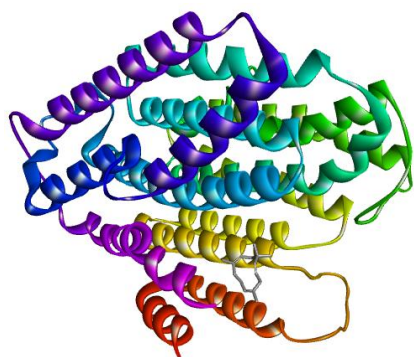


ATP synthase subunit 6 with gamma-eudesmol

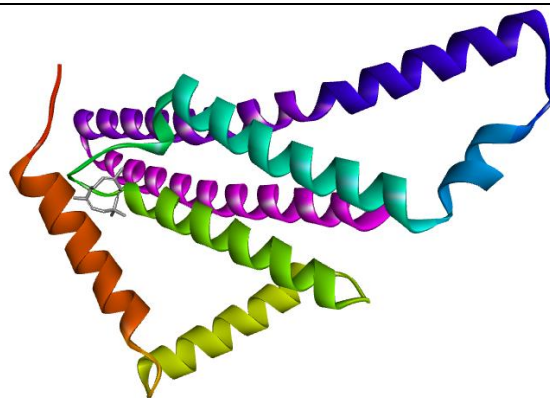
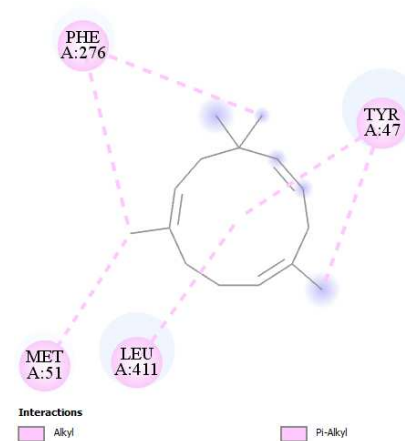


