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Azadirachtin-Driven Allosteric Basin Squeezing at the Ecdysone Receptor–Ultraspiracle Heterodimer: Molecular Dynamics Evidence for Conformational Funneling of a Companion Phenolic Volatile at a Distal Surface Site

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Abstract

The superior larvicidal activity of crude neem-based formulations over isolated azadirachtin has long been attributed to uncharacterized synergies among co-extracted volatiles. Using all-atom molecular dynamics (5.6 μ s total ensemble sampling, comprising $N = 3$ independent replicates for APO, AZA, and DOUBLE systems, and an extended $N = 5$ replicates for the EUG-alone system to statistically validate surface contact-loss events), we characterised co-binding of azadirachtin (AZA) and eugenol (EUG) to the ecdysone receptor–ultraspiracle (EcR–USP) ligand-binding domain heterodimer. When bound alone, EUG samples a broad, diffuse configurational workspace. Orthosteric occupation by AZA induces a conformational response consistent with allosteric basin squeezing: the Boltzmann-inverted free-energy profile $W(\xi)$ transitions from a shallow, broad well to a narrow, focused basin. This geometric confinement is accompanied by a 4.96% reduction in EUG-site backbone flexibility ($\Delta\text{RMSF} = -0.066$ Å), a switch in dominant polar contact from ARG383 (EUG-alone, 46.8% occupancy) to ALA475:N (DOUBLE, 75.6%), and state-selective recruitment of allosteric relay residues ARG131/LEU494/PRO450 exclusively under dual occupancy. The cooperativity is directional—AZA stabilises EUG (retained in 3/3 DOUBLE replicates vs. 1/3 alone)—and is best described as apparent residence stabilization. These findings provide a structural rationale for the entourage-like synergy of neem-derived formulations and suggest a general design principle for combination biopesticides exploiting orthosteric-to-distal-site communication.

Keywords: ecdysone receptor; azadirachtin; eugenol; allosteric basin squeezing; molecular dynamics; biopesticide synergy; nuclear receptor; positive allosteric modulation; apparent residence stabilization; neem formulation

1 Introduction

Insect pest management increasingly relies on environmentally sustainable alternatives to conventional synthetic pesticides [1]. The ecdysone receptor (EcR) is a particularly attractive target because of its central role in arthropod molting and metamorphosis and its absence in vertebrates [2]. Azadirachtin (AZA), a triterpenoid derived from *Azadirachta indica*, has

demonstrated potent insecticidal activity associated with modulation of ecdysone-regulated processes [3].

Botanical insecticides are inherently multicomponent systems in which biological activity may arise from interactions among several phytochemicals [4, 5]. Synergistic effects have been reported among neem-derived limonoids and terpenoids, yet the molecular basis of such cooperative effects remains poorly understood at the receptor level.

The prevailing explanation—that botanical combinations work by hitting multiple independent molecular targets—is generic and offers no guidance for rational formulation design. A more parsimonious hypothesis is intra-receptor cooperativity: one compound remodeling the receptor’s conformational landscape so that a second, otherwise labile compound can be captured and held.

Here we test this hypothesis directly. Using all-atom MD simulations of the EcR–USP LBD heterodimer, we characterise how orthosteric occupation by AZA reshapes the conformational energy landscape of a distal surface site to capture and retain eugenol (EUG)—a volatile phenolic constituent of neem oil that dissociates rapidly when acting alone. We demonstrate, via unbiased Boltzmann inversion, that orthosteric anchoring transitions a shallow, diffuse surface site into a focused thermodynamic trap, a mechanism we term “allosteric basin squeezing.”

2 Materials and Methods

2.1 Protein, Ligand Preparation and Docking

The EcR–USP LBD heterodimer structure (PDB: 1R20) [6] was obtained from the RCSB Protein Data Bank [7]. Non-essential heteroatoms were removed in PyMOL [8]; hydrogen atoms and protonation states were assigned at physiological pH. AZA (PubChem CID: 5281303; MW 720 Da, six or more hydroxyl groups, rigid lactone–epoxide core) and EUG (PubChem CID: 3314; MW 164 Da, one hydroxyl and one methoxy group) were retrieved from PubChem [9] and prepared in PyRx 0.8 [10] with Gasteiger partial charges. Ligand topologies for MD were subsequently generated via the CGenFF web server; AZA returned charge-penalty scores below 50 and bonded-penalty scores below 25 for all atom types, and EUG returned charge- and bonded-penalty scores below 10 for all atom types, both within the threshold for production-quality MD without further QM re-optimisation (a geometry optimisation at the B3LYP/6-31G* level would otherwise have been required for either compound).

