

Chemical profiling and antioxidant potential of the ethanolic extract of the *Odontotermes formosanus* fungus comb: an integrated LC–MS/MS and computational study

Jinxin Liu

Jiangxi University of Traditional Chinese Medicine

Ruiqi Kang

Jiangxi University of Traditional Chinese Medicine

Jiayi Dong

Jiangxi University of Traditional Chinese Medicine

Weina Wang

Jiangxi University of Traditional Chinese Medicine

Zhixin Li

Jiangxi University of Traditional Chinese Medicine

Jia Huang

Jiangxi University of Traditional Chinese Medicine

Qimeng Fan

Jiangxi University of Traditional Chinese Medicine

Liangshan Ming

Jiangxi University of Traditional Chinese Medicine

Hongning Liu

Jiangxi University of Traditional Chinese Medicine

Wei Jiang

Jiangxi University of Traditional Chinese Medicine

Zishu Dong

20211027@jxutcm.edu.cn

Jiangxi University of Traditional Chinese Medicine

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Abstract

Fungus combs of fungus-growing termites are complex symbiotic matrices formed through termite feeding and digestion of plant substrates, followed by continuous transformation by symbiotic *Termitomyces* fungi and associated microorganisms. Although extracts from the fungus combs of some fungus-growing termite species have been reported to exhibit antioxidant activity, the chemical composition and biological properties of the fungus comb of *Odontotermes formosanus* remain poorly characterised. In the present study, untargeted liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used to characterise the ethanolic extract of the *O. formosanus* fungus comb (OFCE), and its antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging and ferric reducing antioxidant power (FRAP) assays. As an exploratory extension, ageing-related database targets were used as a biological context, and target prediction, network analysis, molecular docking, and 100 ns molecular dynamics simulations were integrated to prioritise selected putative annotations and predicted ligand–target relationships. Azelaic acid and N-acetyltryptophan were among the candidates prioritised for further evaluation. Under the computational models and simulation conditions used, the two epidermal growth factor receptor (EGFR)–ligand complexes exhibited conformational compatibility and maintained relatively stable conformational states during portions of the simulations. Collectively, these findings indicate that the *O. formosanus*–*Termitomyces* fungus-comb system contains a chemically diverse pool of ethanol-extractable small molecules accompanied by measurable antioxidant activity, thereby providing candidate leads for subsequent structural confirmation, source attribution, quantitative analysis, and activity validation.

1. Introduction

Normal cellular function depends on redox homeostasis. When reactive oxygen species (ROS) production exceeds the capacity of endogenous antioxidant systems, lipids, proteins and nucleic acids can undergo oxidative damage (1). This has prompted interest in naturally derived compounds with radical-scavenging or reducing activity. Materials transformed by several organisms may contain a wider range of extractable constituents than those obtained from a single plant or microorganism. Fungus combs produced by fungus-growing termites are one such material, shaped by termite processing, symbiotic fungal growth and associated microbial activity.

Fungus-comb formation relies on the continuous processing of plant material by termites, symbiotic fungi and associated microorganisms. Collected plant material passes through the digestive tracts of young workers and is mixed with asexual fungal spores to form the comb, where lignocellulose and other complex constituents undergo further degradation (2). In *O. formosanus*, the comb thus connects plant substrates, termite digestion and microbial metabolism, and may accumulate small molecules from several biological sources.

Extracts of *Macrotermes gilvus* fungus combs scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals (3) and contain organic acids, fatty-acid

derivatives and other constituents (4, 5). Fungus combs from other termite species also contain lipids, sterols and phenolic compounds (6). However, comb composition may vary with termite species and dietary substrate (7), so findings from other species cannot be assumed to apply to *O. formosanus*. The ethanol-soluble small-molecule composition, antioxidant activity and potential biological associations of *O. formosanus* fungus combs remain to be systematically investigated.

Chemical profiling and computational analysis offer complementary ways to examine this complex material. High-resolution liquid chromatography–tandem mass spectrometry (LC–MS/MS) can profile chemical features in natural extracts and help prioritise constituents potentially associated with biological activity (8). Network pharmacology maps predicted associations among compounds, targets and biological processes (9), while molecular docking and molecular dynamics simulations assess the structural plausibility of selected ligand–protein interactions (10)

Accordingly, the present study first employed untargeted LC–MS/MS to characterise the chemical features of the ethanolic extract of the *O. formosanus* fungus comb (OFCE) and evaluated its antioxidant response using DPPH radical-scavenging and ferric reducing antioxidant power (FRAP) assays. These experiments were designed to determine whether the fungus comb contains diverse ethanol-extractable small-molecule features and whether the resulting extract exhibits measurable antioxidant activity under the assay conditions used. Given the intersection between redox imbalance and multiple cellular processes associated with ageing (11, 1), ageing-related database targets were subsequently used as an exploratory biological context. Target prediction, network analysis, functional enrichment, molecular docking, and molecular dynamics simulations were integrated to prioritise selected putative annotations and predicted molecular relationships. This integrated strategy was intended to generate experimentally testable candidate leads from the complex chemical profile and to provide a basis for subsequent structural confirmation, quantitative analysis, and biological validation.

2. Materials and methods

2.1. Reagents and instruments

Fungus combs associated with *O. formosanus* were obtained from Maoming Jingfeng Pest Control Service Co., Ltd. (Maoming, China). DPPH and FRAP assay kits were purchased from Solarbio (Beijing, China). Absolute ethanol was obtained commercially in Shanghai, China.

2.2. Preparation of OFCE

Approximately 200 g of fresh *O. formosanus* fungus-comb material was washed three times with ultrapure water to remove loosely attached surface debris and then homogenised. The material was extracted three times with absolute ethanol under reflux using an electrically heated reflux apparatus, with each extraction conducted for 30 min. The ethanolic extracts obtained from the three successive extractions were combined, filtered, and concentrated under reduced pressure using a rotary evaporator

to remove ethanol, yielding the ethanolic extract of the *O. formosanus* fungus comb (OFCE). The resulting extract was stored at – 20°C until further analysis.

For subsequent antioxidant assays, OFCE was dissolved in absolute ethanol to prepare a 20 mg/mL stock solution. Preliminary experiments were performed using OFCE concentrations of 0.002, 0.02, 0.2, 2, and 20 mg/mL. Based on the observed response range, concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL were selected for the formal assays.

2.3. Untargeted liquid chromatography–tandem mass spectrometry analysis

Untargeted LC–MS/MS analysis of OFCE was performed using a Vanquish ultra-high-performance liquid chromatography system (Thermo Fisher Scientific) equipped with a Waters ACQUITY UPLC BEH Amide column (2.1 mm × 50 mm, 1.7 µm) and coupled to an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific).

