

Research Article

Isolation of Metabolites from *Lawsonia Inermis* and its Prediction as Inhibitor of ABCB1 Mediated Drug Resistance Using Molecular Docking and Molecular Dynamic Simulation

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A B S T R A C T

ABCB1/P-glycoprotein-mediated multidrug resistance (MDR) is a prominent cause of chemotherapy failure in breast cancer and has led to the hunt for natural product-based efflux-pump inhibitors. The ABCB1 inhibitory capability of *Lawsonia inermis* (henna) leaf extract was evaluated using an integrated in silico pipeline (phytochemical profiling, molecular docking, ADMET prediction and molecular dynamics (MD) simulation). The ethanol extraction gave a phenolics (330.84±22.78 mg GAE/g) and flavonoids (30.05±2.79 mg rutin/g) rich extract with 13.036% w/w. GC-MS analysis showed 35 compounds with vanillin, squalene and γ -sitosterol being the major constituents. Docking against the ABCB1 drug-binding cavity (PDB: 7O9W) identified five triterpenoid/sterol ligands, 24-norursa-3,12-diene, γ -sitostenone, lupeol, γ -sitosterol and ergost-5-en-3-ol, with binding free energies ranging from -9.3 to -10.7 kcal/mol, anchored by a conserved hydrophobic residue cluster (Phe303, Ile306, Leu339, Phe343, Phe728). These ligands were above the traditional Lipinski lipophilicity threshold (LogP 7.6–8.6) and preferred partitioning into the membrane facing pocket of ABCB1. A 200 ns MD simulation of the ABCB1- γ -sitosterol complex identified sustained ligand rigidity (RMSD 1.8–2.0 Å), stable hydrogen bond with Tyr307, and water-mediated contacts with Asn721/Asn842, along with progressive expansion of the transporter's radius of gyration, consistent with a steric-wedge mechanism of competitive inhibition. These results suggest *Lawsonia inermis* derived triterpenoids as potential scaffolds for the ABCB1-targeted reversal of multidrug resistance in breast cancer.

Keywords: Breast Cancer, ABCB1, Multidrug Resistance, *Lawsonia inermis*

Introduction

Breast cancer (BC) represents the most frequent cancer among women worldwide and the use of (neo)adjuvant therapy in early stage became state of the art and had a meaningful influence in increasing survival rates throughout the previous few decades¹. Chemotherapy markedly reduces the chance of recurrence and death from early-stage breast cancer, although it can have short-term and long-term side-effects. Lack of data on prevention or treatment of long-term side effects means there is an urgent need to identify and develop effective techniques to manage this large group of disabling symptoms. The number of breast cancer survivors is growing and they endure distinct long-term physical, psychological and emotional changes that can have a significant impact on their quality of life. For more than a decade, a mixture of an anthracycline and a taxane was regarded as the best treatment for women with early-stage breast cancer². Despite tremendous progress in cancer treatment in recent decades, chemotherapy remains the major approach to treating cancer. Unfortunately, a single chemotherapy treatment does not fit all kinds of breast cancer.

On the other hand, one of the failures for chemotherapy is multi-drug resistance generated by ABC transporters like P-gp, MRP1 and BCRP, which accounts for >90% of cancer patient mortality treated with conventional chemotherapeutics or new targeted medicines³. ABCB1 overexpression remains widely reported in breast cancer that includes triple-negative breast cancer (TNBC) and is associated with a weak chemotherapeutic response, decreased rate of progression free survival and worse overall result⁴. Despite the development of three generations of synthetically produced P-glycoprotein inhibitors including first-generation drugs such as verapamil and cyclosporin A, second-generation analogues and third-generation compounds such as tariquidar, zosuquidar and elacridar, none of these compounds has been approved for clinical use mainly due to pharmacokinetic drug-drug interactions, off-target toxicity and poor tolerability at the concentrations required for effective P-glycoprotein inhibition^{5,6}. This unmet need has focused the attention towards plant-derived natural products as MDR-reversing drugs that can be an alternative and potentially safer, more selective and better tolerated source.⁷

Since ancient times people have always used plants as medicine across the globe and they remain the main source of drugs in traditional medication. These plants are significant for pharmacological research and drug development, since not only plant ingredients are utilized directly as therapeutic agents, but also as basic raw materials for drug production or as standardized models

for pharmacologically active molecules. Medicinal plants are contributing a lot to health care systems because of their extensive therapeutic potential, affordable prices, and more substantial safety margins.^{8,9} *Lawsonia inermis* L. is a traditional medicinal and dye plant used for wound healing, dermatological and antibacterial applications in South Asia, the Middle East and North Africa^{10,11}. Its leaves contain phenolics, flavonoids, naphthoquinones (especially lawsone), tannins, coumarins and terpenoids. Its ethanolic/methanolic extracts have been repeatedly shown to have strong antioxidant, antimicrobial and antiproliferative activity due to phenolic and flavonoid content.¹²

The antioxidant, antimicrobial and general anticancer properties of *L. inermis* are relatively well documented and flavonoids/phenolics are established as a class of P-gp modulators. However, no study has yet profiled the GC-MS identifiable constituents of the ethanolic leaf extract of *L. inermis* for computationally evaluating their interaction with the ABCB1 drug-binding cavity in the context of breast cancer multidrug resistance. Hence, the present study was undertaken to assess the potential of the ethanolic extract of *L. inermis* and to integrate GC-MS profiling with molecular docking and MD modeling against ABCB1 to discover constituents that may inhibit efflux-mediated MDR in breast cancer.