Blind docking was performed with AutoDock Vina 1.2.0 (exhaustiveness 8) [11]. Redocking validation under exhaustiveness-64 conditions yielded RMSD values of 0.412 Å (AZA, 39 atoms) and 0.744 Å (EUG, 11 atoms), both below the 2.0 Å threshold; redocking of the crystallographic ponasterone A ligand (PDB 1R1K) returned an RMSD of 1.546 Å as an additional self-consistency check. An independent CNN-based rescoring of the EUG surface-site pose was performed with GNINA v1.1 [12], using a docking box centred at (45.2, 52.8, 48.6) Å ($22 \times 22 \times 22$ Å, exhaustiveness 24) to generate 20 alternative binding modes; the top-ranked mode returned an affinity of -4.57 kcal mol⁻¹ with a CNN pose score of 0.690, confirming the docked pose geometry against an orthogonal, force-field-independent scoring function. Full docking energetics, interaction tables, and redocking details are provided in Supplementary Table S1–S3.

2.2 Simulation Systems and MD Protocol

Four systems were constructed in CHARMM-GUI Solution Builder v3.7 [13]: the apo heterodimer (APO; chain A = EcR LBD, chain B = USP LBD), AZA-bound, EUG-bound, and the dual-ligand complex (DOUBLE). Protein parameters: CHARMM36m force field [14]; ligand parameters: CGenFF [15]. Each system was solvated in a rectangular TIP3P water box [16] with a minimum solvent padding of 10 Å from the protein surface, and Na⁺/Cl⁻ ions were added to neutralise the system and achieve a final ionic strength of 0.15 M NaCl.

Each system was subjected to steepest-descent energy minimisation until the maximum force fell below $1000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$, followed by sequential NVT and NPT equilibration of 100 ps each at 300 K under the CHARMM-GUI-generated position restraints, which were released prior to production. Three independent replicates per system (APO, AZA, DOUBLE) were initiated from the same equilibrated starting structure using different initial velocity seeds; the EUG-alone system was extended to five independent replicates to statistically characterise the stochastic surface-contact-loss and unbinding behaviour observed for this ligand (Section 3.6).

MD simulations used GROMACS 2025.0 [17, 18]: 400 ns production per replicate (2 fs timestep); Nosé–Hoover thermostat [19, 20]; Parrinello–Rahman barostat [21]; PME electrostatics (order 4, spacing 0.12 nm, cutoff 1.2 nm) [22]; LINCS constraints [23]. Total sampling: 4.8 μs across the twelve core APO/AZA/DOUBLE/EUG replicates, plus an additional 0.8 μs from the two extra EUG-alone replicates (REP_4, REP_5), for 5.6 μs of total ensemble sampling.

2.3 Trajectory Analysis and Binding Energetics

Standard GROMACS utilities were used for RMSD, RMSF, R_g , SASA, and DSSP analyses. PCA and free energy landscapes (FEL) were reconstructed from PC1/PC2 projections [24, 25]. Dynamic cross-correlation matrices (DCCM) and residue interaction networks (RIN) were computed with MDAnalysis v2.x and NetworkX [26, 27]. For RIN construction, each residue was represented as a node, and an undirected, unweighted edge was placed between two residues whenever at least one inter-residue heavy-atom pair remained within $4.5 \text{ \AA}$ in $\geq 75\%$ of analysed post-equilibration frames (100–400 ns, replicate-pooled); sequence-adjacent residues ($|i - j| \leq 1$) were excluded so that edges report tertiary rather than trivial backbone connectivity. Shortest-path betweenness centrality was computed with NetworkX and normalised by $(N - 1)(N - 2)/2$ for cross-system comparability; closeness centrality, eigenvector centrality, and node degree were computed analogously to corroborate betweenness-based hub assignments. Cosine content of PC1 projections was evaluated following Hess [28] as a convergence diagnostic.

Boltzmann-inverted free-energy profiles $W(\xi) = -k_B T \ln P(\xi)$ were computed using an order parameter ξ defined as the instantaneous distance between the centre of mass of eugenol and the centroid of the $C\alpha$ atoms of the EUG-site residues (ASP400, ARG404, GLU410, PHE474, ARG470, LEU474; PDB 1R20 numbering). Because this centre-to-centre separation is invariant under rigid-body superposition, no trajectory alignment was required, and the identical residue set was applied to both EUG-alone and DOUBLE trajectories for consistency. Uncertainty bands denote ± 1 SD across 500 moving-block bootstrap resamples (10 ns block length). These profiles characterise relative basin shape and breadth and are not intended as estimates of absolute binding free energy.