The mobile phases consisted of water containing 25 mmol/L ammonium acetate and 25 mmol/L ammonium hydroxide (pH 9.75) as mobile phase A and acetonitrile as mobile phase B. The autosampler was maintained at 4°C, and the injection volume was 2 µL. MS and MS/MS spectra were acquired using Xcalibur software (version 4.4, Thermo Fisher Scientific) in information-dependent acquisition (IDA) mode.

The electrospray ionisation parameters were as follows: sheath gas flow rate, 50 arbitrary units; auxiliary gas flow rate, 15 arbitrary units; capillary temperature, 320°C; full-scan MS resolution, 60,000; MS/MS resolution, 15,000; and stepped normalised collision energies of 20, 30, and 40. The spray voltages were set to 3.8 kV in positive-ion mode and – 3.4 kV in negative-ion mode.

2.4. Data preprocessing and metabolite annotation

Raw LC–MS/MS data were converted to mzXML format using ProteoWizard (version 3.0.24054) (12). Peak detection, extraction, alignment, and integration were subsequently performed using an in-house R-based workflow.

Metabolite identification and annotation were performed by comparison with an internal spectral library containing retention-time (RT), precursor-ion (MS1), and MS/MS spectral information. Annotation confidence was classified according to the Metabolomics Standards Initiative (MSI) framework (13). Level 1 identification required agreement of RT, MS1, and MS2 information with those of authentic standards analysed under the same experimental conditions. Level 2 corresponded to putative compound annotations based on physicochemical properties and/or spectral-library matching without confirmation using authentic standards under identical analytical conditions. Level 3 represented putative compound-class annotations, whereas a specific chemical structure was not assigned solely on the basis of theoretical database matching. Unmatched molecular features were classified as Level 4.

The MS2 matching score generated by the original analytical workflow was used as a parameter for subsequent candidate screening but was not considered equivalent to an MSI confidence level. Because absolute concentrations of individual metabolites were not determined in the untargeted analysis, LC–MS peak areas were interpreted as relative analytical responses rather than absolute compound concentrations.

2.5. DPPH radical-scavenging assay

DPPH radical-scavenging activity was determined according to the manufacturer's instructions and with reference to the classical DPPH method (14). Briefly, 10 μ L of OFCE solution at 1.25, 2.5, 5, 10, or 20 mg/mL was mixed with 190 μ L of DPPH working solution. After incubation for 30 min at room temperature in the dark, absorbance was measured at 515 nm.

A solvent blank was included, and the intrinsic absorbance of OFCE was corrected using a sample-background control. Vitamin C (0.015625–0.300 mg/mL) was used as a positive control. Each concentration was measured in technical triplicate.

DPPH radical-scavenging activity was calculated as follows:

$$\text{DPPH scavenging activity (\%)} = [A_0 - (A_1 - A_2)] / A_0 \times 100\%$$

where A_0 represents the absorbance of the solvent blank, A_1 the absorbance of the sample reaction mixture, and A_2 the background absorbance of the sample.

2.6. Ferric reducing antioxidant power assay

Ferric reducing antioxidant power was determined according to the manufacturer's instructions and with reference to the classical FRAP method (15). FRAP reagents I, II, and III were mixed at a volume ratio of 7:1:1 immediately before use.

The reaction mixture contained 180 μ L of FRAP working solution, 6 μ L of OFCE solution at 1.25, 2.5, 5, 10, or 20 mg/mL, and 18 μ L of distilled water. For the blank, 24 μ L of distilled water was used in place of the sample. After incubation for 10 min at room temperature in the dark, 200 μ L of the reaction mixture was transferred for absorbance measurement at 593 nm.

A standard curve was established using FeSO_4 . Each concentration was measured in technical triplicate, and the results were expressed as $\mu\text{mol Fe}^{2+}$ equivalents per gram of extract.

2.7. Exploratory screening of annotation-derived candidates

A total of 691 unique putative annotations with MS2 matching scores > 3.5 were included in exploratory computational screening. Compounds indexed in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP) were screened using thresholds of oral bioavailability $\geq 30\%$ and drug-likeness ≥ 0.18 (16).

For compounds not indexed in TCMSP, canonical SMILES strings were retrieved from PubChem (17). SwissADME was then used to retain compounds predicted to have high gastrointestinal absorption and to satisfy at least two of the Lipinski, Ghose, Veber, Egan, and Muegge drug-likeness criteria (18).

Following screening, 397 candidate entries were retained. Their potential human protein targets were predicted using SwissTargetPrediction, and predictions with probabilities > 0 were retained (19). All computational screening procedures were used solely for candidate prioritisation.

2.8. Collection of ageing-related targets and overlap analysis

Ageing-related human targets were retrieved from GeneCards (20), OMIM (21), and DrugBank (22) using the search term “anti-ageing” and restricting the species to *Homo sapiens*.

Venny 2.1 (23) was used to identify overlapping targets between predicted candidate targets and ageing-related targets. Cytoscape 3.10.3 was subsequently used to construct a putative compound–shared-target network (24).

2.9. Protein–protein interaction network analysis

The overlapping targets were submitted to the STRING database (25), with the organism restricted to *Homo sapiens* and the minimum interaction score set to > 0.700.

The resulting network was imported into Cytoscape 3.10.3. Degree centrality, betweenness centrality, and closeness centrality were calculated using CentiScaPe (26). Nodes satisfying the thresholds of degree > 15.1768, betweenness centrality > 624.7195, and closeness centrality > 0.0010845 were retained. Seven highly connected targets were selected for subsequent structural modelling.

2.10. GO and KEGG enrichment analyses

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using DAVID (27), with *Homo sapiens* specified as the background species. GO enrichment included biological process, cellular component, and molecular function categories (28), whereas KEGG pathway enrichment was conducted according to the KEGG database (29).

Terms with nominal $P < 0.05$ were retained, and the 20 KEGG pathways with the lowest P values were displayed.

2.11. Molecular docking

Six prioritised candidate ligands—Phe-Val, azelaic acid, Thr-Leu, milrinone, N-acetyltryptophan, and betaxolol—were docked against seven prioritised proteins: AKT1, IL6, TP53, STAT3, EGFR, SRC, and CTNNB1.

Ligand structures were obtained from PubChem and converted using Open Babel 3.1.1 (30). Protein structures were retrieved from the RCSB Protein Data Bank (PDB) (31) using the following PDB entries: AKT1 (8UW7), IL6 (1ALU), TP53 (9SPQ), STAT3 (6NJS), EGFR (9DF4), SRC (8VCG), and CTNNB1 (8RU3).

Protein and ligand structures were prepared using AutoDockTools 1.5.7 (32), including removal of water molecules, addition of hydrogen atoms, and definition of rotatable ligand bonds. Docking results were visualised using PyMOL 2.6 and Discovery Studio, and docking scores were summarised using GraphPad Prism 10.1.2.