Materials and Methods

Plant material and preparation of ethanolic extract

Fresh, mature and fully expanded leaves of *L. inermis* were collected from Jamia Millia Islamia campus followed by botanical authentication and sample submission in the relevant lab (Lab500) of Department of Biotechnology. Leaves were dried in a hot-air oven at 45°C till weight stabilization and powdered for the preparation of ethanolic extract. 50 gm of powder was dissolved in 200 mL of ethanol and kept at shaker for 24-48 hours. The extract was filtered through muslin cloth and then Whatman No. 1, concentrated by drying naturally and the percentage yield was calculated as (weight of dried extract / weight of powder used) × 100. The dried extract was stored at 4 °C until further use.

Estimation of total phenolic content (TPC)

TPC was estimated by the Folin-Ciocalteu method.¹³ Briefly, 0.5 mL of extract or standard was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent, followed by the addition of 2.5 mL of sodium carbonate. After incubation (45 min, dark, room temperature), absorbance was read at 765 nm. TPC was expressed as mg gallic acid equivalents (GAE)/g dry extract using a gallic acid standard curve. The samples were taken in triplicate.

Estimation of total flavonoid content (TFC)

TFC was determined by the aluminum chloride colorimetric method.¹³ 0.5 mL extract was mixed with 2% aluminum chloride and potassium acetate/sodium nitrite as applicable, incubated for 60 minutes and absorbance was measured at 415 nm. TFC was expressed as mg rutin equivalents (RE)/g dry extract using a rutin standard curve.

GC-MS analysis

GC-MS analysis was performed on a Agilent 7890A series (Agilent, Santa Clara, CA, USA) equipped with a CTC-PAL autosampler connected to an HP-5MS (5% phenyl polydimethylsiloxane) capillary column (30 m × 0.25 mm I.D and 0.25 µm film thickness) and a mass detector (Agilent 5975C inert XL EI/CI MSD with Triple-Axis Detector, Agilent Technology, USA).¹⁴ The instrument was programmed at 50 °C for 5 min, then elevated to 280 °C at a rate of 10 °C/min for 7 min. The entire run time was 42 min. Compounds were tentatively identified by comparing mass spectra and retention indices with the NIST/WILEY spectral library.

Selection and preparation of the target protein (ABCB1)

The three-dimensional structure of human ABCB1 (PDB ID: 7O9W) was obtained from the Protein Data Bank. The protein structure was prepared by removing crystallographic water molecules and heteroatoms not relevant to the binding site, adding polar hydrogens, assigning Kollman charges and energy-minimizing the structure using standard protein-preparation protocols like AutoDock Vina¹⁵, Discovery Studio¹⁶, PyMOL¹⁷ etc.

Ligand preparation

The 3D structures of the GC-MS identified phytoconstituents were retrieved from PubChem in SDF format. The downloaded structures were converted to pdbqt format using Open Babel¹⁸ and saved for further use.

Molecular docking studies

Molecular docking was performed on the GCMS metabolites of *Lawsonia inermis* using AutoDock Vina, targeting the transmembrane drug-binding cavity of ABCB1 as defined by residues previously implicated in substrate/inhibitor binding by mutagenesis and structural studies.¹⁹ The docking/binding score of the binding poses was graded and the top scoring posture of each ligand was examined for hydrogen bonding, hydrophobic and π -visualizing stacking interactions with critical ABCB1 binding site residues using visualization tools e.g. Discovery Studio Visualizer/PyMOL.

In silico drug-likeness and ADMET prediction

The best ranked phytoconstituents were evaluated for their drug-likeness (Lipinski's rule of five) and predicted

absorption, distribution, metabolism, excretion and toxicity (ADMET) profiles using tools ADMETlab 3.0²⁰ and RDKit²¹ wherever applicable, to support their pharmacological plausibility as lead candidates.

Molecular dynamics (MD) simulation

The best ranked ligand-ABCB1 complex from docking was subjected to all-atom MD simulation using Desmond plugin on Schrodinger suite -2024-4 version in order to evaluate the stability and dynamic behaviour of the predicted binding mode²². Trajectory studies were performed using root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), and hydrogen bond occupancy along the simulated trajectory.

Results

Percentage Yield

The percentage yield (w/w) of the ethanolic extract of *Lawsonia inermis* leaf was 13.036% w/w.

Estimation of total phenolic and flavonoid content

TPC and TFC of the extract are summarized in Table 2. The total phenolic content of ethanolic extract of *Lawsonia inermis* was analyzed by using gallic acid standard calibration curve ($y=0.0049x+0.0597$; $R=0.9969$) and expressed as milligram equivalents of gallic acid per gram of dry extract (mg GAE/g). The total phenolic content of the extract was 330.84 ± 22.78 mg GAE/g of ethanolic extract of *L. inermis* ($n = 3$). The overall flavonoid concentration was quantified utilizing a standard calibration curve of rutin ($y = 0.0052x+0.0049$; $R=0.9921$), and the findings were reported as milligram equivalents of rutin per gram of dry extract. The extract exhibited a total flavonoid content of 30.05 ± 2.79 mg rutin/g of ethanolic extract of *L. inermis* ($n=3$). This plant contains high phenolic levels which are linked to important health-promoting properties. Phenolic and flavonoids compounds are known to reverse multidrug resistance (MDR) in cancer by blocking ABC efflux transporters such as P-glycoprotein (ABCB1), MRP1 and BCRP23. *L. inermis* is a rich source of phenolic and flavonoid components that have been found to have anticancer potential¹⁰. Our results indicate that *L. inermis* ethanolic leaf extract had high total phenolic and flavonoid content and hence can have a great potential to block the ABC transporter-mediated drug efflux and overcome the multidrug resistance in cancer cells.

