Bioactivity of melianone against *Salmonella* and in silico prediction of a membrane protein target

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Received: 8 April 2020 / Accepted: 15 September 2020 / Published online: 30 September 2020
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Abstract

Melianone, the protolimonoid (24, 25-epoxyflindissone), was isolated from the medicinal tree species, *Swietenia mahagoni* (L.) JACQ (Meliaceae). The compound isolated from petroleum ether leaf extracts (5.39%) was quantified using high-performance thin-layer chromatography (HPTLC) method. In antimicrobial assays melianone inhibited *Salmonella* ser. Typhi with an MIC of 0.053 μM . Induced Fit Docking (IFD) of the ligand, melianone, with proteins involved in anaerobic virulence of the pathogen, revealed that it binds with FocA (a transport protein of formate ions) at its “periplasmic opening” with a glide energy of $-51.8576 \text{ kcal mol}^{-1}$. Melianone altered the overall conformation of the protein (protomer A) by 0.347 Å RMSD. It induced a notable protein topology (Ω loop region) shift in the channel from an intermediate-open to a closed-state conformation and was supported by molecular dynamic simulations performed. FocA, a protein that contributes to its survival under anaerobic conditions, was further evaluated experimentally, after exposure of *Salmonella* ser. Typhi to melianone, resulting in the altered homeostasis of formate.

Keywords *Swietenia mahagoni* · Triterpenoid · Melianone · Anti-salmonella MIC · Molecular docking · Formate ion channel · Molecular dynamic simulations

Introduction

A member of the Meliaceae, *Swietenia mahagoni* (L.) Jacq. is a valuable timber tree with ethnomedical importance (Champagne et al. 1992). Several types of limonoids and

triterpenoids were isolated and structurally elucidated from *S. mahagoni* (Chen et al. 2007; Abdelgaleil et al. 2006; Govindachari et al. 1999; Ekimoto et al. 1991). The triterpenoid, melianone was first reported from its non-polar extracts (leaves and twigs, Basak et al. 1970) and was also found in other species belonging to Simaroubaceae, Rutaceae, and Meliaceae (Online Resource 1). A review of the functional potentials of mahogany family presented a shortcoming in their antimicrobial properties (Paritala et al. 2015) with inconclusive effects against *Salmonella* species (vide, Online Resource 2). In the present study, a systematic antimicrobial bioassay-guided fractionation of the petroleum ether leaf extracts of *S. mahagoni* was conducted. Fractionation led to the isolation and structural determination of the protolimonoid, melianone (24, 25-epoxyflindissone). Melianone with anti-*Salmonella* activity was then virtually screened (21 potential protein targets from PDB) using induced fit docking analysis (IFD method) to select putative proteins involved in anaerobic virulence of this pathogen. FocA (3Q7K), an important formate transport channel protein of *S. Typhi* (Weichert and Beitz 2017), was one of the membrane target proteins for melianone involved in the survival of the pathogen under anaerobic conditions (Lu

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13205-020-02441-9>) contains supplementary material, which is available to authorized users.

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et al. 2012). Anaerobic metabolism during pathogenesis produces formate, a known source of high-energy compound and reducing power (Online Resource 3 adapted from Lu et al. 2011) that was assayed using colorimetry (Maeda and Wood 2008).

Thus, the objectives of this study was to investigate the bioactivity of melianone against *Salmonella* ser. Typhi using experimental antimicrobial assays, in silico screening for putative target virulence proteins, and its involvement in antibiosis.

Material and methods

Extraction, isolation and compound identification

Leaf samples of *Swietenia mahagoni* (authenticated) were collected from 10-year-old trees, grown within our Deemed-to-be-University campus, Chennai (13.0395° N 80.1427° E). The shade-dried and powdered leaves, (880 g) were extracted with 4 L of petroleum ether by exhaustive percolation for 3 days with intermittent stirring for every 16 h at room temperature. The mixture was filtered and the combined menstruum upon concentration in a rotary evaporator (IKA Werke® HB4 Basic, Germany) yielded 28 g of crude petroleum ether leaf extract of *S. mahagoni* (PESM). Ten grams of the PESM was chromatographed over a silica gel column in an elutropic series with hexane and ethylacetate. The hexane: ethylacetate (50:50, v/v) fractions furnished a white amorphous compound (480 mg). The structure was identified using ^1H and ^{13}C NMR (Bruker, Ultra Ascend 400 MHz, USA). The compound was also subjected to a High-Resolution Mass Spectrometry (HRMS) analyses (Jeol GC Mate II).