2.12. Molecular dynamics simulations

Molecular dynamics (MD) simulations of the EGFR–azelaic acid and EGFR–N-acetyltryptophan complexes were performed for 100 ns using GROMACS 2022.2 (33).

The Amber99SB-ILDN force field was applied to the protein (34). Ligand topologies were generated using Sobotop 1.0 dev3.1 with GAFF2 parameters (35, 36) and RESP charges (37). Each complex was placed in a cubic simulation box extending 1 nm from the solute and solvated using the TIP3P water model (38). After neutralisation, the NaCl concentration was adjusted to 0.15 M.

Long-range electrostatic interactions were treated using the particle mesh Ewald method (39). Both electrostatic and van der Waals cutoffs were set to 1.2 nm. Covalent bonds involving hydrogen atoms were constrained using the LINCS algorithm (40), and a 2 fs integration time step was used.

Energy minimisation was performed using sequential steepest-descent and conjugate-gradient algorithms, followed by 1 ns of NVT equilibration and 1 ns of NPT equilibration. The temperature was maintained at 300 K using velocity-rescaling temperature coupling with a coupling constant of 1 ps (41). Pressure was maintained at 1 bar using the Parrinello–Rahman barostat with a coupling constant of 1 ps (42).

The trajectories were analysed in terms of root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration, solvent-accessible surface area, intermolecular hydrogen bonding, principal component analysis (PCA), and free-energy landscapes.

2.13. Statistical analysis

DPPH radical-scavenging activity and FRAP values are presented as the mean $\pm$ standard deviation (SD). Each concentration was measured in technical triplicate ($n = 3$) using the pooled OFCE sample. Data processing and graphical presentation were performed using GraphPad Prism 10.1.2, and error bars represent the SD. No inferential statistical tests were performed for between-group comparisons.

3. Results

3.1. LC–MS/MS analysis

The preparation of OFCE and the overall experimental workflow are shown in Fig. 1a. Total ion chromatograms acquired in positive- and negative-ion modes revealed a complex LC–MS feature profile (Fig. 1b).

Among the putative annotations, organic acids and derivatives, nitrogen-containing heterocyclic compounds, aromatic compounds, amino acids and derivatives, and peptides accounted for 15.46%, 12.70%, 9.39%, 6.68%, and 6.26%, respectively, with the remaining categories shown in Fig. 1c. These percentages represent the distribution of annotated features rather than quantitative chemical composition.

The correlations among quality-control (QC) samples and the QC-relative standard deviation (RSD) distribution supported analytical reproducibility (Fig. 1d–e). A total of 691 unique annotation names with MS2 matching scores > 3.5 were obtained (Table S1). Representative prioritised entries included azelaic acid, N-acetyltryptophan, Phe-Val, Thr-Leu, milrinone, and betaxolol.

Because confirmation using authentic standards and absolute quantification were not performed, all compound-level results are reported as putative annotations.

3.2. OFCE exhibited increasing antioxidant responses across the tested concentration range

DPPH radical-scavenging activity increased across the tested OFCE concentration range, from $27.31 \pm 7.13\%$ at 1.25 mg/mL to $52.93 \pm 3.23\%$ at 20 mg/mL (Fig. 1f). The corresponding vitamin C control results are shown in Fig. 1g.

Similarly, FRAP values increased from 8.04 ± 1.28 to 39.93 ± 2.88 $\mu\text{mol Fe}^{2+}$ equivalents g^{-1} extract over the same OFCE concentration range (Fig. 1h). Results for the intermediate concentrations are presented in the Fig .

Preparation, putative chemical composition, and antioxidant activity of OFCE. (a) Experimental workflow; (b) total ion chromatograms acquired in positive- and negative-ion modes; (c) distribution of putatively annotated metabolite classes; (d) correlations among QC samples; (e) cumulative distribution of QC-RSD values; (f) DPPH radical-scavenging activity of OFCE; (g) vitamin C positive control; and (h) FRAP values of OFCE. OFCE, ethanolic extract of the *O. formosanus* fungus comb; QC, quality control; RSD, relative standard deviation; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power

3.3. Predicted targets overlapping with ageing-related targets

Following absorption, distribution, metabolism, and excretion (ADME) and drug-likeness screening, 397 candidate entries were retained. Comparison of their predicted targets with 1,221 ageing-related targets identified 338 overlapping targets (Fig. 2a–b).

All edges in the compound–target network represent database-derived predictions and do not indicate that the candidate compounds were experimentally confirmed, possess demonstrated bioavailability, or have established functional relationships with the corresponding targets.

The candidate compounds were associated with a total of 1,927 predicted targets. Network-topology analysis prioritised Phe-Val, azelaic acid, Thr-Leu, milrinone, N-acetyltryptophan, and betaxolol. The top 20 candidate annotations are listed in Table 1.

Table 1
Topological characteristics of prioritised putative annotations in the
OFCE compound–target network

No.	Putative annotation	Degree	BC	CC
1	Phe-Val	21	0.0868	0.3761
2	Azelaic acid	16	0.0654	0.373
3	Thr-Leu	16	0.0654	0.373
4	Milrinone	14	0.0568	0.3718
5	N-Acetyltryptophan	12	0.0482	0.3705
6	Betaxolol	12	0.0482	0.3705
7	DEHP	12	0.0482	0.3705
8	12-Hydroxystearic acid	11	0.0439	0.3699
9	Thymidine	10	0.0395	0.3693
10	Maculosin	10	0.0395	0.3693
11	N-acetyldopamine	10	0.0395	0.3693
12	3-Methylcrotonylglycine	9	0.0352	0.3687
13	Pseudolaric Acid B	8	0.0308	0.3681
14	N-Acetylhistidine	7	0.02645	0.3675
15	Phenylacetylglutamine	7	0.0264	0.3675
16	Eicosapentaenoic acid	7	0.0264	0.3675
17	Harmine	7	0.0264	0.3675
18	4-Hydroxybenzoic acid	6	0.022	0.3669
19	Pyruvate	6	0.022	0.3669
20	Hydroxyphenyllactic acid	5	0.0176	0.3663

BC, betweenness centrality; CC, closeness centrality.

3.4. Protein–protein interaction network analysis

The initial protein–protein interaction (PPI) network comprised 338 nodes and 2,489 edges. After centrality-based filtering, 56 nodes and 587 edges were retained (Fig. 2c).

AKT1, IL6, TP53, STAT3, EGFR, SRC, and CTNNB1 were selected as prioritised targets for subsequent analysis. The topological parameters of the top 20 hub targets are shown in Table 2.