GC-MS Compound Profile

GC-MS analysis of the extract identified 35 compounds given in Table 3, representing major chemical classes, e.g., phenolics, fatty acid esters, terpenoids. The most abundant constituents found are Vanillin (30.29%), Squalene 921.07%), gamma-Sitosterol (10.67%), followed by others.

Molecular Docking Results

All the compounds discovered by GC-MS were docked against the ABCB1 drug-binding cavity (PDB ID:7O9W). Table 4 summarizes the binding affinities and critical interactions for the top ranked compounds. To provide deeper insight into the initial binding affinities and the specific interactions of the potential inhibitors in the ABCB1 transmembrane cavity, molecular docking studies were performed. The five phytochemical ligands showed a favorable binding free energy range of -9.3 to -10.7 kcal/mol. The interaction patterns reveal that the key component contributing to the stability of each of the complexes is hydrophobic in character. For each of the complexes, a set of non-polar residues that were discovered to be non-variable were phenylalanine 303, isoleucine 306, leucine 339, phenylalanine 343 and phenylalanine 728. These residues interact with the bulky sterol and triterpene backbones of each of the phytochemical ligands via close range interactions significantly of the van der Waals type as well as pi-alkyl and pi-sigma interactions. This implies that the core cavity of the transporter may accommodate a variety of large and complicated hydrophobic molecules of different nature.

ADMET Properties

Physicochemical properties of the compounds were investigated to determine their pharmacokinetic and membrane-partitioning profiles for certain phytochemical inhibitors. As shown in Table 5, all 5 compounds are of low molecular weight (394.69 to 426.73 g/mol) and a low number of hydrogen bond acceptors (0-1) and donors (0-1). Their high lipophilicity (LogP: 7.63 to 8.58) which is greater than the classical Lipinski threshold, which normally denotes a critical limitation, is in this case a critical advantage in the case of ABCB1 inhibition, because it promotes the spontaneous partition of the bilayer with the transporters poly specific pocket. Moreover, low topological polar surface area (TPSA: 0 to 20.23Å²) values are responsible for deep core desolvation and low number of rotatable bonds (0-6) provide the required structural stiffness to build up stable steric wedges.

Molecular Dynamics Simulation

Root Mean Square Deviation(RMSD)

To assess the flexibility of the transporter, the dynamics of the ABCB1- γ -sitosterol complex were compared to the apo structure using molecular dynamics simulations. Analysis of the C α atom backbone in the ligand-bound complex indicated a modest structural transition that ultimately resulted in a divergence of ~8.0 Å. Most importantly, the comparison with the apo structure shows that this degree of structural drift is intrinsic to the transporter, rather than any ligand-induced destabilization, and highlights

the significant conformational changes that need to be achieved in the catalytic process of ABCB1 transporters. γ -sitosterol demonstrated a structural adaptation in this flexible environment, followed by a plateau of 7.0-8.0 Å, which was constant from 30 ns. Rather than dissociating the ligand formed a sustained dynamic equilibrium which shows that it can tolerate the great conformational flexibility of the poly-specific binding site of the transporter.

Root Mean Square Fluctuation(RMSF) & Radius of Gyration(Rg)

The residue level flexibility of both apo and γ -sitosterol-bound versions of ABCB1 was investigated utilizing root-mean square fluctuation (RMSF) of C α atoms. Both compounds displayed hard regions with the baseline RMSF between 1.0 Å and 2.0 Å, but also extremely flexible loop topologies. From the comparison of the two trajectories, we found the presence of local modifications in the molecule upon ligand binding. In the apo molecule, the most flexibility was observed in the N-terminal region (residues 50-100) and in residues 1050-1100, where the RMSF value exceeded 6.0 Å in both cases. The peaks occur about 4.0 Å and 5.0 Å, respectively, when the ligand γ -sitosterol is bound, indicating a decrease in the flexibility of these particular areas. On the other hand, the structure formed by residues 580-600, which shows a mild RMSF peak of roughly 5.2 Å, undergoes a large rise in fluctuations, with a peak value above 8.0 Å.

The global spatial properties of the transporter were investigated by measuring the radius of gyration (Rg) values in the simulation trajectories. After the first equilibrium stage both apo and holo trajectories started to show a well-defined conformational characteristic. In particular, the apo form preserved its compact structure and its Rg values varied mostly in the lower range of 39.25 Å to 40.0 Å. At the same time, the ligand binding caused a steady global expansion of the complex with the Rg values reaching a stable plateau in the range of 40.0 to 40.5 Å after 100 ns. Such spatial expansion on a macroscopic scale suggests the involvement of the ligand as a steric wedge inside the core transmembrane cavity. The continued expansion suggests that the bulky ligand γ -sitosterol binds the transporter in a competitive manner and so prevents large-scale domain closure such as NBD dimerization, rather than stabilizing the transporter structure in a fixed configuration.

Protein-Ligand Interaction

The ligand dynamics makes the molecule γ -sitosterol a very stable competitive inhibitor in the ABCB1 pocket. The interaction profile shows a very effective bipartite interaction. A stable polar network is formed, consisting of a direct hydrogen link with Tyr307 and a required water bridge to Asn721 and Asn842, stabilizing the ligand orientation.