High-performance thin layer chromatography (HPTLC) quantification

Quantification of the pure compound in crude leaf extract PESM was performed using the photo-densitometric HPTLC method (Camag, Switzerland). Known concentrations of pure compound and crude extract PESM were spotted on HPTLC plates with a CAMAG Automatic TLC Sampler 4 (ATS4) and developed using chloroform:methanol (90:10, v/v). The HPTLC plates were derivatized with methanol:sulphuric acid (90:10, v/v) reagent and chromatograms scanned at 502 nm. The calibration curve for linearity of melianone was obtained by plotting peak area vs. concentration of compound (Xie et al. 2014). The data analysed by the linear least square regression method were used to estimate the percentage of pure compound in the crude leaf extract sample (PESM). Detection limit (DL) and quantification limit (QL) were estimated statistically for three

determinations of the samples as per the guidelines (ICH Q14 2018).

Anti-*Salmonella* activity assayed using thin-layer agar diffusion

Bacterial isolates (ATCC 6539) of *Salmonella enterica* subsp. *enterica* ser. Typhi (*Salmonella* ser. Typhi; nomenclature vide, CDC, Brenner et al. 2000) were resourced from the Department of Medical Microbiology, Sri Ramachandra Medical College and Research Institute (DU) and maintained on Muller Hinton (MH) agar. All biosafety protocols for handling and disposing pathogenic strains were observed as per the Institutional Biosafety procedures.

Thin layer Muller Hinton (MH) agar, seeded (Hultmark et al. 1982) with cells of *Salmonella* ser. Typhi (10^6 CFU mL^{-1} ; ATCC 6539), were used to test different concentrations of pure compound and crude PESM. The pure compound was tested at 20, 30, 40 and 50 $\mu\text{g well}^{-1}$ and crude PESM of 500, 1000, 1500, 2000 and 2500 $\mu\text{g well}^{-1}$ concentrations in triplicate. Ciprofloxacin (4 $\mu\text{g/mL}$) served as antibiotic control and methanol (10 μL) as solvent control. After incubation for 18 h at 37 °C, the antibacterial activity of compound was detected as zones of inhibition in millimetres (mm) for each concentration. Measurements were made using transparent graduated scale placed on the back of the petriplate against a lit box. Data are presented as mean diameter (mm) $\pm$ SD of the inhibition zones and are representative of five independent experiments to ascertain their reproducibility.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) microbroth dilution method

The MIC for the pure compound and extracts against *Salmonella* ser. Typhi was examined by microbroth dilution method (CLSI 2000) in a 96-well titre plate (Tarsons, India). The compound and crude PESM (working stock of 10 mg/mL) were dissolved in methanol and serially double diluted in MH broth from the initial test concentrations of 100 $\mu\text{g/mL}$ and 2.5 mg/mL, respectively. All the wells, except the broth control well, were inoculated with 5 μL of the diluted *S. Typhi* culture (10^6 CFU/mL). Additional controls viz., positive control, ciprofloxacin (30 $\mu\text{g/mL}$), un-inoculated well with methanol in MH broth (1:1) and inoculated wells with 100 μL of MH broth, were maintained. Visual detection using 10 μL of 0.1% 2,3,5-triphenyltetrazolium chloride [TTC] (HiMedia) was employed at the end of the incubation period. Duplicates of each treatment were maintained. The titre plate containing the experimental setup was incubated for 18 h at 37 °C to determine the MIC. MIC was defined as the lowest concentration of the compound that inhibited

the visible growth (pink color formazan) of the microorganism after overnight incubation. The assay was independently performed five times for reproducibility.

To determine the MBC, the contents in each well after determining the MIC through visual observation (after 18 h incubation) were thoroughly mixed. Ten microliters of the sample from each well was serially diluted, and suitably diluted samples were streaked on to a fresh MH agar solid medium and incubated for another 18–24 h. The concentration of the test compound inhibiting 99.9% of bacterial growth (yielding no bacterial colonies) on the solid medium was taken as MBC (CLSI 1999, 2000).

Protein target identification-induced fit docking (IFD)

Protein coordinates were retrieved from the Protein Data Bank (PDB) using the keyword “*Salmonella enterica* subspecies *enterica* serovar Typhi”. The retrieved proteins were then screened for redundancy, resolution and hypothetical proteins (unknown function) and then those with better resolution values were selected for the in silico analysis. Each target protein was prepared using the protein preparation wizard module from the Schrodinger’s package. The hydrogen atoms were added and missing residues constructed to favour good hydrogen bond interactions, following the minimization step using OPLS_2005 force field. The NMR solved structure of the compound serving as the ligand molecule was prepared in ChemsSketch 10.0 and saved in (.mol) format. Later the structure was converted to 3D form in Maestro environment and saved in (.pdb) format. The ligand molecule was subjected to the Ligand Preparation workflow for energy minimization using OPLS2005, force-field optimization through conjugate-gradient algorithm for about 1000 cycles. IFD analysis was performed for the ligand against 21 selected target proteins of *Salmonella* ser. Typhi from PDB using Glide 6.0 (Schrodinger suite). Both protein and ligand were kept flexible in a relaxed state and glide docking parameters were set to default. The grid was set around the active site residues taken from the literature (*vide*, Online Resource 4). From the docking calculations performed using IFD, the protein–ligand complex with the best binding conformation was selected based on their Glide energy (kcal mol^{-1}).