Table 2
Topological characteristics of prioritised hub targets in the PPI network

Target	Description	UniProt	DC	BC	CC
AKT1	RAC-alpha serine/threonine-protein kinase	P31749	41	3685.91	0.0015
IL6	Interleukin-6	P05231	39	5663.95	0.0015
TP53	Cellular tumor antigen p53	P04637	39	13525.08	0.00159
STAT3	Signal transducer and activator of transcription 3	P40763	39	2805.83	0.00145
EGFR	Epidermal growth factor receptor	P00533	38	4264.62	0.00151
SRC	Proto-oncogene tyrosine-protein kinase Src	P12931	37	5169.56	0.00151
CTNNB1	Catenin beta-1	P35222	37	6691.93	0.00153
TNF	Tumor necrosis factor	P01375	34	4439.85	0.00145
JUN	Transcription factor Jun	P05412	34	1605.41	0.00144
IL1B	Interleukin-1 beta	P01584	33	2632.59	0.00144
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	P04406	32	3498.88	0.00142
BCL2	Apoptosis regulator Bcl-2	P10415	32	2110.33	0.00142
HSP90AA1	Heat shock protein HSP 90-alpha	P07900	32	4166.66	0.00144
ESR1	Estrogen receptor	P03372	30	2987.54	0.0014
HIF1A	Hypoxia-inducible factor 1-alpha	Q16665	29	988.32	0.00138
CASP3	Caspase-3	P42574	29	2872.56	0.00143
MAPK1	Mitogen-activated protein kinase 1	P28482	29	726.11	0.00137
MAPK3	Serine/threonine-protein	H0YDH9	28	840.82	0.00137
PTGS2	Prostaglandin G/H synthase 2	P35354	26	1346.38	0.00135
MMP9	Matrix metalloproteinase-9	P14780	26	1344.07	0.00137

BC, betweenness centrality; CC, closeness centrality; DC, degree centrality.

3.5. GO and KEGG enrichment analyses

GO enrichment analysis indicated that the overlapping targets were mainly associated with biological processes involving MAPK and ERK1/2 cascades, positive regulation of PI3K–AKT signalling, cell migration and proliferation, negative regulation of apoptosis, and responses to hypoxia.

The targets were primarily associated with cellular components including the plasma membrane, cell surface, receptor complexes, and cytoplasm, and were enriched in molecular functions such as protein kinase activity, protein tyrosine kinase activity, and ATP binding (Fig. 2d).

KEGG enrichment analysis identified pathways including PI3K–AKT, MAPK, HIF-1, apoptosis, and AGE–RAGE signalling, which are associated with cellular stress and ageing-related processes (Fig. 2e). These predictions do not establish the direction of pathway regulation or demonstrate an anti-ageing mechanism.

Exploratory network analysis of OFCE candidate compounds and ageing-related targets. (a) Candidate compound–target network; (b) overlap between predicted candidate targets and ageing-related targets; (c) PPI network of overlapping targets; (d) GO enrichment analysis, showing biological process, cellular component, and molecular function categories from top to bottom; and (e) KEGG pathway enrichment analysis. OFCE, ethanolic extract of the *O. formosanus* fungus comb; PPI, protein–protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes

3.6. Molecular docking analysis

Among the six candidate ligands and seven prioritised proteins, the most favourable docking scores for each ligand were obtained for Phe-Val–EGFR ($-6.94 \text{ kcal mol}^{-1}$), azelaic acid–EGFR ($-6.64 \text{ kcal mol}^{-1}$), N-acetyltryptophan–TP53 ($-6.63 \text{ kcal mol}^{-1}$), Thr-Leu–IL6 ($-6.48 \text{ kcal mol}^{-1}$), milrinone–EGFR ($-5.82 \text{ kcal mol}^{-1}$), and betaxolol–EGFR ($-4.96 \text{ kcal mol}^{-1}$) (Fig. 3a).

The distributions of docking scores across the individual targets and the corresponding mean scores are shown in Fig. 3b–c. EGFR showed the lowest mean docking score ($-6.11 \text{ kcal mol}^{-1}$) within the present computational dataset and was therefore selected for subsequent molecular dynamics simulations.

Representative predicted binding conformations are shown in Fig. 3d–i. Predicted contact residues for the azelaic acid–EGFR complex included Lys157, Lys163, Gln102, Thr101, and Arg87, whereas those for the N-acetyltryptophan–EGFR complex included Val80, Arg87, Lys157, and Gln102.

Molecular docking of prioritised candidate compounds with predicted targets. (a) Most favourable docking score obtained for each ligand; (b) distribution of docking scores across the selected targets; (c) mean docking score for each target; and (d–i) representative three-dimensional and two-dimensional ligand–target interaction conformations

3.7. Molecular dynamics characterisation of two EGFR–ligand models

Both EGFR–ligand complexes were subjected to 100 ns molecular dynamics simulations.

For the EGFR–azelaic acid system, the trajectories entered a relatively stable region after approximately 45 ns, with RMSD values of approximately 0.24, 0.18, and 0.15 nm for EGFR, the protein–ligand complex, and the ligand, respectively. The EGFR–N-acetyltryptophan system showed a comparatively stable trajectory after approximately 55 ns, with corresponding RMSD values of approximately 0.30, 0.23, and 0.15 nm (Fig. 4a–b).

RMSF analysis showed relatively low fluctuations for most structured residues, whereas greater flexibility was mainly observed at terminal and localised regions (Fig. 4c–d). Intermolecular hydrogen bonds formed and dissociated dynamically throughout both simulations (Fig. 4e–f). The corresponding free-energy landscapes showed relatively concentrated low-energy conformational basins (Fig. 5).

These observations support the conformational feasibility of the two modeled complexes under the present simulation conditions but do not independently demonstrate binding affinity or functional regulation of EGFR. The complete trajectories are provided in Fig. S1.

Molecular dynamics simulation analysis of the EGFR–azelaic acid and EGFR–N-acetyltryptophan complexes over 100 ns. (a–b) RMSD trajectories of EGFR, the ligand, and the protein–ligand complex in the EGFR–azelaic acid (a) and EGFR–N-acetyltryptophan (b) systems; (c–d) RMSF of EGFR residues in the EGFR–azelaic acid (c) and EGFR–N-acetyltryptophan (d) complexes; and (e–f) changes in the number of intermolecular hydrogen bonds between EGFR and the corresponding ligand throughout the simulation in the EGFR–azelaic acid (e) and EGFR–N-acetyltryptophan (f) systems. EGFR, epidermal growth factor receptor; RMSD, root-mean-square deviation; RMSF, root-mean-square fluctuation

Free-energy landscapes derived from molecular dynamics simulations. (a–b) Two-dimensional free-energy landscapes of the two core complexes shown in Fig. 4, projected onto principal component 1 (PC1) and principal component 2 (PC2); (c–d) corresponding three-dimensional free-energy landscapes. Colours represent relative free-energy levels, with blue regions indicating lower-energy conformational basins

4. Discussion

In the present study, the ethanolic extract of an *O. formosanus*–*Termitomyces*-associated fungus comb was characterised using untargeted chemical profiling, and increasing DPPH radical-scavenging and FRAP reducing responses were observed across the tested concentration range. These findings provide preliminary answers to the two principal questions raised in the Introduction.