At the same time, several hydrophobic interactions, mostly with Phe343, hold the bulky steroid skeleton. This is well illustrated by the timeline analysis where the critical interactions remain continuous throughout the whole 200-nanosecond simulation time.

Ligand and its Properties

The intrinsic ligand properties are analyzed to further improve the idea of competitive inhibition, known as the 'steric wedge' hypothesis. A 200ns molecular dynamics simulation is used to characterize competitive inhibition (Fig.7). For a chemical to impede the transport cycle of ABCB1, it needs to retain its volume as the transport protein undergoes a conformational shift. According to the analysis, the ligand γ -sitosterol is a rigid, volumetric, structural block.

Root Mean Square Deviation (RMSD) equilibrates swiftly and stabilizes at 1.8-2.0 Å which is an indication of intrinsic structural rigidity of the compound. The entirely inflexible (and in this case viscous) structural backbone is described as the total absence of intramolecular hydrogen bonding. Furthermore, the chemical reduces the solvent accessible surface area (SASA) to a constant value of 20-30 Å², while occupying the core, nonpolar cavity of the receptor. The molecular (MolSA, ~415 Å²) surface area and (PSA, ~48 Å²) values are steady, indicating there are no substantial internal aberrations. Finally, the stiffness of the compound and the deep burial of its pocket also permit the compound to keep a volumetric resistance and avoid the closure of the transmembrane domain. This resistance results in functional inhibition of the ABCB1 protein.

Table 1. Percent Yield

S.no	Medicinal Plant	Extraction method	Initial weight (g)	Yield extracted (g)	% Yield
1.	<i>Lawsonia inermis</i>	Ethanolic	50	6.518	13.036

Table 2. Total phenolic and flavonoid content of the ethanolic *L. inermis* leaf extract

Parameter	Value (mean $\pm$ SD)	Standard Used
Total phenolic content (TPC)	330.84 $\pm$ 22.78 mg GAE/g extract	Gallic acid
Total flavonoid content (TFC)	30.05 $\pm$ 2.79 mg RE/g extract	Rutin

Table 3. Phytocompound identified in ethanolic extract of *Lawsonia inermis* by GC-MS

S.No.	RT	Bioactive Compounds	Formula	Mass (g/mol)	Area %
1.	4.912	2-Hydroxy-gamma-butyrolactone	C ₄ H ₆ O ₃	102.09	1.24
2.	6.572	Cyclopropylmethanol	C ₄ H ₈ O	72.11	0.80
3.	8.143	Ethyl maltol	C ₇ H ₈ O ₃	140.14	5.37
4.	8.441	2,3-Dihydro-benzofuran	C ₈ H ₈ O	120.15	2.61
5.	9.848	2-Methoxy-4-vinylphenol	C ₉ H ₁₀ O ₂	150.17	0.30
6.	10.489	Decanoic acid	C ₁₀ H ₂₀ O ₂	172.26	0.55
7.	11.031	Vanillin	C ₈ H ₈ O ₃	152.15	30.29
8.	12.154	Levoglucofan	C ₆ H ₁₀ O ₅	162.14	0.69
9.	14.211	Bicyclo[4.3.0]nonan-1-ol, 7,9-bis (methylene)-2,2,6 trimethyl-	C ₁₄ H ₂₂ O	206.32	0.74
10.	15.525	2-Hydroxy-1,1,10-trimethyl-6,9-epidioxydecalin	C ₁₃ H ₂₂ O ₃	226.31	0.67
11.	15.695	Dehydrovomifoliol	C ₁₃ H ₁₈ O ₃	222	0.45
12.	16.182	Neophytadiene	C ₂₀ H ₃₈	278.5	1.66
13.	16.630	2,6,10-Trimethyl,14 ethylene-14-pentadecene	C ₂₀ H ₃₈	278	0.67
14.	17.778	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, ethyl ester	C ₁₉ H ₃₀ O ₃	306.4	1.20
15.	18.875	Phytol	C ₂₀ H ₄₀ O	296.5	6.03
16.	19.387	Linolenic acid, ethyl ester	C ₂₀ H ₃₄ O ₂	306.5	0.48
17.	19.793	Phytol acetate	C ₂₂ H ₄₂ O ₂	338.6	0.29

18.	21.003	Fumaric acid, decyl 4-heptyl ester	$C_{21}H_{38}O_4$	354.5	0.21
19.	22.297	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330.5	0.20
20.	23.729	2-Methylhexacosane	$C_{27}H_{56}$	380.7	0.29
21.	24.576	Squalene	$C_{30}H_{50}$	410.7	21.07
22.	25.313	Solanesol	$C_{45}H_{74}O$	631.1	1.16
23.	25.824	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2, 6,10,15,19,23-hexamethyl-, (all-E)-(.+/-.)	$C_{30}H_{50}O$	426.7	0.28
24.	25.897	Geranyl-linalool isomer	$C_{20}H_{34}O$	290.5	0.31
25.	25.968	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2, 6,10,15,19,23-hexamethyl-, (all-E)-(.+/-.)	$C_{30}H_{50}O$	426.7	0.35
26.	26.797	Octatriacontyl trifluoroacetate	$C_{40}H_{77}F_3O_2$	647	1.17
27.	27.078	Vitamin E	$C_{29}H_{50}O_2$	430.7	6.71
28.	28.237	Ergost-5-en-3-ol, (3.beta.,24R)-	$C_{28}H_{48}O$	400.7	0.60
29.	28.529	Stigmasta-5,22-dien-3-ol, (3.beta.,22E)-	$C_{29}H_{48}O$	412.7	0.58
30.	29.289	.gamma.-Sitosterol	$C_{29}H_{50}O$	414.7	10.67
31.	30.416	Cycloartane-3.beta.,25-diol	$C_{30}H_{52}O_2$	444.7	0.25
32.	30.602	Lupeol	$C_{30}H_{50}O$	426.7	0.49
33.	31.144	gamma.-Sitostenone	$C_{29}H_{48}O$	412.7	0.63
34.	31.946	24-Norursa-3,12-diene	$C_{29}H_{46}$	394.7	0.21
35.	32.779	Phytyl stearate	$C_{38}H_{74}O_2$	563	0.79