Molecular dynamic simulations

To study the dynamic behaviour of the hybrid, protein–ligand complex in the system, we used Gromacs 4.6.5 software (Pronk et al. 2013). The forcefield used for the parameterization of protein file was GROMOS96 43A1 (Chiu et al. 2009). The ligand topology was generated using PRODRG server (Schuttelkopf et al. 2004). The

complete system was calibrated to be in equilibrium at a constant temperature, volume and pressure. The trajectory files obtained at the end of the molecular dynamics simulation were analysed using the in-built functions of Gromacs, i.e. RMSD, RMSF, SASA, Radius of Gyration and HBOND. The final trajectory files obtained were plotted using XMGRACE, a graphical plotting tool.

Indirect colorimetric detection of formate in melianone treated *Salmonella* ser. Typhi cells

The *Salmonella* ser. Typhi cells (ATCC 6539) were grown anaerobically in complex formate (CF) broth at 37 °C overnight. The cells were harvested and washed with 100 mM phosphate mineral buffer (PMB) pH 6.5 and resuspended in PMB (Maeda and Wood 2008). To the resuspended cells (10^3 cells mL^{-1}), different concentrations [0.0256 μM ; 0.053 μM and 0.106 μM and corresponding to $\frac{1}{2} \times \text{MIC}$; $1 \times \text{MIC}$ and $2 \times \text{MIC}$, respectively] of the pure compound, melianone were added. Another set of resuspended cells were provided with 100 mM of formate along with the three concentrations of pure compound. Supplementation of 100 mM formate in the medium for growth of *Salmonella* ser. Typhi cells under anaerobic conditions was used to induce the expression of FocA protein (Suppmann and Sawers 1994; Weichert and Beitz 2017). A set of cells that were unexposed to the compound served as controls. Triplicates for each treatment were maintained.

After 42 h of anaerobic incubation at 37 °C in PMB, 2 mL of the cell suspension from each treatment and control groups was centrifuged at $16,000 \times g$ for 1 min. The supernatant (1 mL) from each group was collected and mixed with 1 mL of 1 mM potassium permanganate. The optical densities, $A_{540\text{nm}}$, were measured within 10 s (Maeda and Wood 2008) using a colorimeter. The concentrations of formate in treatment and control groups were determined from the titration of known amounts of formate with KMnO_4 (calibration graph, Online Resource 5). The results were expressed as mM formate detected in the broth for the two groups of treated cultures. This would help in determining the final amount of formate ions found in the external growth medium after 42 h of anaerobic growth. The assay was made in triplicate and the means and standard deviations were calculated.

The viability of the cells from treatment and control groups was scored by re-growing 20 μL of the appropriate, diluted cell suspension in CF agar plates under anaerobic conditions. The CFU/ mL was calculated using the following formula:

$$\text{CFU/mL} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{volume of culture plated}}$$

The results are presented as percentage of reduction (%) in CFU/mL using the following formula (Microchem Laboratory 2015):

$$\text{Percentage of reduction(\%)} = 100 \times \frac{(\text{CFU before treatment} - \text{CFU after treatment})}{\text{CFU before treatment}}$$

The percentage reduction (%) in colonies was used to estimate inhibition by melianone during anaerobiosis relative to the accumulation of formate in the external medium. The assay was independently performed thrice with triplicates for reproducibility.

Statistics

Means and standard deviations shown are deduced from one representative experiment in which triplicates for a treatment were maintained. The assays were performed, independently, three to five times for reproducibility. Statistical significance with (***) that denotes *P* value less than 0.0001 was determined by Student's *t* test using the Graphpad Prism 5.

Results

Compound identification

Petroleum ether extract of *S. mahagoni* (PESM) purified by column chromatography yielded a pure compound (*vide*, 2.1) with an R_f of 0.6 on TLC (*vide*, 2.2). The ^1H -NMR and ^{13}C -NMR data obtained for this compound (Online Resource 6) were in accordance with the literature (Polonsky et al. 1977) and the compound was identified as melianone

($\text{C}_{30}\text{H}_{46}\text{O}_4$). The melting point of the compound was determined to be 222–224 °C (from literature 223–224 °C; Lavie et al. 1967) and the mass was 470.6837 m/z^+ (expected $m/z^+ = 470.6838$). The mass spectrum of melianone with its embedded chemical structure is given in Fig. 1.