Consistent with previous observations of chemical diversity and antioxidant activity in fungus combs produced by other fungus-growing termite species (6, 4, 5, 3), the present results extend this evidence to the fungus comb of *O. formosanus*. However, differences among studies in termite species, plant substrates, extraction procedures, and assay conditions preclude direct comparisons of antioxidant potency or conclusions regarding species-specific chemical composition.

In addition, the present study analysed the intact fungus comb rather than isolated fungal material. The putatively annotated metabolites may therefore originate from plant substrates, termites, *Termitomyces*, associated microorganisms, or their combined biotransformation processes (2). Accordingly, these chemical signals can be used to describe the overall chemical phenotype of the *Termitomyces*-associated fungus-comb system but cannot currently be attributed specifically to the symbiotic fungus itself. The structures, abundances, origins, and contributions of the major candidate compounds to the overall activity of the extract remain to be established.

The multicomponent nature of OFCE suggests that its antioxidant activity may reflect contributions from compounds with different redox properties. Azelaic acid has previously been reported to reduce certain forms of ROS generation in human neutrophils (43). Its disodium salt has also been shown to scavenge hydroxyl radicals and inhibit lipid peroxidation under the reported experimental conditions, although it did not scavenge superoxide generated by the xanthine–xanthine oxidase system (44). N-Acetyltryptophan has likewise been reported to exhibit radical-scavenging activity in the ABTS assay (45).

The redox behaviour of several other candidate annotations appears to be more strongly dependent on biological origin and experimental context. N-Acetyldopamine is an important metabolite involved in insect cuticle sclerotisation (46), making its biological origin potentially relevant to the termite-associated matrix investigated here. The isolated compound has been reported to inhibit lipopolysaccharide-induced lipid peroxidation in rat brain homogenates (47), whereas other studies have described context-dependent effects on hydroxyl-radical formation in iron-mediated Fenton systems (48). These observations further illustrate that the redox behaviour of an individual compound may vary substantially among experimental environments.

Interpretation of putative annotations must also account for uncertainty in chemical structure assignment. For example, hydroxyphenyllactic acid was annotated in the present dataset, whereas relatively direct evidence relating this compound class to oxidative stress has been reported mainly for p-hydroxyphenyllactic acid. This microbial metabolite has been shown to reduce ROS generation in isolated mitochondria and neutrophils (49). Whether these findings are directly applicable to the LC–MS signal observed here depends on further confirmation of the corresponding isomeric identity. Similarly, the TEAC antioxidant activity of 4-hydroxybenzoic acid has been shown to depend on pH conditions (50).

Taken together, these previous studies do not demonstrate that the putatively annotated compounds reported here are the principal contributors to the antioxidant activity of OFCE. Rather, they indicate that some of the putative annotations obtained in the present study have previously reported redox-related

properties that warrant further investigation. Their actual contributions will need to be evaluated after compound-identity confirmation and in the context of abundance and assay conditions.

The computational analyses and the DPPH/FRAP assays performed in this study address different levels of biological information. The former used ageing-related database targets as an exploratory background to prioritise candidate molecules derived from LC–MS/MS putative annotations, whereas the latter measured only the radical-scavenging and ferric-reducing capacities of the extract in the antioxidant assays used here.

The network analysis prioritised EGFR, AKT1, TP53, STAT3, IL6, SRC, and CTNNB1, together with enriched pathways including PI3K–AKT, MAPK, HIF-1, apoptosis, and AGE–RAGE signalling. These results were derived from the intersection between predicted compound targets and ageing-related database targets. Several of these processes are also involved in stress responses, inflammation, and redox regulation (51, 52, 53, 54), thereby providing experimentally testable directions for future investigation.

However, overlap with these pathways does not demonstrate that the corresponding nodes mediate the DPPH or FRAP responses of OFCE, nor does it establish an anti-ageing effect of OFCE or define the direction of pathway regulation.

Within this predictive framework, molecular docking and molecular dynamics simulations were used only to evaluate the structural feasibility of selected candidate ligand–target relationships under the specified computational models (55, 56). EGFR was selected for further simulation based on its priority in the predicted network and its relative docking performance with several candidate ligands.

The EGFR–azelaic acid and EGFR–N-acetyltryptophan complexes underwent conformational adjustment during the 100 ns simulations and maintained relatively stable conformational states during portions of the trajectories. These observations indicate that neither modeled complex exhibited obvious immediate conformational incompatibility under the present simulation conditions.

Nevertheless, these computational results are not equivalent to experimentally measured binding affinity and do not demonstrate that either ligand modulates EGFR function. Establishing a relationship between these predicted molecular interactions and the antioxidant phenotype of OFCE will require confirmation and quantification of the candidate compounds, activity testing of individual compounds and defined combinations, direct binding or receptor-activity assays, and validation of cellular ROS, oxidative-damage, and related signalling endpoints under conditions of EGFR perturbation.

5. Conclusion

In summary, the *O. formosanus*–*Termitomyces*-associated fungus comb exhibited a diverse profile of ethanol-extractable small-molecule features, while OFCE showed increasing antioxidant responses across the tested concentration range in the DPPH and FRAP assays. Several putative annotations have

previously reported redox-related properties (45, 47, 44, 57) and may therefore serve as candidate chemical leads for interpreting the observed phenotype; however, their identities, abundances, origins, and actual contributions remain unresolved.

The network analysis conducted using ageing-related database targets, together with subsequent docking and MD simulations, further generated several candidate molecular relationships for future evaluation. Their current significance is limited to candidate prioritisation and hypothesis generation.

Future studies involving confirmation with authentic standards, quantitative analysis, source-matched controls, bioactivity-guided fractionation, testing of individual compounds and defined combinations, and cellular or other biological validation of candidate targets will be required to distinguish the relationships among fungus-comb chemical composition, the antioxidant phenotype of OFCE, and the predicted molecular associations.

Declarations

Competing Interests

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

Ethics Approval

Not applicable. This study involved only invertebrate insects and fungus comb materials and did not involve human participants, human-derived materials, or vertebrate animals. Therefore, ethical approval was not required.

Potential reviewers

Lucas S. Solidade: lucassolidade@estudante.ufscar.br. Brazil;

Tae-Jin Oh: tjoh3782@sunmoon.ac.kr. South Korea;

Amr H. Hashem: amr.hosny86@azhar.edu.eg. Egypt.