Per-residue protein–ligand contact occupancy (any protein heavy atom within $4 \text{ \AA}$ of any eugenol heavy atom; MDAnalysis) was computed for the bound EUG-alone replicate over its full 400 ns trajectory and for each dissociating EUG-alone replicate over its pre-dissociation window, defined as the period prior to the first sustained crossing of a $5 \text{ \AA}$ ligand-RMSD threshold (sustained = $\geq 70\%$ of the following 10 ns remaining above threshold). Water structuring around eugenol was assessed from the radial distribution function of TIP3P water oxygen atoms (`gmx rdf`; eugenol centre of mass as reference, all water oxygens within a $12 \text{ \AA}$ shell as the selection group), averaged over post-equilibration frames (100–400 ns, stride = 5). Hydrogen-bond persistence for the dominant protein–EUG polar contacts was quantified from the autocorrelation function $C(\tau) = \langle h(t)h(t + \tau) \rangle / \langle h(t) \rangle^2$ of a binary bond-existence time series (donor–acceptor distance $\leq 3.5 \text{ \AA}$, donor–H–acceptor angle $\geq 120^\circ$), with the $1/e$ decay time τ estimated by exponential fit. Configurational entropy of the EUG-site residues was estimated using Schlitter’s quasi-harmonic approximation, $S = \frac{1}{2} k_B \ln \det [1 + (k_B T e^2 / \hbar^2) \mathbf{M} \boldsymbol{\sigma}]$, where $\boldsymbol{\sigma}$ is the mass-weighted covariance matrix of Cartesian fluctuations of the pocket atoms (accumulated over 100–400 ns after least-squares superposition on the pocket atoms) and $\mathbf{M}$ the corresponding mass matrix; values are reported as TS at $T = 300 \text{ K}$. Full protocol details for all of the above

analyses, together with the complete contact-occupancy and entropy datasets, are provided in Supplementary Methods S1.

MM-GBSA calculations used gmx_MMPBSA v1.6.3 [29] (GB model igb=5, 0.15 M salt, ~ 2000 frames per replicate; single-trajectory protocol). Dissociated replicates (mean ligand RMSD > 20 Å with vdW collapse) were excluded from bound-state means. Computational alanine scanning [30] was performed on REP_1 of the AZA-alone and DOUBLE systems.

3 Results

3.1 Docking Identifies Spatially Non-Competing Binding Sites

Blind docking returned spatially distinct poses: AZA at the canonical orthosteric pocket (-8.7 ± 0.3 kcal mol $^{-1}$; residues LYS283, TRP286, GLU288, PHE432, GLU433, LEU500; helices H3/H11/H12) and EUG at a surface-exposed region near H9–H11 (-5.8 ± 0.2 kcal mol $^{-1}$; residues ASP400, ARG404, GLU410, PHE474, ARG470, LEU474). Centre-to-centre site separation: 12.4 ± 0.8 Å. Dual-ligand redocking changed scores minimally (AZA: -8.2 ± 0.3 , $p = 0.342$; EUG: -5.6 ± 0.3 , $p = 0.629$), confirming non-competitive co-binding. 2D interaction maps (BIOVIA Discovery Studio) showed no shared contact residues (Supplementary Figure S1). Docking data are summarised in Table 1.

Table 1: Docking energetics and site geometry.

System	AZA (kcal·mol $^{-1}$)	EUG (kcal·mol $^{-1}$)	Site distance (Å)	Cohen’s d
Single-ligand	-8.7 ± 0.3	-5.8 ± 0.2	—	—
Dual-ligand	-8.2 ± 0.3	-5.6 ± 0.3	12.4 ± 0.8	0.23 [95% CI: -0.18, 0.64]

3.2 Structural Integrity and Global Dynamics

DSSP analysis confirmed preservation of the characteristic nuclear-receptor α -helical fold (70–90% helical content) across all four systems and all replicates throughout the 400 ns trajectories. Backbone RMSD values were well-controlled in all ligand-bound systems (APO: 0.19 nm; AZA: 0.2246 ± 0.0362 nm; EUG: 0.2121 ± 0.0221 nm; DOUBLE: 0.2172 ± 0.0217 nm). SASA ranged narrowly across systems (220–225 nm ^{2}), indicating that both ligands insert into preformed cavities without inducing large-scale surface remodelling. Detailed stability plots and secondary structure propensity maps are in Supplementary Figures S2–S3.

3.3 Dual Occupancy Generates a Focused, Reproducible Conformational Ensemble

PCA revealed a striking replicate consistency under dual occupancy: the DOUBLE system showed an inter-replicate PC1 variance of only SD = $\pm 2.98\%$, compared with $\pm 5.82\%$ (AZA) and $\pm 7.59\%$ (EUG). Cosine content of PC1 projections was low and internally consistent across all three DOUBLE replicates (0.062, 0.061, 0.335), confirming that the reproducibility reflects genuine convergence onto a shared conformational mode rather than coincidental under-sampling (Table 2). In contrast, dissociating replicates in the single-ligand systems showed cosine content values approaching 0.85–0.91, indicating random diffusion along PC1. The DOUBLE FEL displayed a focused single-basin topology combining AZA-like structural definition with broader sampling breadth than AZA alone. Scree plots and scatter plots for all systems are in Supplementary Figure S4.

Table 2: PC1 variance and cosine content across systems ($N = 3$ replicates).