Table 4. Molecules with top 5 docking score and interactions between the major metabolites of *Lawsonia inermis* and ABCBI's drug-binding domain evaluated by the molecular docking investigation

S.No	Compounds	Binding free energy(kcal/mol)	Total interaction residues	Hydrogen bonds	Vander Waals	Pi -Pi Stacked/Pi-Pi shaped	Pi-Sigma/ Pi-Alkyl
1	24-Norursa-3,12-diene	-10.7	Met986 Asn842 Asn721 Ser766 Gln725 Tyr307 Phe303 Phe728 Phe343 Ile306 Tyr310 Leu339 Phe770 Leu724 Leu339	NO H-BONDS	Met986 Asn842 Asn721 Ser766 Gln725 Tyr307	—	Phe303 Phe728 Phe343 Ile306 Tyr310 Leu339 Phe770 Leu724 Leu339
2	γ-sitostenone	-9.7	Ile340 Met69 Tyr953 Phe72 Leu975 Phe732 Phe728 Ser979 Tyr310 Phe343 Phe983 Phe303 Trp232 Leu339 Ile306 Phe336	NO H-BONDS	Ile340 Met69 Tyr953 Phe72 Leu975 Phe732 Phe728 Ser979 Tyr310	Phe343 Phe983	Phe303 Trp232 Leu339 Ile306 Phe336

3	Lupeol	-9.7	Gln725 Tyr310 Phe770 Tyr307 Asn842 Ala987 Gln990 Phe343 Ile340 Leu339 Ile306 Phe303 Leu724	1 H-BOND WITH Gln725	Tyr310 Phe770 Tyr307 Asn842 Ala987 Gln990	Phe343	Ile340 Leu339 Ile306 Phe303 Leu724
4	γ -sitosterol	-9.3	Leu339 Tyr310 Ser979 Phe732 Asn721 Gln838 Asn842 Val991 Gln725 Phe983 Ile306 Phe728 Phe336 Phe303 Phe336 Ala987 Phe770	2 H-BOND WITH Tyr307 and Asn721	Leu339 Tyr310 Ser979 Phe732 Gln838 Asn842 Val991 Gln725	Phe983	Ile306 Phe728 Phe336 Phe303 Phe336 Ala987 Phe770
5	Ergost-5-en-3-ol	-9.3	Gln838 Tyr310 Phe732 Ser979 Gln725 Tyr307 Asn721 Gly722 Val991 Asn842 Phe336 Phe983 Phe728 Ala987 Phe770 Leu724 Phe303	1-H BOND WITH Gln838	Gln838 Tyr310 Phe732 Ser979 Gln725 Tyr307 Asn721 Gly722 Val991 Asn842	—	Phe336 Phe983 Phe728 Ala987 Phe770 Leu724 Phe303

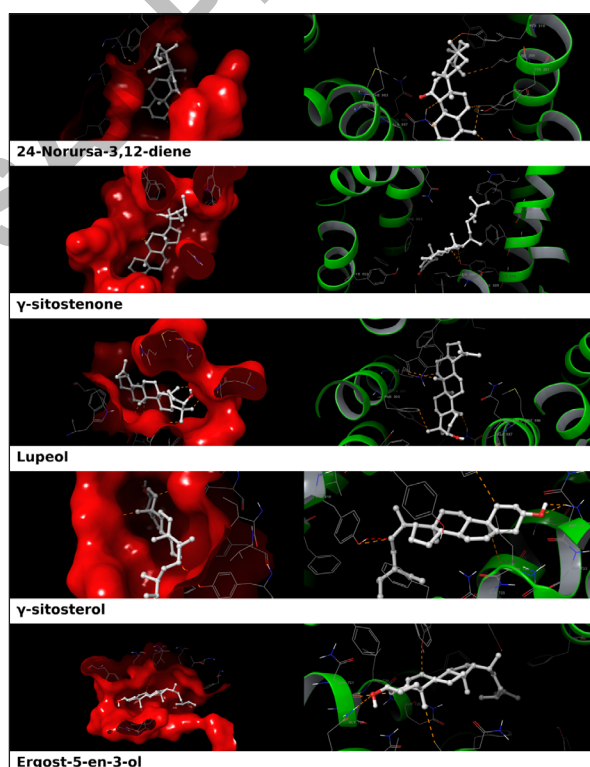


Figure 1.Figure showing ligand in binding pocket of the protein (ABCB1) its binding pose and ligand interacting residues along with their solid surface image of Protein's binding pocket bound with ligand