β -tubulin with humulene

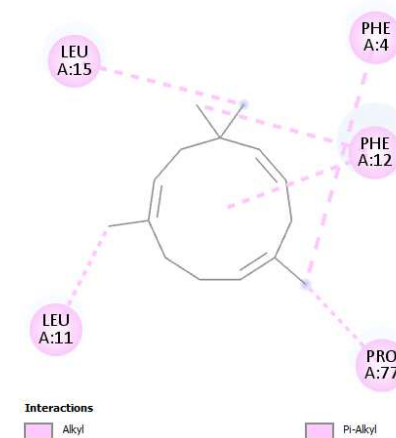


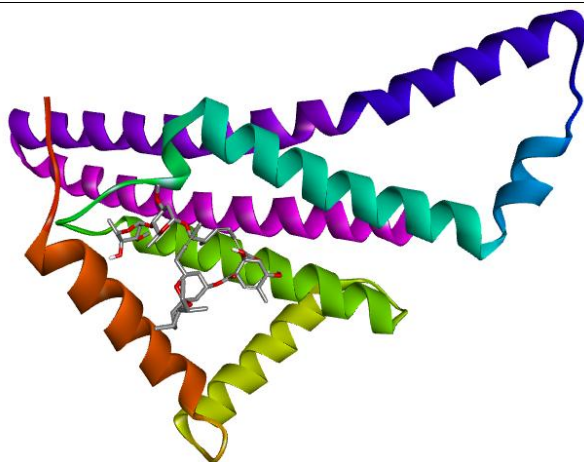


NADH-ubiquinone oxidoreductase chain 4 with humulene

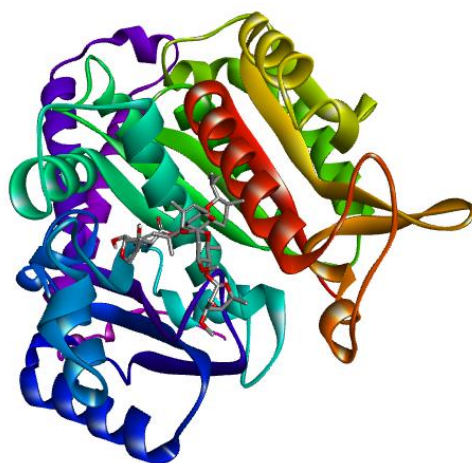
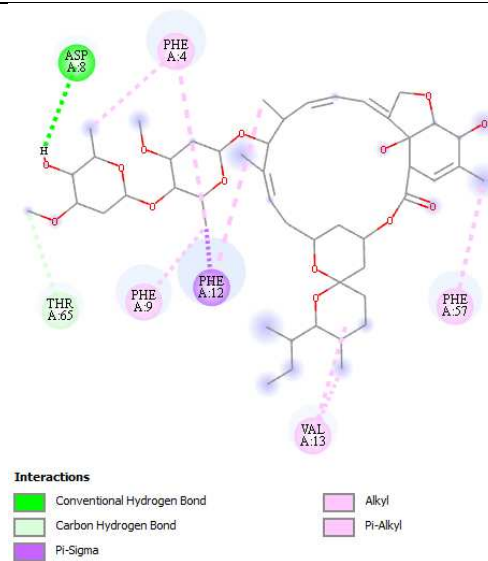