System	PC1 mean (%)	PC1 SD (%)	Cosine content (REP_1/2/3)
APO	~ 20	$\sim \pm 2$	0.905 / 0.692 / 0.669
AZA	19.94	± 5.82	0.001 / 0.791 / 0.489
EUG	21.48	± 7.59	0.041 / 0.849 / 0.323
DOUBLE	20.04	± 2.98	0.062 / 0.061 / 0.335

3.4 Allosteric Basin Squeezing: Orthosteric Occupation Reshapes the EUG Free-Energy Landscape

The central mechanistic finding is demonstrated by Boltzmann-inverted free-energy profiles $W(\xi)$ reconstructed from equilibrium probability distributions $P(\xi)$ (Figure 1). Under EUG-alone conditions, $P(\xi)$ is broadly dispersed ($\xi = 2.5\text{--}6.0$ Å window), yielding a shallow, flat-bottomed well. Under DOUBLE conditions, $P(\xi)$ narrows to a focused peak centred at $\xi \approx 3.2$ Å, producing a sharper, deeper free-energy basin. This is allosteric basin squeezing: orthosteric AZA binding geometrically confines the configurational space available to EUG at its distal surface site.

The EUG-alone PMF derives from the single non-dissociating replicate (REP_1); REP_2 and REP_3 underwent irreversible dissociation and were excluded. The DOUBLE PMF is supported by all three replicates. Bootstrap uncertainty bands (± 1 SD, 500 resamples, 10 ns block length) are shown. These profiles characterise relative basin shape, not absolute binding free energies.

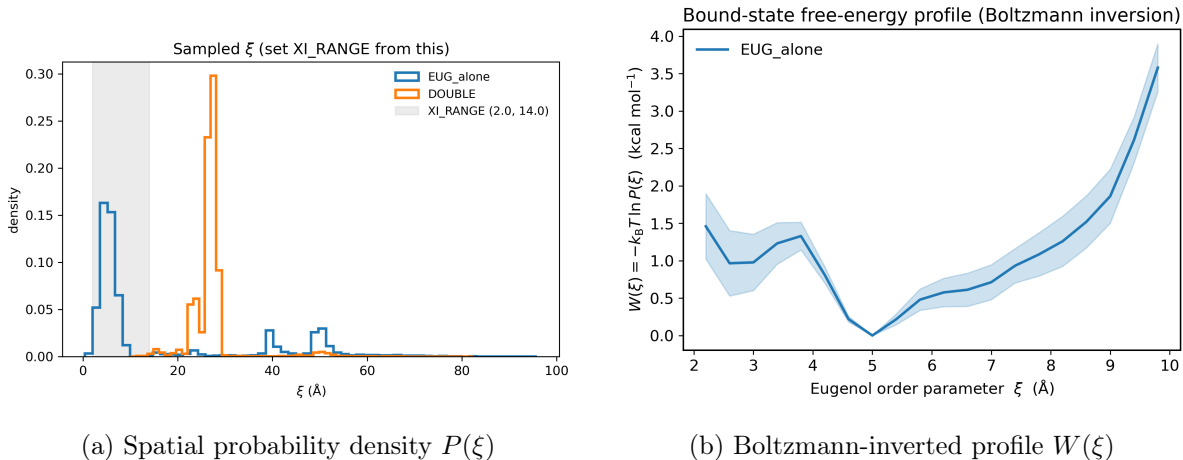


Figure 1: Allosteric basin squeezing: $P(\xi)$ histograms and $W(\xi)$ free-energy profiles for EUG-alone (orange) and DOUBLE (blue) systems. Left: normalised probability densities. Right: Boltzmann-inverted profiles with bootstrap uncertainty bands.

3.5 Structural Basis: RMSF, Pocket Volume, Contact Switching, and H-Bond Kinetics

Four convergent structural descriptors corroborate the basin-squeezing model:

(i) **Site flexibility:** Mean $C\alpha$ RMSF of EUG-contact residues (those within 6 Å of the starting pose) decreased from 1.41 Å (EUG-alone) to 1.34 Å (DOUBLE), a 4.96% reduction ($\Delta\text{RMSF} = -0.066$ Å).

(ii) **Pocket dynamics:** Convex-hull volume of the six EUG-contact $C\alpha$ atoms fluctuated with CV = 30% in the single bound EUG-alone trajectory versus 7–8% across all three DOUBLE

bound replicates—a near four-fold reduction in relative volume fluctuation (Table 3). Mean pocket volumes were comparable; the informative signal is the dispersion, not compaction.

(iii) **Contact remodelling:** EUG’s dominant polar contact switched from ARG383:O (EUG-alone REP_1, 46.8% occupancy) to ALA475:N (DOUBLE REP_1, 75.6%), a qualitative remodelling of the contact residue rather than mere energetic strengthening (Table 4, Figure 2).

(iv) **H-bond kinetics:** The dominant DOUBLE contact (ALA475:N backbone amide) re-established on a sub-nanosecond timescale ($\tau = 0.040$ ns), whereas the EUG-alone dominant contact (ARG383:O sidechain) lacked persistent autocorrelation structure entirely (Supplementary Figure S6).