Table 5. Physiochemical and ADMET Properties of selected ligands

S.No	Ligands	MW (g/mol)	LogP	HBA	HBD	RotB	TPSA ($\text{\AA}^2$)
1	24-Norursa-3,12-diene	394.687	8.5840	0	0	0	0
2	γ -sitostenone	412.702	8.2330	1	0	6	17.07
3	Lupeol	426.729	8.0248	1	1	1	20.23
4	γ -sitosterol	414.718	8.0248	1	1	6	20.23
5	Ergost-5-en-3-ol	400.691-	7.6347	1	1	5	20.23

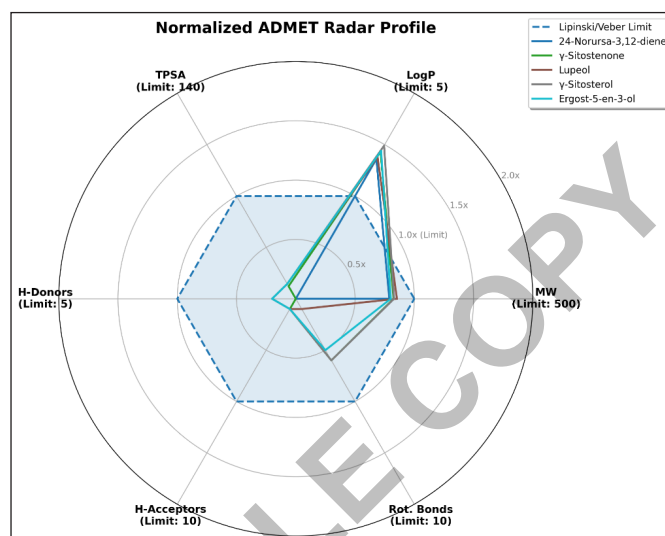


Figure 2. Radar plot showing different ADMET properties of our best docked compounds

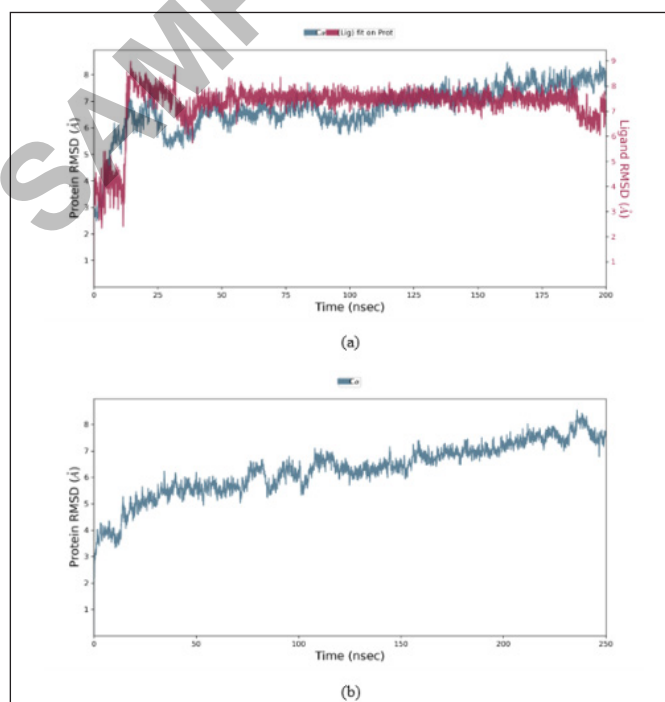


Figure 3. RMSD plot depicting the C(α) deviation of ABCBI protein when bound to ligand and deviation of ligand fitted with protein. (a) RMSD of protein C(α) and ligand fit on protein (b) RMSD of APO ABCBI i.e. when not bound to any ligand

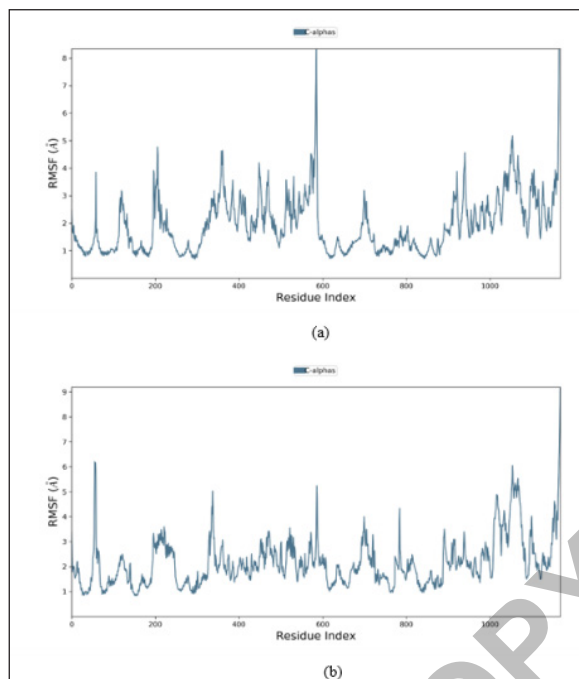


Figure 4. Plot Showing RMSF of protein when bound with ligand and Apo form of protein (a) RMSF of protein residues when bound with ligand Gama-Sitosterol (b) RMSF of ABCB1 residues in its Apo form