ATP synthase subunit 6 with humulene

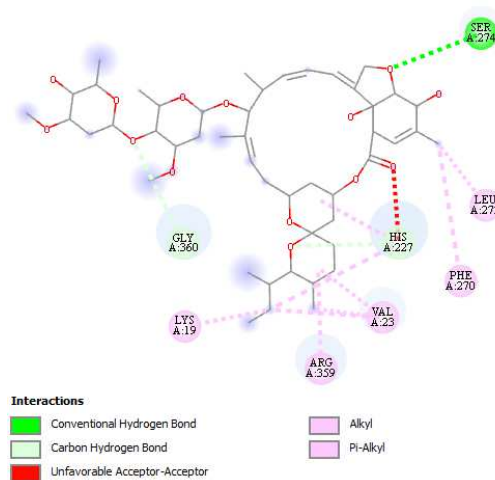


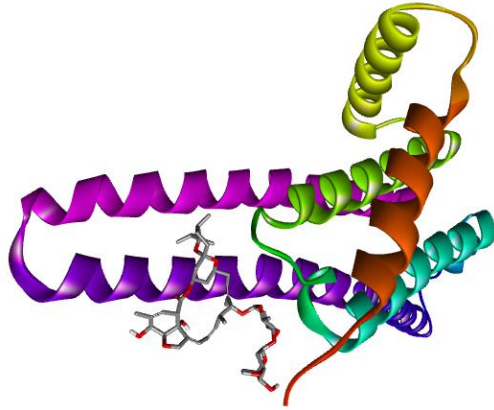


NADH-ubiquinone oxidoreductase chain 4 with ivermectin

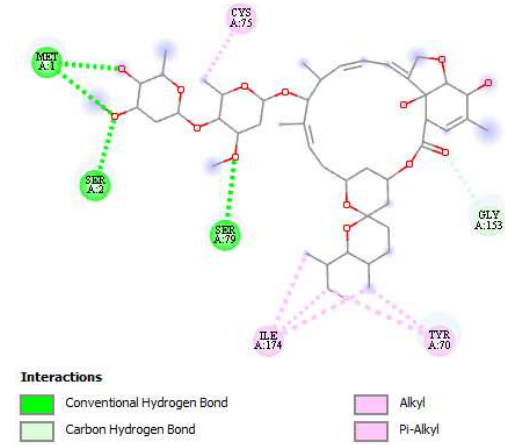


β -tubulin with ivermectin

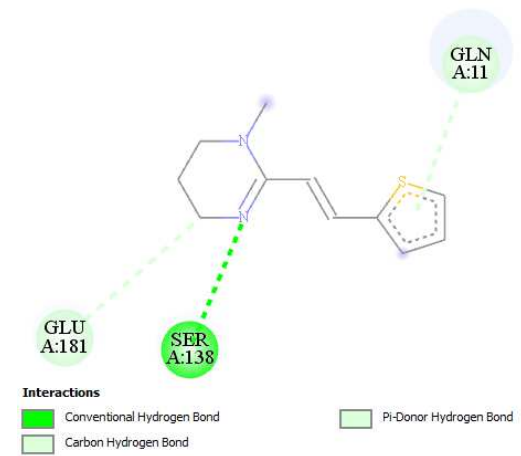


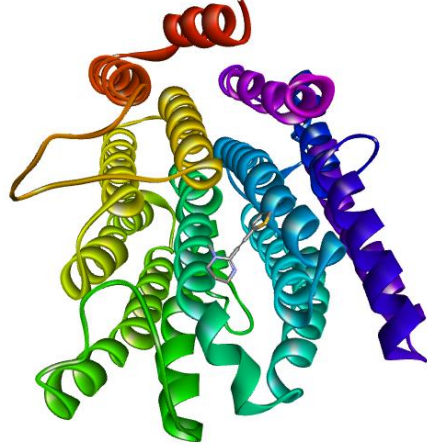


ATP synthase subunit 6 with ivermectin

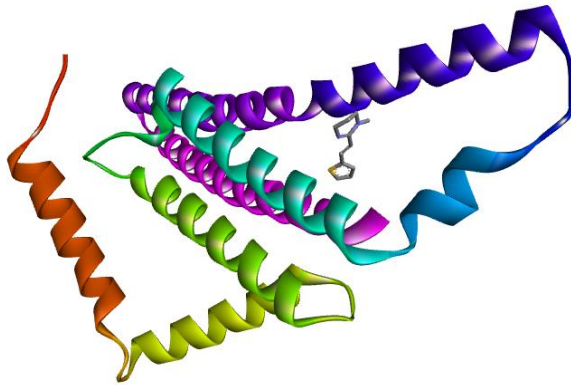
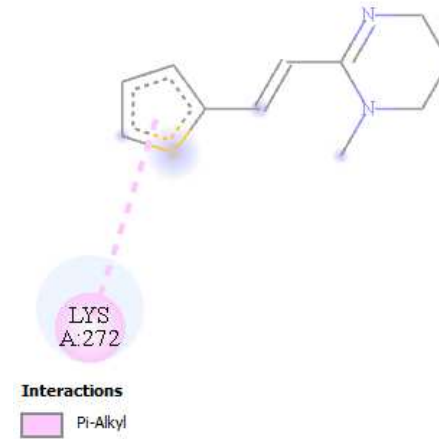