Quantification of melianone contents

A simple, high-performance thin-layer chromatography was developed and validated for quantifying the compound, melianone, using densitometric analysis of the band obtained at R_f of 0.6 ± 0.02 in crude extracts (Xie et al. 2014). The calibration curve plotted with peak area of melianone versus the concentration was found to be linear ($Y = 0.367 + 0.119 \times X$; correlation coefficient (r) = 0.99960; $\text{sdv} = 1.66\%$; Online Resource 7) in the concentration range 100–1000 ng per spot. The limits of detection and quantification were determined as 46.033 and 139.495 ng per spot, respectively, with a detection range of 200–1000 ng.

Based on the peak area for melianone, it was estimated that 269.53 ng of melianone was found in 5000 ng of crude sample (PESM) corresponding to 5.39% of melianone.

Anti-Salmonella activity

The crude extract PESM inhibited the enteric pathogen *Salmonella* ser. Typhi by producing the inhibition zones ranging from 7 ± 0.0 to 10 ± 0.2 mm for 500 to 2500 $\mu\text{g well}^{-1}$ concentrations (Fig. 2b). The pure compound, melianone, produced 5.5 ± 0.07 to 7.5 ± 0.0 mm zones of inhibition at 20–50 $\mu\text{g well}^{-1}$ (Fig. 2a). Ciprofloxacin (4 $\mu\text{g/mL}$), as positive control showed 14 mm zone.

The MIC for melianone was 25 $\mu\text{g mL}^{-1}$ (0.053 μM) against *Salmonella* ser. Typhi (Fig. 3a), while for crude PESM it was 2.5 mg mL^{-1} (Fig. 3b). For MBC, the highest dilution of melianone and PESM in which no re-growth of

Fig. 1 Mass spectrum and chemical structure of melianone isolated from petroleum ether leaf extracts of *Swietenia mahagoni*. (The spectrum shows m/z of melianone only from 200 to 470.5)

Fig. 2 Antibacterial activity of the isolated melianone (a) and *Swietenia mahagoni* extracts (PESM, b) showed clear zones of inhibition in thin-layer agar diffusion assay against *Salmonella* ser. Typhi. The diameter of inhibition zones (mm; mean $\pm$ standard deviation) for the compound melianone and PESM (c) are given in the table below

Salmonella ser. Typhi occurred on fresh agar medium was 25 µg mL⁻¹ (Fig. 3c) and 2.5 mg mL⁻¹ (Fig. 3d), respectively.

Induced fit docking (IFD) analysis of protein: melianone complex

Melianone with anti-*Salmonella* activity was virtually screened to find potential *Salmonella* ser. Typhi protein targets using IFD method. The 21 target proteins (5 membrane and 16 enzymatic proteins) were docked with melianone individually and the complexes whose glide energy values ≤ -50 kcal/mol were selected. The docking statistics for the top ten target hits given in Table 1 showed two membrane proteins and eight enzymatic proteins. For example, the proteins CDP-Tyvelose epimerase (1ORR) and CDP-D-glucose 4,6-dehydratase (1WVG) formed complexes with melianone with glide energy values of -68.3581 and -58.2577 kcal mol⁻¹, respectively, and were involved in the formation of tyvelose sugar, found in the O-antigen of LPS.

Out of the five membrane proteins screened, melianone was able to dock well with only two membrane proteins, the formate channel protein, FocA (3Q7K) and the outer membrane protein, OmpF (4KR4), with low glide energy of

-51.8576 and -50.868 kcal mol⁻¹. Best docking conformations were visualized using CHIMERA (Pettersen et al. 2004) and the H-bond distances were calculated using Lig-Plot+ (Laskowski and Swindells 2011). The best conformation for each target was selected based on the glide energy value, number of H-bond interactions and hydrophobic interactions. Of these two likely membrane target proteins, FocA was further evaluated experimentally because of its major role as a transport protein of *Salmonella* ser. Typhi involved in the survival of the pathogen under anaerobic conditions (Lu et al. 2011). The in silico search for putative protein targets indicated more than one site of action for this triterpenoid, suggesting that the mechanism of action for melianone is not restricted to Foc A alone.

Melianone-FocA complex

Melianone, serving as the ligand or inhibitor, docked itself with the formate channel protein, FocA (3Q7K, A chain), with low glide energy of -51.8576 kcal mol⁻¹ and -7.57 docking score. In the best docking conformation, it docked at the periplasmic opening of the axial passage (Fig. 4a) forming five H-bond and seven hydrophobic interactions

Fig. 3 Minimum inhibitory concentrations (MIC) of melianone (a) and *S. mahagoni* leaf extracts, PESM (b) against *Salmonella* ser. Typhi (arrows indicate MIC values) was determined using the micro-

broth dilution assay. Minimum bactericidal concentrations (MBCs) for melianone (25 µg/ mL; c) and PESM (2.5 mg/ mL; d) was determined by the absence of regrowth in MH agar plates after treatment

including the essential invariant Lys156. The interacting FocA residues are shown in Fig. 4b. This is in agreement with FocA chain A, (3Q7K) co-crystallized with formate substrate molecule showing hydrogen bonding with the invariant Lys156 (Weichert and Beitz 2017).