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Author Contribution

Jinxin Liu: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft. Ruiqi Kang: Methodology, Investigation, Data curation, Formal analysis, Validation, Writing – original draft. Jiayi Dong: Investigation, Data curation, Validation. Weina Wang: Investigation, Methodology, Visualization. Zhixin Li: Investigation, Data curation, Validation. Jia Huang: Investigation, Resources, Validation. Qimeng Fan: Investigation, Data curation, Visualization. Liangshan Ming: Software, Formal analysis, Visualization. Hongning Liu: Formal analysis, Validation. Wei Jiang: Supervision, Project administration, Funding acquisition, Writing – review & editing. Zishu Dong: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary information material.

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Figures

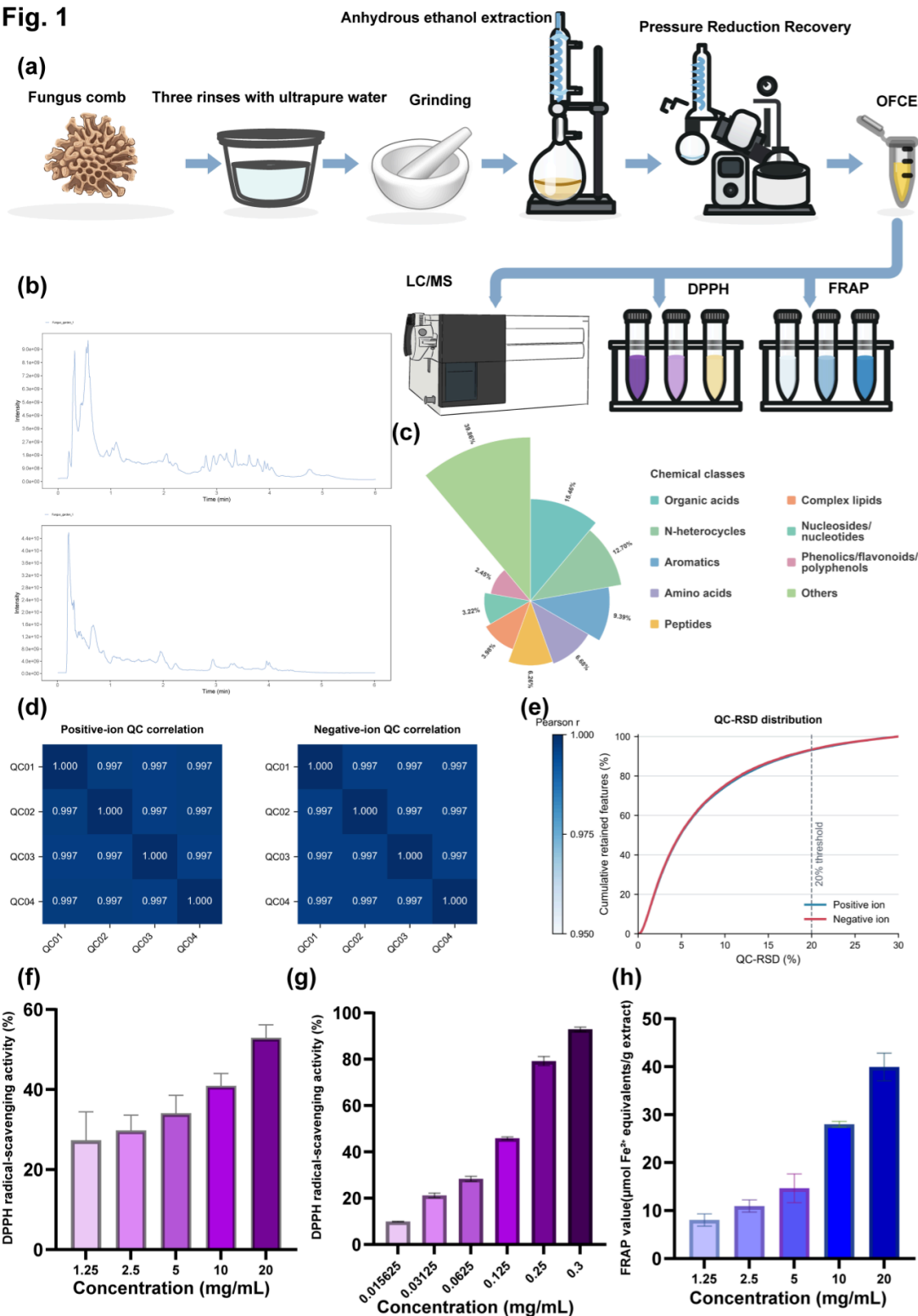


Figure 1

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Fig. 2