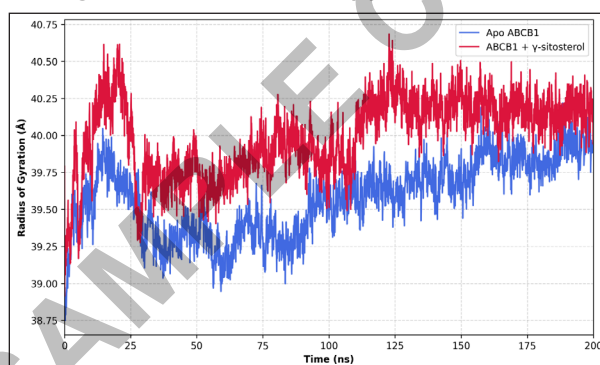


Figure 5. Radius of Gyration of Protein ABCB1 shown here when in unbound state (i.e. Apo) and when bound to ligand (γ-sitosterol)

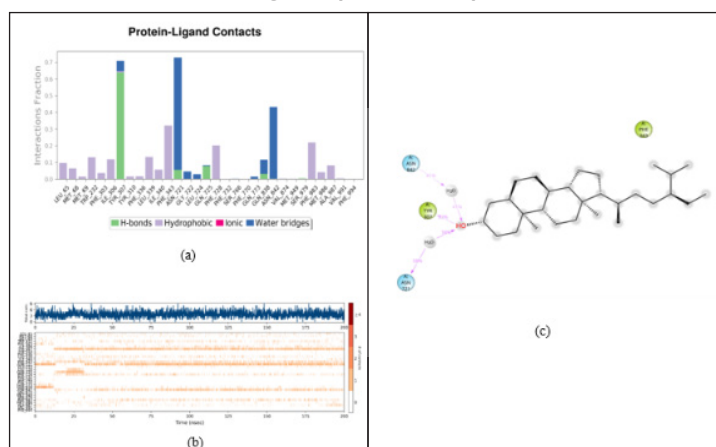


Figure 6. Figures showing protein-ligand contact over period of simulation (a) Bar plot showing different types of bond formed during the length of the simulation and their interaction strength. (b) Total number of interaction amino acid residues made during the molecular dynamic simulation. (c) 2-D interaction plot of ligand contact with protein and its residues

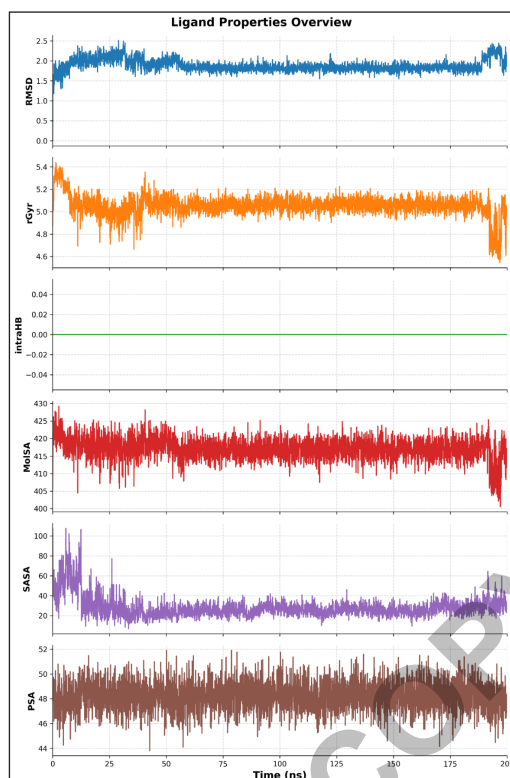


Figure 7. Combined figure for all the ligand properties such as its RMSD, Radius of Gyration(rGyr), MolSA, SASA, PSA

Discussion

One well-characterized mechanism of MDR in breast cancer is the overexpression of ABCB1 at the plasma membrane of tumour cells, allowing for the ATP-dependent efflux of structurally diverse chemotherapeutics including anthracyclines, taxanes and vinca alkaloids before they reach cytotoxic intracellular concentrations. Synthetic first and second-generation P-glycoprotein inhibitors (verapamil, cyclosporine A, tariquidar) have largely failed in the clinic due to pharmacokinetic interference and off-target toxicity. Plant-derived polyphenols and terpenoids have therefore re-emerged as attractive, better-tolerated scaffolds for efflux-pump modulation. Based on this, *Lawsonia inermis* was picked as it was known to have ethnopharmacological use and documented phenolic/flavonoid richness, making it a plausible option to test for ABCB1-directed action.

The high total phenolic (330.84 ± 22.78 mg GAE/g) and flavonoid (30.05 ± 2.79 mg rutin/g) content obtained from the ethanolic extract is consistent with the wider literature associating these classes of compounds with ABC-transporter inhibition and offers a chemical explanation as to why *L. inermis* extract could potentially interfere with drug efflux prior to the study of any individual constituent in isolation. However, the GC-MS profile indicates that the bioactivity of the extract is unlikely to be phenolic-based alone. The most abundant individual constituents were vanillin and squalene, while the compounds that ultimately dominated

the docking results were triterpenoid and phytosterol-type molecules (lupeol, γ -sitosterol, γ -sitostenone, ergost-5-en-3-ol, 24-norursa-3,12-diene). This discrepancy is important from a molecular point of view- it shows that the ABCB1-modulating capability of the extract is more likely due to its bulky, hydrophobic terpenoid fraction than to its smaller phenolic acids, which are normally responsible for its antioxidant activity.