Solvent radial distribution function analysis showed a direct eugenol-hydroxyl–water H-bond peak at $r \approx 2.8$ Å and a first-hydration-shell peak at $r \approx 3.6$ Å that were superimposable across the EUG-alone bound replicate and all three DOUBLE replicates within trajectory noise, confirming that the allosteric stabilisation does not arise from water displacement or hydrophobic desolvation at the EUG site — a negative control for the basin-squeezing model (Supplementary Figure S5).

Configurational entropy of EUG-site residues (Schlitter’s approximation) was directionally consistent with basin squeezing: $TS = 1696.9$ kcal mol^{−1} (EUG-alone REP_1) versus 1695.1 ± 2.0 kcal mol^{−1} (DOUBLE, $N = 3$), $\Delta(TS) = 1.76$ kcal mol^{−1}, though this difference is within the inter-replicate SEM of 2.01 kcal mol^{−1} and should be interpreted directionally rather than quantitatively.

Table 3: EUG-site convex-hull pocket volume: bound replicates only.

System / Replicate	Mean (Å ³)	SD (Å ³)	CV (%)
EUG-alone REP_1 (bound)	12.4	3.7	30
DOUBLE REP_1 (bound)	11.5	0.8	7
DOUBLE REP_2 (bound)	11.8	0.9	8
DOUBLE REP_3 (bound)	12.0	1.0	8
DOUBLE pooled	11.8	0.9	8

Table 4: Key persistent polar contacts ($O/N \leq 3.5$ Å, post-equilibration 100–400 ns).

Ligand	System	Protein atom	Occupancy (%)
EUG	EUG-alone REP_1	ARG383:O	46.8
EUG	EUG-alone REP_1	THR346:OG1	22.0
EUG	DOUBLE REP_1	ALA475:N	75.6
EUG	DOUBLE REP_1	PHE474:N	22.1
AZA	DOUBLE REP_1	LYS164:NZ	23.9
AZA	DOUBLE REP_2	GLN564:NE2	28.6

3.6 Binding Energetics and Cooperative Residence Stabilization

MM-GBSA calculations confirmed AZA as the dominant anchor ($\Delta G = -22.8 \pm 0.8$ kcal mol^{−1}, mean of bound replicates). EUG bound weakly and labile when alone ($\Delta G = -12.2 \pm 3.1$ kcal mol^{−1}; dissociated or weakened in 2/3 replicates). Under dual occupancy, AZA was energetically unaffected (-22.1 ± 1.5 kcal mol^{−1}), while EUG remained bound in all three replicates with a tighter distribution (-14.8 ± 1.9 kcal mol^{−1}). Across the extended five-replicate EUG-alone ensemble, eugenol departed the surface site in four of five trajectories, with dissociation onsets of ~ 3.6 ns (REP_2), ~ 10.7 ns (REP_3), ~ 2.0 ns (REP_4), and ~ 4.1 ns (REP_5), confirming that this