Melianone impacted changes in FocA

FocA is a homopentamer transport protein with individual substrate channel in each protomer. Structurally, each protomer has six transmembrane segments (TMs) and N-terminal helices that undergo structural rearrangements during

transport rendering a open or close or an intermediate conformation to each protomer (Lu et al. 2013).

The secondary structure, topology sketches for the chains-A, B and E (intermediate, open and closed state) of FocA (3Q7K) generated from the online database PDBsum (Laskowski 2001), are shown in Fig. 5, and their sketches were in accordance with literature (Lu et al. 2011). Similarly, a topology sketch was generated for FocA–melianone complex in PDBsum, to study the inhibitor-mediated topological changes in protomer A (Fig. 5d). Topology sketches obtained for the protomer A before docking (intermediate open state) and after docking with melianone at the periplasmic opening

Table 1 *Salmonella* ser. Typhi protein target (PDB) docking statistics with the ligand melianone is presented along with their Glide energy

PDB ID	Type of protein	Protein name	Glide energy (kcal/mol)	Docking Score	H-bonds	Protein atom	Ligand atom	H-bond distance (Å)	Hydrophobic interacting residues
3Q7K	Membrane protein	Formate channel (FocA)	-51.8576	-7.57	5	TYR64:OH LYS68:NZ TYR52:OH LYS68:NZ TYR64:OH	O1 O1 O2 O4 O4	3.05 3.01 2.81 3.22 2.59	Q151, T152, H155, K156, H159, L167, G206
4KR4	Membrane protein	<i>Salmonella typhi</i> OmpF complex with Ampicillin	-50.1868	-4.08	2	ARG130:NH1 TYR301:OH	O2 O3	2.9 3.3	D108, A110, P111, S114, G115, D116, G120, A121, N307
1ORR	Enzymatic protein	CDP-tyvelose 2-epimerase complexed with NAD and CDP	-68.3581	-9.46	3	LEU13:N ARG237:NE ARG237:NH2	O4 O3 O3	3.17 3.10 2.78	G8, G11, R36, L80, V84, M86, K127, Y165, S194, M195, Q200, G207, W208, R299
1WVG	Enzymatic protein	CDP-D-glucose 4,6-dehydratase	-58.2577	-7.71	2	GLN1092:NE2 LYS1163:NZ	O3 O4	2.93 3.27	G1018, G021, K1023, L1043, D1044, P1046, M1089, A1091, Q1092, P1093, I1132, T1133, S1134, Y1159, K1163, A1195
1PG4	Enzymatic protein	Acetyl CoA synthetase	-62.742	-8.93	3	LEU13:N ARG237:NE ARG237:NH2	O4 O3 O3	3.17 3.10 2.78	G8, G11, R36, L80, V84, M86, K127, Y165, S194, M195, Q200, G207, W208, R299
2P2F	Enzymatic protein	Acetyl CoA synthetase with acetate, AMP and CoA bound	-52.0743	-8.45	2	THR311:OG1 THR311:OG1	O1 O4	2.91 2.96	T304, A305, D306, W309, V310, V333, A357, V386, T416, S523, G524, H525
1WVC	Enzymatic protein	Alpha-D-glucose-1-phosphate cytidyl transferase complexed with CTP	-55.750	-6.81	3	GLY10:N ARG111:NE TYR129:OH	O1 O4 O3	3.15 2.97 3.08	L8, A9, G11, L12, R15, S106, M107, T108, D131, N188, G189, Q210, W232
3G1T	Enzymatic protein	Short chain dehydrogenase	-62.6416	-6.09	2	ARG46:O3 TYR167:OH	O3 O4	2.83 2.72	G21, D24, G25, I26, R46, K50, N101, A102, G103, S154, Y167, P197, G198, T200, T202, S203, M204
3Q58	Enzymatic protein	N-Acetylmannosamine-6-phosphate epimerase	-58.825	-5.65	2	SER208:N ARG212:NE	O4 O1	2.77 2.85	S20, N22, K74, R83, R153, L154, Y157, E184, G185, R186, N188, T205, G207
4EZE	Enzymatic protein	Had family hydrolase t0658	-52.6224	-3.57	1	HIS282:NE2	O4	2.80	T9, N44, P160, K266, N280, Y281, H282, G283 (Chain A); I43, N44, T45, A99, P160, G161, W265, K266, Y281, H282, G283, D285

Fig. 5 The topology sketches depicting the TM arrangements for different chains of native FocA protein [PDBsum]. **a** Intermediate channel state topology of protomer A; **b** closed channel state topology of protomer B; **c** open channel state topology of protomer E; **d** the topol-

ogy of melianone docked FocA (protomer A) with intermediate state after docking with melianone showed rearranged TM3-4 and is likely to affect formate transport

complexed structures achieved the equilibrium state and the convergence was observed for both plots at 4 ns (Fig. 7a). The RMSF demonstrates the fluctuations in the amino acid residues upon the time scale (Fig. 7b). From the RMSF plots, we could observe that the complex has a higher fluctuation than the native structure with the higher fluctuation observed mainly in about ~60 to ~115 residues.