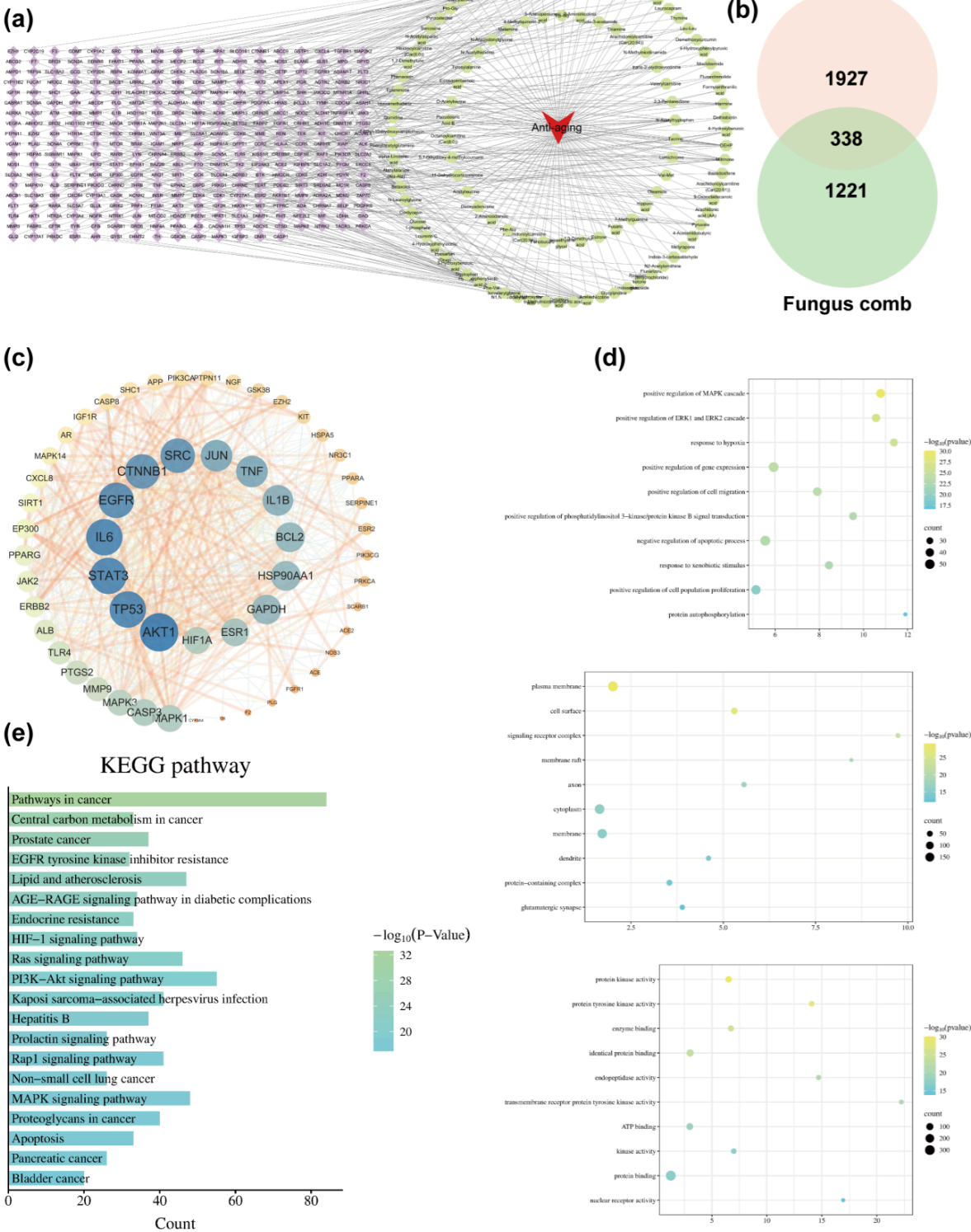


Figure 2

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Fig. 3

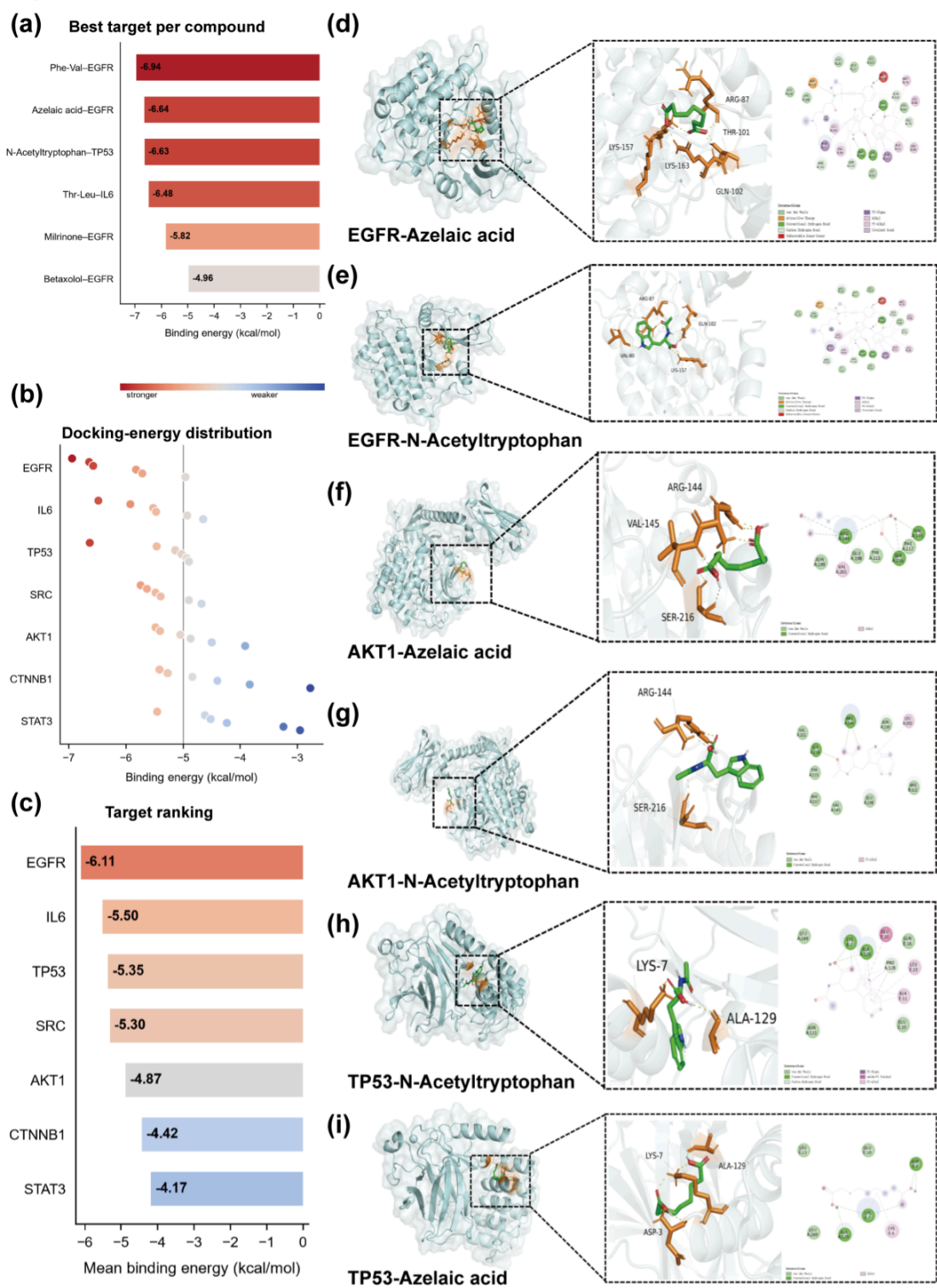


Figure 3

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Fig. 4

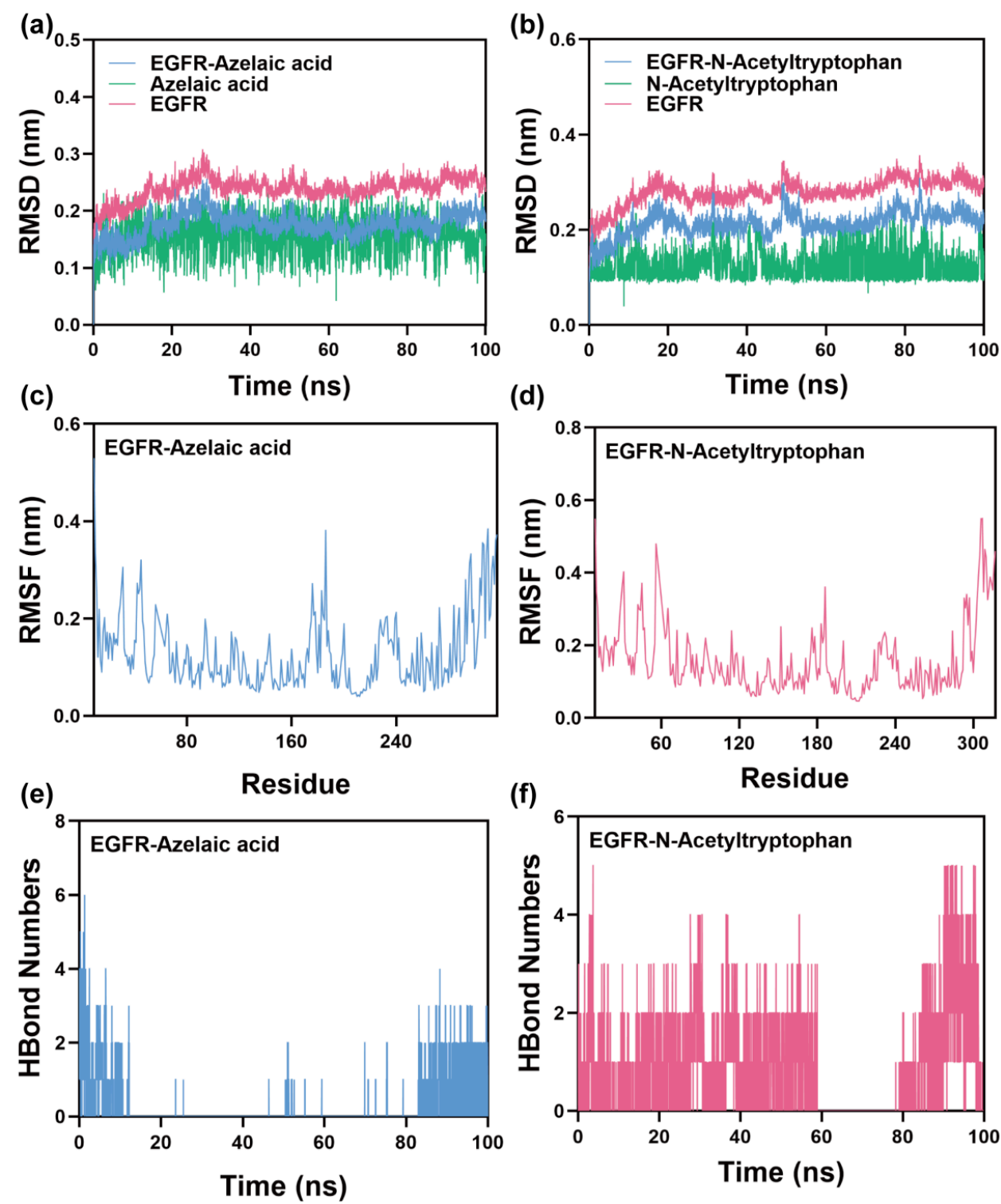


Figure 4

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Fig. 5

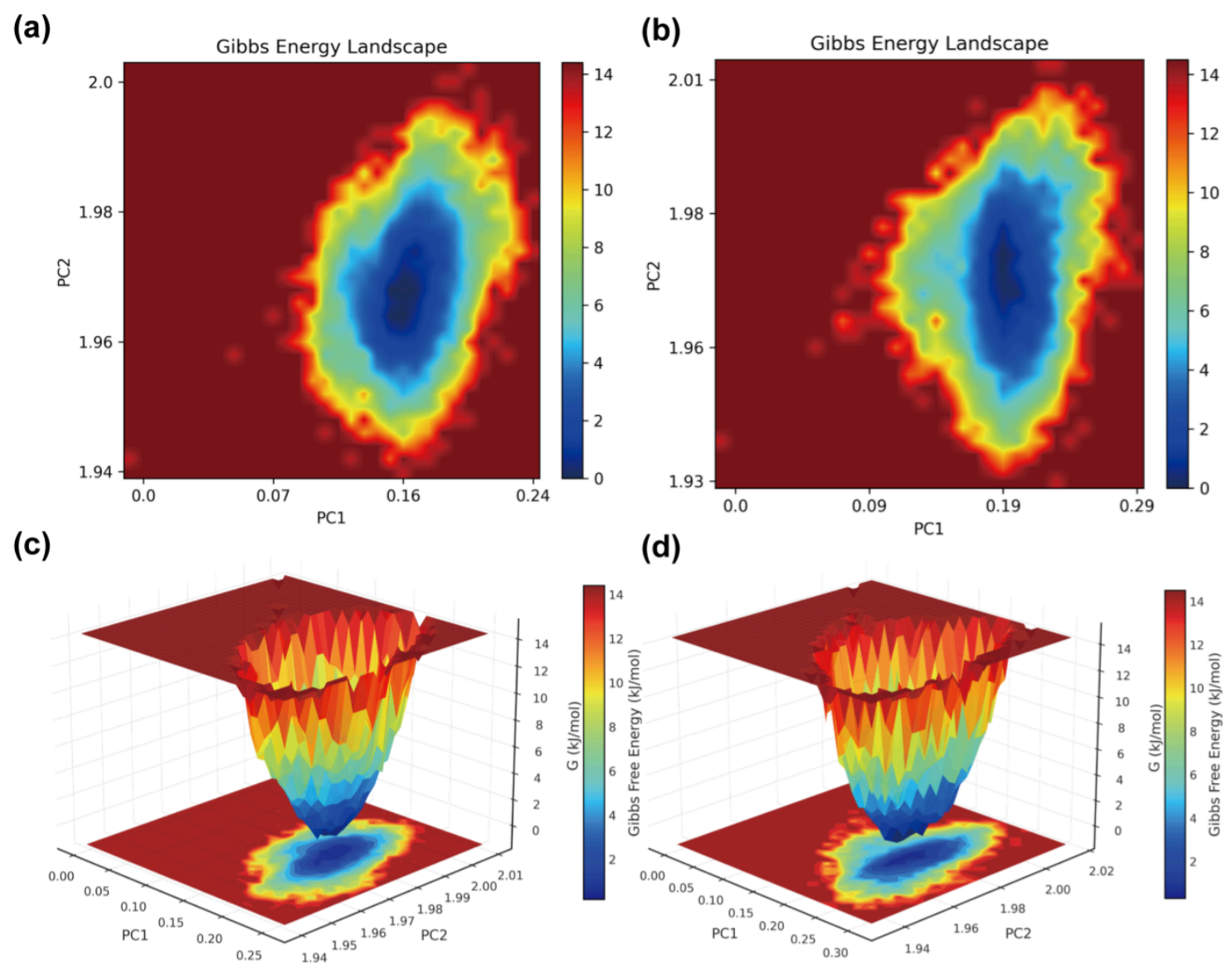


Figure 5

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