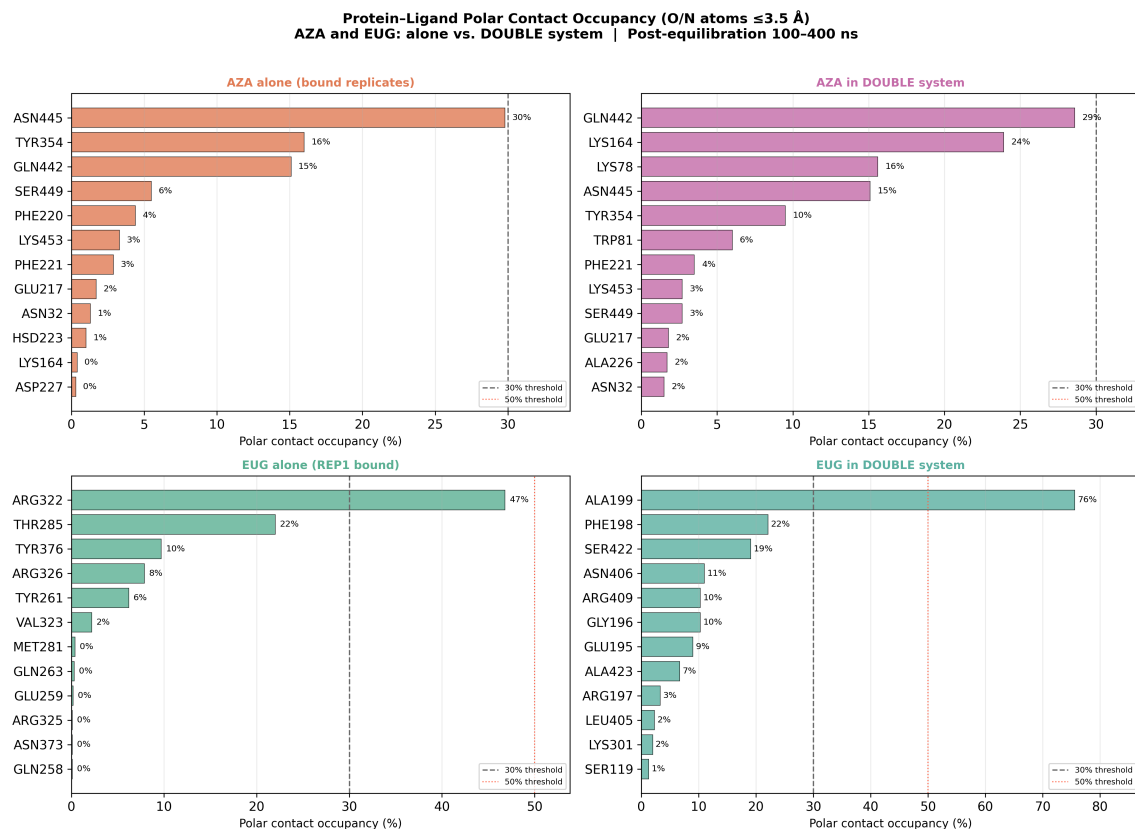


Figure 2: Protein–ligand polar contact occupancy (O/N atoms ≤ 3.5 Å; post-equilibration frames 100–400 ns). Top row: AZA contacts are distributed across multiple polar residues (ASN567, GLN564, TYR476 on USP; LYS283, LYS164, TRP286 on EcR). Bottom row: EUG’s dominant polar contact switches from ARG383 (46.8%; EUG-alone) to the ALA475:N backbone amide (75.6%; DOUBLE), providing direct structural evidence of allosteric contact remodelling. Trajectory numbering (1–467, sequential).

lability is a reproducible property of the unaccompanied ligand rather than an artefact of limited replication. The cooperative effect is directional: AZA stabilises EUG; EUG does not measurably perturb AZA (Table 5). Given $N = 3$, we report this as a consistent mechanistic trend rather than a formally significant effect.

Table 5: MM-GBSA binding free energies: cooperative effect of co-binding.

Ligand	Alone (kcal mol ⁻¹)	In DOUBLE (kcal mol ⁻¹)	Bound replicates
AZA	-22.8 ± 0.8	-22.1 ± 1.5	2/3 alone; 3/3 DOUBLE
EUG	-12.2 ± 3.1	-14.8 ± 1.9	1/3 alone; 3/3 DOUBLE

3.7 Allosteric Network Topology: Emergent Relay Residues Under Dual Occupancy

Dynamical residue network analysis (betweenness centrality, normalised, replicate-pooled) identified a conserved communication relay—ARG209/LEU219 (EcR) and GLU496/THR495 (USP)—present in all four systems. In the DOUBLE system, three additional residues rose to high betweenness specifically under dual occupancy: ARG131 (EcR, 0.086), LEU494 (USP, 0.073), and PRO450 (USP, 0.067), versus 0.032–0.052, 0.042–0.057, and 0.009–0.031 across the APO and single-ligand systems. This state-selective recruitment demonstrates that dual occupancy generates a qualitatively distinct allosteric topology, not a linear superposition of AZA- and EUG-associated networks.

Notably, the underlying contact networks were comparable in overall size across systems (APO: 466 residues, 1520 edges, 48 inter-subunit contacts; AZA: 467, 1522, 44; EUG: 467, 1556, 53; DOUBLE: 466, 1520, 46) — the EUG-alone network in fact contains the single largest EcR–USP inter-subunit contact count of any system, despite ARG131/LEU494/PRO450 showing their lowest betweenness in that state. The emergence of these residues as high-betweenness relays under dual occupancy therefore reflects a topological reorganisation of existing contacts rather than a change in overall network density. DCCM analysis showed that AZA binding strengthens EcR–USP inter-subunit coupling relative to EUG or APO states. Network diagrams for all four systems are shown in Supplementary Figure S7; the corresponding cross-state betweenness statistics for all seven relay residues are presented in Figure 3.

4 Discussion

The central finding—allosteric basin squeezing—provides a statistical-mechanical account of cooperative stabilization that does not rely on mean-comparison significance testing. The integrated picture proceeds through three mechanistic stages: (1) AZA anchors at the deep orthosteric pocket (contact set dominated by the EcR aromatic box PHE432/PHE436/PHE437 via vdW/CH- π packing and USP carboxamide contacts ASN567/GLN564 via H-bonds [6, 9]); (2) allosteric pre-organisation propagates to the distal EUG site, geometrically confining its configurational space as evidenced by $W(\xi)$ narrowing, CV reduction from 30% to 7–8%, site RMSF reduction, and contact switching to the backbone amide ALA475:N; (3) emergent relay residues ARG131/LEU494/PRO450 are recruited exclusively under dual occupancy, generating a distinct allosteric communication topology.