β -tubulin with levamisole

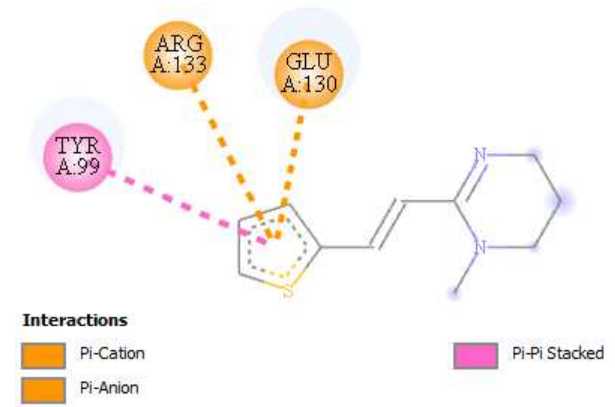


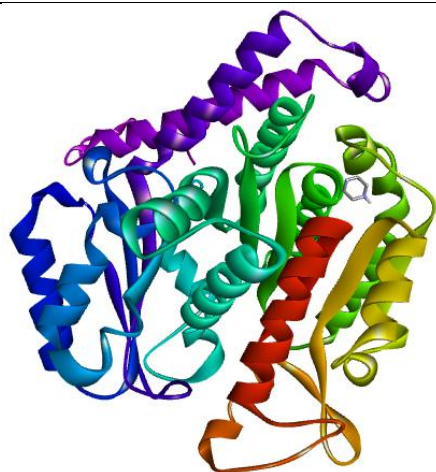


NADH-ubiquinone oxidoreductase chain 4 with Levamisole

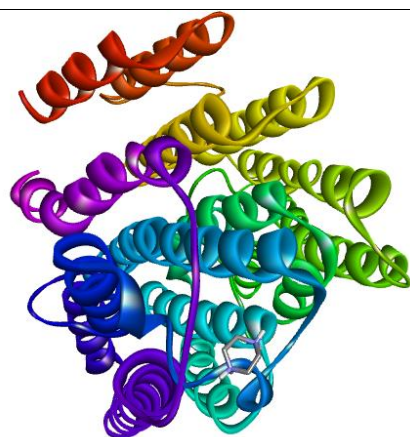
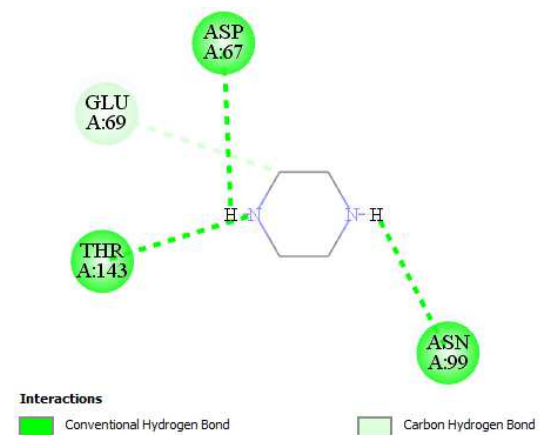


ATP synthase subunit 6 with levamisole

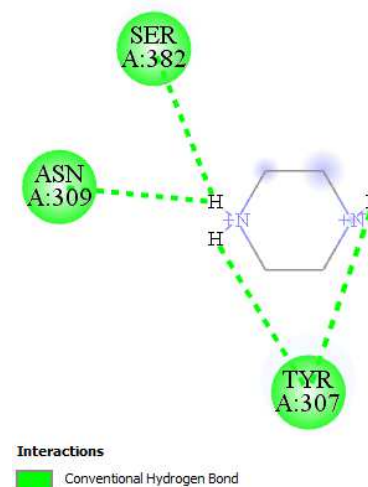


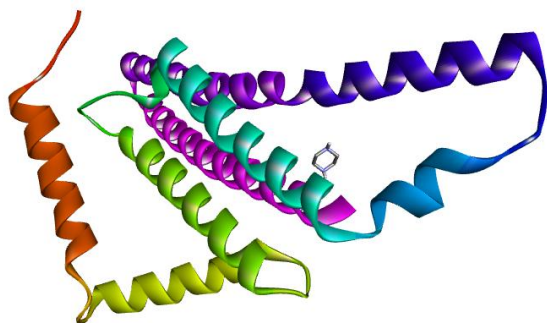


β -tubulin with piperazine

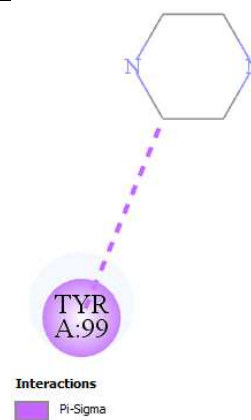


NADH-ubiquinone oxidoreductase chain 4 with piperazine





ATP synthase subunit 6 with piperazine



Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [2Agarwood.pdf](#)
- [SupplementaryTSFS.docx](#)