The SASA analysis revealed the constant fluctuation starting from ~95 nm to a profound reduction of ~90 nm for the native structure, whereas the complexed structure started ranging from ~95 nm and declined till ~80 nm. The plot of solvent accessible surface area has been depicted in Fig. 7c. From SASA we infer that the complex structure is having a higher tendency towards the ligand than that

of the native structure. The compactness, depicted as Rg, for both the native and the complex had similarity in its fluctuation. The Rg of native structure was maintained in a constant value, i.e. ~1.9 nm, whereas the complexed protein FocA-melianone showed the variation of compactness starting from ~1.85 nm and maintaining constant fluctuation at ~1.8 nm as shown in Fig. 7d. The hydrogen bonding pattern revealed the formation of hydrogen bonds sharing the impact of compactness of the complex. The native structure has the hydrogen bonds forming within the range of ~175 numbers and maintained across the time period, whereas the complex structure forms more number of hydrogen bonds, i.e. more than ~200 numbers shown in Fig. 7e. To support our findings on the FocA-melianone complex, the hydrogen

Fig. 6 Superimposition of FocA–melianone complex (pink–blue) over FocA, A chain (yellow). The residues forming channel pore are represented as surface model (**a**). The residues deviating at the periplasmic opening (PO), central vestibule (CV) and the cytoplasmic

opening (CO) are shown as sticks; **b** the binding of melianone in the periplasmic opening of the axial passage of FocA brings about conformational changes both to the backbone and the side chain atoms of crucial residues

bonding pattern was plotted between the protein and ligand structures as shown in Fig. 7f. We observed prominent formation of hydrogen bonds across the time scale. The maximum number of hydrogen bonds formed were three and the consistent H-bonds were two in number showing the extent of strong bonding pattern of the ligand to the protein structure. The trajectory analysis revealed that the ligand, melianone, had a close proximity of binding toward the periplasmic opening of the formate channel protein (FocA).

Hence, these *in silico* predictions of conformational changes in the key residues lining the axial passage of FocA protein could alter the regulation of the metabolic flow of formate ions in and out of the cell.

Melianone alters formate homeostasis in *Salmonella*

A standard graph plotted for absorbance values proportional to the known concentrations of formate (0, 25, 50, 75, 100, 150, 200, 250 and 300 mM) with 1 mM KMnO_4 was found to be linear (Online Resource 5). With increase in concentrations of formate, there was a gradual decrease in the absorbance denoting the reduction of KMnO_4 . With the help of this

standard graph, the formate concentrations present in the test samples were determined.

Cells were treated with melianone both in the presence and absence of formate supplement. Addition of 100 mM formate to one set of treatment samples served as a reference initial concentration (Maeda and Wood 2008) and also induced expression of the channel protein in anaerobic bacteria. The effect of melianone (0.0256; 0.053 and 0.106 μM) on the formate transport and the viability of anaerobically grown *Salmonella* ser. Typhi cells are presented in Table 2 and Fig. 8. Typhi cells after treatment with melianone with and without formate supplement showed a decrease in populations over their respective controls (Fig. 8a and d). The untreated controls showed large numbers of colonies (too numerous to count, ‘TNTC’; CFU/mL) recovered from appropriate dilutions plated. Melianone reduced viable populations by 87% (at 0.053 μM) to 100% (at 0.106 μM) during anaerobiosis.

The *Salmonella* ser. Typhi cells grown under anaerobic conditions with or without 100 mM formate and treated with various inhibitory concentrations of melianone (0.0256; 0.053 and 0.106 μM) showed a gradual increase in the formate accumulated in the medium. The formate

Fig. 7 Molecular dynamic simulations assessed the impact of melianone binding to FocA channel protein [unbound (black) and the bound (red)] residues. Fluctuations were recorded when the ligand bound protein was compared to the native form for the following parameters: final trajectories of RMSD (**a**) for backbone protein to the ligand, melianone; final trajectories of RMSF (**b**) of C α for protein to the ligand, melianone; solvent accessible surface area (SASA) (**c**); the Rg score (**d**) of compacted conformation protein; the H-bond plot between the unbound and bound protein (**e**); and the hydrogen bonding pattern (**f**)

concentrations detected in the melianone-treated groups were found to be statistically significant when compared with untreated group. The pH of the medium determined after 42 h melianone treatment in anaerobic cells did not show much variation (data not shown).