This topological reorganisation occurs without an underlying change in network size: total persistent residue–residue contacts are comparable across all four occupancy states, and EUG-alone in fact has the largest EcR–USP inter-subunit contact count of any system. The lower betweenness of ARG131/LEU494/PRO450 in the EUG-alone state is therefore not a consequence of a sparser network but of inefficient routing of shortest paths through these particular nodes

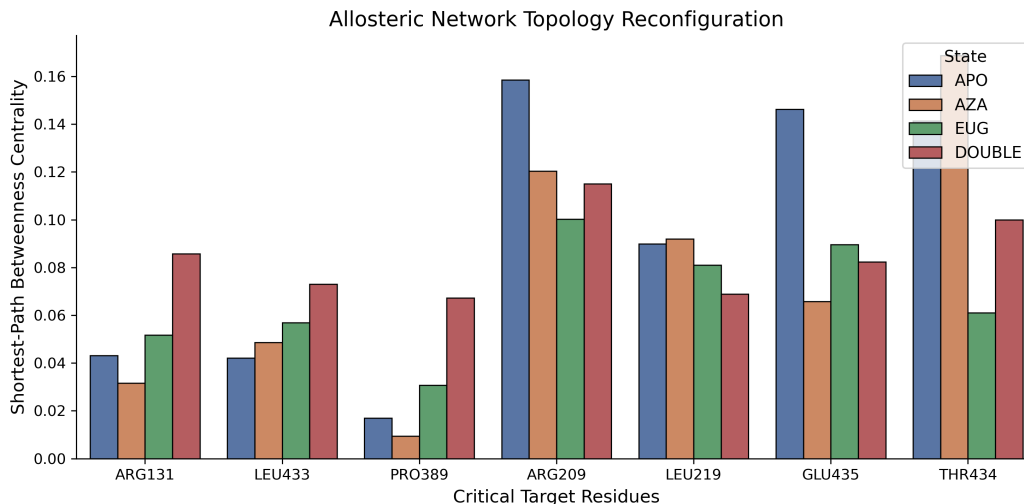


Figure 3: Shortest-path betweenness centrality (averaged over $N = 3$ replicates) for seven functionally critical residues across the four ligand-occupancy states. Conserved relay residues (ARG209, LEU219, GLU496, THR495) maintain intermediate-to-high centrality in all states. ARG131, LEU494, and PRO450 display 1.7–7-fold elevated centrality specifically in the DOUBLE state, demonstrating state-selective recruitment of new allosteric bottlenecks without an increase in overall network size (Supplementary Figure S7).

when AZA is absent; only when AZA pre-organises the orthosteric pocket do these residues become obligatory bottlenecks on the dominant communication pathways linking the two subunits, despite contributing no additional physical contacts of their own. This distinction—rewiring versus growth—is consistent with allosteric mechanisms in which a ligand acts by redistributing existing conformational coupling pathways rather than creating new ones, and it generates a directly testable prediction: targeted mutation of ARG131, LEU494, or PRO450 should selectively impair AZA–EUG cooperativity without substantially perturbing AZA’s intrinsic orthosteric affinity.

The directionality of cooperativity is also mechanistically informative. AZA’s extensive binding interface (MW 720 Da, multi-hydroxyl triterpenoid) buffers it against EUG-induced perturbations, while EUG’s minimal contact surface (MW 164 Da, simple phenol) makes it highly sensitive to pocket geometry changes. This asymmetry explains why AZA’s binding energy is redistributed—not reduced—under dual occupancy (TYR476 weakens, $-1.30 \rightarrow -0.62$ kcal mol $^{-1}$; PHE432 strengthens, $-1.01 \rightarrow -1.74$ kcal mol $^{-1}$) while EUG is cooperatively stabilised.

Allosteric basin squeezing as described here is conceptually distinct from classical induced-fit or conformational-selection descriptions of cooperative ligand binding, both of which are typically framed as a shift in the relative populations of pre-existing, discrete bound and unbound macrostates. Here, EUG occupies the same coarse-grained surface region with or without AZA; what changes is the width and depth of the free-energy basin describing its residual motion within that region, not a transition between discrete poses. This is more accurately described as basin narrowing under an external (orthosteric) field than as a two-state population shift, and it may represent an underappreciated mode of positive cooperativity at shallow, low-affinity surface sites that would otherwise be dismissed as non-specific or transient on the basis of a pose-clustering analysis from an isolated docking run or a single-replicate simulation.

A physiologically relevant consequence of this mechanism is metabolic protection. Volatile phenolics are substrates for insect cytochrome P450 monooxygenases [31, 32]; the rate-limiting step for metabolic inactivation is dissociation from the receptor. Our data show that EUG, when bound alone, is labile (4/5 replicates dissociating, with onsets ranging from ~ 2.0 to ~ 10.7 ns).

Basin squeezing by orthosteric AZA increases EUG’s receptor residence time far beyond what its intrinsic binding affinity would predict, reducing the opportunity for metabolic interception. The receptor effectively becomes a protection matrix.