The docking results support this interpretation. All five top-ranked ligands converged on a similar hydrophobic sub-pocket lined by Phe303, Ile306, Leu339, Phe343 and Phe728 and engaged it largely by van der Waals, π -alkyl and π -sigma contacts rather than hydrogen bonding. Such a pattern is consistent with the well-documented versatility of the ABCB1 drug-binding cavity, which is lined almost exclusively by aromatic and aliphatic residues suitable to accommodate big, non-polar substrates. The fact that the binding free energies of five structurally similar sterols/ triterpenes lie in a small range (-9.3 to -10.7 kcal/mol) further suggests that shape and bulk complementarity to this hydrophobic groove, rather than any particular polar anchor, is the major determinant of affinity for this class of ligands.

The ADMET profile of the short-listed compounds is initially unfavorable by conventional criteria for oral drug-likeness, with all five ligands exceeding Lipinsky's LogP threshold (7.6-8.6). This apparent liability may however

be mechanistically advantageous in the specific context of ABCB1 inhibition rather than simply a developability concern. ABCB1 recognizes many substrates and translocate them predominantly from the inner leaflet of the plasma membrane, so a ligand's capacity to partition into the lipid bilayer and access the transporter's transmembrane cavity is arguably as relevant here as is its water solubility. The very low topological polar surface area (0-20.23 Å²), low hydrogen bond donor/acceptor counts (0-1) and limited number of rotatable bonds observed for these ligands are consistent with efficient desolvation upon entry into the hydrophobic pocket and the conformational rigidity required to physically block the transport cycle, as has been described for P-glycoprotein substrates and inhibitors (but has not been experimentally confirmed for these compounds). The same properties also imply a clear translational limitation: high lipophilicity is usually associated with poor aqueous solubility, high plasma protein binding and increased risk of off-target membrane interactions so these leads would likely require formulation strategies (e.g., nanoparticle or lipid-based delivery) and dedicated selectivity profiling prior to proceeding beyond the discovery stage.

The molecular dynamics results most strongly support a real inhibitory rather than a transitory docking contact. The best ranked ligand, γ -sitosterol, showed a low and stable RMSD (1.8-2.0 Å) over 200 ns and an almost rigid, non-hydrogen bonded backbone, while preserving a direct hydrogen bond to Tyr307 and water-bridged contacts to Asn721 and Asn842 throughout the simulation window, suggesting a persistently stable, non-dissociating pose instead of a fluctuating or loosely bound one. Simultaneously, ligand binding had a significant impact on the intrinsic dynamics of the protein itself: RMSF analysis indicated decreased flexibility in the N-terminal region (residues 50–100), which is usually mobile, and in the 1050-1100 region, and an emerging region of high fluctuation around residues 580-600, consistent with an allosteric redistribution of flexibility away from the ligand-occupied cavity. Most importantly, the radius of gyration of the transporter exhibited a gradual increase from the apo baseline (39.25-40.0 Å) to a higher stable plateau (40.0-40.5 Å) in the ligand-bound state. Given ABCB1's catalytic cycle requires large-scale conformational closure driven by nucleotide-binding-domain (NBD) dimerization, a ligand that instead drives sustained global expansion is best interpreted as a physical 'steric wedge' that occupies the transmembrane cavity and sterically opposes the domain closure required for transport- a competitive, non-ATP-site mechanism distinct from that of many synthetic inhibitors. These results must be understood in the context of a fully computational investigation. Docking scores are an

estimate of binding affinity and not a measure of binding thermodynamics. Moreover, the investigation was limited to one cryo-EM/crystal conformation of ABCB1 (PDB: 7O9W) which does not account for the conformational ensemble in which the transporter samples during its transport cycle. The extract itself is a complicated mixture and there has been no testing of the contribution of the shortlisted compounds above and beyond the remaining 30 GC-MS constituents or possible synergy between them. Before the findings can be considered more than a robust computational hypothesis, functional validation including rhodamine-123 or calcein-AM efflux assays, cytotoxicity and combination-index studies with standard chemotherapeutics such as doxorubicin or paclitaxel in MDR breast cancer models such as MCF-7/ADR or MDA-MB-231/TX400, and confirmation of target engagement will be required.

Conclusion

This study highlights the potential of *Lawsonia inermis* leaf extract, particularly its triterpenoid/phytosterol fraction, as a natural source of ABCB1-directed multidrug resistance modulators in breast cancer. Phytochemical profiling confirmed a phenolic- and flavonoid-rich extract and molecular docking against the ABCB1 drug-binding cavity (PDB: 7O9W) identified five hydrophobic ligands, 24-norursa-3,12-diene, γ -sitostenone, lupeol, γ -sitosterol and ergost-5-en-3-ol, which demonstrated strong and consistent binding to a conserved aromatic/aliphatic sub-pocket. Although they are not typical of orally druggable small compounds, their high lipophilicity and low polarity seem suitable with engagement of this membrane-embedded transporter. Among them, γ -sitosterol was the top mechanistically validated candidate, as a 200 ns molecular dynamics simulation of the ABCB1- γ -sitosterol complex captured a rigid, low-RMSD binding pose with a stable hydrogen bond to Tyr307 and water-bridged contacts to Asn721/Asn842. This sustained engagement changed ABCB1 dynamics and gradually increased the transporter's radius of gyration consistent with a steric-wedge mechanism of competitive inhibition. Taken together, these results support γ -sitosterol, in addition to the other *L. inermis*-derived triterpenoids, as a candidate lead scaffold for reversing ABCB1-mediated multidrug resistance and justify prioritizing it for in vitro efflux and cytotoxicity assays, followed by in vivo evaluation, as the next steps towards its development as a chemosensitizing adjunct in breast cancer therapy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

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