Discussion

The triterpenoid isolated from the petroleum ether leaf extract of *S. mahagoni* (Meliaceae) was identified as melianone (C₃₀H₄₆O₄) (Lavie et al. 1967) through the HRMS

Table 2 Reduced viability (% reduction) of anaerobically grown *Salmonella* ser. Typhi treated with melianone and formate estimated in the external medium [F[±] indicates medium supplemented (100 mM) or not with formate before the assay; TNTC-too numerous to count]

Melianone concentration (μM)	Formate detected (mM)		Viability score % reduction	
	[F ⁻]	[F ⁺]	F ⁻	F ⁺
UnTreated	54.5 ± 4	143.5 ± 0***	TNTC	TNTC
½ × MIC; 0.0256	67 ± 1	168.5 ± 0***	TNTC	70
1 × MIC; 0.053	71 ± 4	181 ± 0***	87	81
2 × MIC; 0.106	90 ± 5	201.6 ± 7***	100	100
Methanol control	54.5 ± 4	143.75 ± 0***	TNTC	TNTC

Absolute formate concentrations are mean ± sem (*n* = 3); representative data of experiments carried out three times independently

***Denotes *P* value less than 0.0001; statistically significant when compared with F-group

Fig. 8 Representation of the colonies recovered from the treated broth (melianone ±) at the end of the formate assay. The reduction in population correlated with accumulation of formate in the medium when treated with melianone. Absence of colonies in 2 × MIC (a); colonies seen in ½ × MIC (b); 1 × MIC (d) and in control (c)

and NMR data (Fig. 1 and Online Resource 6). Melianone isolated from different plant species showed a wide range of biological activities like cytotoxic, antiproliferative, nematocidal, viricidal, insecticidal and antibacterial (Online Resource 1 and 2). Qualitative antibacterial activity was reported for the *S. mahagoni* extracts (petroleum ether, chloroform, ethyl acetate, seed oil and methanol) (Haque et al. 2009; Kader et al. 2009). These extracts marginally inhibited the activity of Gram positive pathogens like *E. faecalis*, *S. epidermis*, *B. subtilis*, etc. and Gram

negative pathogens like *E. coli*, *Salmonella* ser. Typhi, *Shigella*, etc. In the present study, the isolated triterpenoid, melianone, was tested for its antimicrobial activity against the Gram negative pathogenic bacteria *Salmonella* ser. Typhi using the thin-layer agar diffusion assay (Fig. 2). The MIC and MBC of melianone were determined to be 25 μg mL⁻¹ (0.053 μM) against *Salmonella* ser. Typhi (Fig. 3). Melianone could, therefore, be considered as a putative antibacterial agent as its MBC was not more than four times the MIC (French 2006). At MIC concentration of 25 μg mL⁻¹ melianone showed 3% haemolysis of the red blood cells [data not presented] within the limits acceptable for biomaterials (Szymonowicz et al. 2007), while for direct contact with blood the limit stated is 1% (Szelest-Lewandowska et al. 2007). Using the purified melianone, a HPTLC photodensitometric quantification of the compound was attempted and it was found that 5.39% of melianone was present in the crude leaf extract of *Swietenia mahagoni* (PESM) (Online Resource 7).

An in silico search for protein targets of the ligand, melianone performed through IFD resulted in obtaining 10 best putative protein targets with negative glide energy value ≤ 50. Amongst them, melianone bound to the formate channel protein FocA (3Q7K) at its periplasmic opening by making at least 5 H-bonds with a glide energy of (-)51.8576 kcal mol⁻¹ (Table 1). There has been a recent growing interest on FocA as a good target for inhibition (Online Resource 3) because of its pivotal role in transport of the toxic formate ions across the plasma membrane in bacteria (StFocA) (Das et al. 2009) and transport of lactate in the intraerythrocytic malaria parasite (PfFNT) (Hapuarachchi et al. 2017). Binding of melianone at the periplasmic opening of FocA protomer resulted in the conformational changes of key residues of the protein (Fig. 5). One major impact was the topological modifications in the transmembrane (TM) helices of FocA protomer. In general, the protomers (A, B, C, D, E) of the pentameric protein FocA protein (3Q7K) are known to exist in different channel conformations (open state, intermediate state and closed state) based on the positions of their N-terminal helix and Ω loop (Lu et al. 2011). Interestingly, the topological sketches obtained from PDBsum for the melianone-FocA complex revealed striking re-arrangements in the TM helices of TM2a, TM2b and TM3-4 at the N-terminal end compared to the unbound protein (Fig. 5a and d). Alterations found in the signature Ω loop with a change in its position from DOWN to UP due to melianone binding configured the protomer from open state to the closed state. This significant Ω loop is also speculated to be responsible for the flexibility of the protomer rendering a gating mechanism for the formate channel (Waight et al. 2010; Wang et al. 2009). In addition, the superimposition studies performed for FocA-melianone complex upon FocA-formate ion complex