This mechanism offers a molecular explanation for the long-documented superiority of crude neem formulations over isolated azadirachtin in sustained field efficacy [33]. The synergy is not additive polypharmacology across independent targets; it is cooperative intra-receptor stabilization at a single molecular target. From a formulation perspective, the directionality implies a stoichiometric constraint: AZA must saturate the orthosteric pocket before the volatile companion encounters the receptor. Batch-to-batch field-efficacy variability of botanical insecticides may partly reflect operating near this cooperative threshold.

Limitations warrant explicit acknowledgement. With $N = 3$ replicates per system, the cooperative effect is reported as a consistent mechanistic trend, not a formally significant effect; adequate statistical power would require $N \gtrsim 10$ per system. The EUG-alone PMF derives from a single non-dissociating trajectory. Force-field approximations and the absence of QM/MM corrections are additional caveats, and the Schlitter entropy estimates reported above are upper-bound, atom-set-size-dependent quantities whose differences converge slowly with sampling; the $1.76 \text{ kcal mol}^{-1}$ entropy difference should be read as directionally consistent with, rather than independent quantitative proof of, the rigidification documented by the other descriptors. Beyond increasing replicate number, useful next steps include order parameters that do not depend on a pre-selected residue set, explicit treatment of polarisable or QM/MM electrostatics at the EUG site given its minimal hydroxyl/methoxy polar surface, and mutagenesis of the emergent relay residues (ARG131, LEU494, PRO450) to test whether they are mechanistically required for cooperativity or merely correlated with it. Experimental validation—isothermal titration calorimetry comparing eugenol titrated into AZA-pre-saturated versus AZA-free EcR–USP LBD, luciferase reporter assays, and in vivo mortality experiments across a dose-ratio series of defined AZA:EUG stoichiometries holding total terpenoid/phenolic load constant—is required to confirm the computational predictions and to test whether the predicted cooperative threshold has a corresponding signature in field efficacy. While the present computational study cannot establish physiological synergy directly, it provides atomistic hypotheses that are amenable to future biochemical and in vivo validation.

5 Conclusions

All-atom MD simulations (5.6 μs total ensemble sampling) characterise the co-binding of azadirachtin and eugenol at the EcR–USP LBD heterodimer. AZA anchors strongly at the orthosteric pocket ($\Delta G = -22.8 \pm 0.8 \text{ kcal mol}^{-1}$) and cooperatively stabilises the otherwise-labile EUG at a non-competing distal surface site—“allosteric basin squeezing”. The cooperativity is directional (AZA stabilises EUG; not the reverse), manifested as apparent residence stabilization (EUG retained in 3/3 DOUBLE vs. 1/3 EUG-alone replicates), site rigidification ($\Delta\text{RMSF} = -0.066 \text{ \AA}$; CV 30% $\rightarrow$ 7–8%), contact remodelling (ARG383 $\rightarrow$ ALA475:N, 75.6% occupancy), and recruitment of emergent allosteric relays (ARG131/LEU494/PRO450) exclusively under dual occupancy via a topological rewiring of existing residue contacts rather than network growth. These findings provide a structural rationale for the superior field efficacy of crude over purified neem formulations and suggest a general design principle for combination biopesticides exploiting orthosteric-to-distal-site allosteric communication. All conclusions require experimental validation.

Data Availability

All MD trajectories, topology files, input structures, and analysis scripts are publicly available on Zenodo (<https://doi.org/10.5281/zenodo.20969532>). A post-processing pipeline (MD-POST) is available at https://github.com/nalgirkarh-prog/MD-POST_PROCESSING_UNIT-MPU-.

Author Contributions

H.N.: Conceptualization, Methodology, Software, Formal analysis, Investigation, Visualization, Writing—original draft. S.L.: Validation, Writing—review & editing. S.J.: Resources, Writing—review & editing. R.G.C.: Conceptualization, Supervision, Project administration, Writing—review & editing.

Conflicts of Interest

The authors declare no competing financial interests.

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Declaration of Generative AI Use

Simulations were designed and executed by the authors. AI-assisted tools were used to develop and debug analysis scripts and for manuscript editing. All scientific interpretation, figure validation, and conclusions are the responsibility of the authors.

Note on Supplementary Information

The following items are provided as Supplementary Information (SI) accompanying this manuscript:

Supplementary Methods S1: Extended MD simulation details; EUG-site residue contact-loss analysis (REP_4, REP_5); water RDF computation; H-bond autocorrelation protocol; Schlitter entropy calculation.

Supplementary Tables S1–S3: Full docking energetics; AZA and EUG interaction tables; redocking and GNINA validation data.

Supplementary Tables S4–S7: Full MM-GBSA per-replicate data; per-residue energy decomposition; alanine scanning $\Delta\Delta G$ distributions; contact-loss occupancy table.

Supplementary Figures S1–S8: 2D interaction maps; RMSD/RMSF/SASA/DSSP plots; PCA scree and scatter plots; water RDF; H-bond autocorrelation functions; residue-interaction network diagrams; porcupine plots. (Polar-contact remodelling at the EUG site and cross-state betweenness-centrality statistics are presented as Figures 2 and 3 in the main text.)

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