(3Q7K, protomerA) showed r.m.s deviation (Fig. 6). This deviation was due to the melianone-induced conformational changes in some of the residues like Lys68, Asp88, Thr91, Lys156, His159, Val175, Glu208 and His209 lining the central vestibule of FocA (Fig. 6 and Online Resource 3). These residues appear as highly conserved among the homologues across several species (Waight et al. 2010). The RMSF plot obtained through molecular dynamic simulations also showed deviations in the trajectories for these amino acids (Fig. 7) and the deviations were found to be very specific towards the residues belonging to Ω loop (86–108) TM2a (63–86), TM2b (86–108) and TM3-4 (135–159). Thus the findings from simulation analysis further prove the binding ability of melianone and the mediated changes in FocA protomer.

Similar reports are available for the aqua glyceroporins (AQP1 and AQP3), a channel protein of human RBC with structural similarity to FNT proteins exhibiting similar periplasmic pocket binding with aphen, audien and their monohydroxo derivatives [Au(III)]. Some derivatives approaching closer to the AQP3 SF domain (similar to the central vestibule of FocA channel) were claimed to favour their proximate binding to the channel's constriction pore, thereby hindering the ion transport (Martins et al. 2012).

The predicted potential target protein, FocA (3Q7K), was also important for the virulence of *Salmonella* ser. Typhi during anaerobic pathogenesis while maintaining the formate homeostasis. The experimental determination of any imbalance reported, when the antibacterial melianone is added to the culture, was demonstrated using a simple, colorimetric-based formate detection method. The anaerobic *Salmonella* ser. Typhi cells treated with various inhibitory concentrations of melianone (0.053 and 0.106 μ M) showed an increased titrated formate ion concentration in the medium while cell populations decreased (Fig. 8 and Table 2).

Feng et al. (2012) and Suppmann and Sawers (1994), showed that, in order to survive the anaerobic condition and escape the internal acidosis (accumulation of formate ions inside the cell), *Salmonella* cleverly employs various strategies like exporting the formate ions via the FocA channel for periplasmic reduction by formate dehydrogenases (FDH) (Lv et al. 2013) or disproportionating to H_2 and CO_2 by the cytoplasmic formate–hydrogen lyase complex (FHL) (Lu et al. 2012; vide, Online Resource 3). The formate detection assay results showed a dose-dependent increase in both bacterial cell death and detectable formate ions when treated with melianone (Table 2). This correlated with our in silico results when melianone binding to the periplasmic opening of formate channel protomer-A led to the alterations in the topology of the passage from intermediate open state towards the closed state (Fig. 5). The closed state conformational shift of the FocA passage probably hinders the

expulsion of formate ions (Sawers 2005) from the cytosol leading to internal cellular acidosis that could lead to cell death (Wang et al. 2009).

In the present study, the lipophilic, triterpenoid melianone may act by binding to the periplasmic opening of FocA channel protein leading to an increase in formate in the milieu and reduction in bacterial cell populations (Table 2). Alternately, the triterpenoid is likely to cause massive disruption of the cell membrane itself which is not specific to *Salmonella*. Further confirmation could come from purifying the protein and testing them in vesicles or heterologous systems (Martins et al. 2012). A protein crystallization study may also provide better insight into the mechanism of action or mutations of the conserved residues leading to lowering of the H-bonds. To the best of our knowledge, this is the first report for the anti-*Salmonella* activity of melianone from *S. mahagoni*. Further investigations on proving its activity on other tentative targets will be useful in studying this versatile plant compound with multiple targets.

Acknowledgements A. Veni is thankful to the management of Sri Ramachandra Institute of Higher Education and Research (Deemed-to-be University) for the award of SRU-Chancellor fellowship for this study. Plants were authenticated by Prof (Retd.) P. Jayaraman, Director, Institute of Herbal Botany, Plant Anatomy Research Centre, West Tambaram, Chennai-45 (Reg. No of the certificate: PARC/2014/2059). We sincerely acknowledge the contributions of other reviewers for their constructive criticisms. We record with gratitude the invaluable suggestions from Prof. V Ganesh, Dept. of Human Genetics (SRIHER, DU) towards improvement of this manuscript.

Funding (Information that explains whether and by whom the research was supported). A. Veni is thankful to the management of Sri Ramachandra Institute of Higher Education and Research (Deemed-to-be University) for the award of SRU-Chancellor fellowship for this study.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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