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Research Article

4-Hydroxy-α-Tetralone and its Derivative as Drug Resistance Reversal Agents in Multi Drug Resistant *Escherichia coli*

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The purpose of present investigation was to understand the drug resistance reversal mechanism of 4-hydroxy-α-tetralone (1) isolated from *Ammannia* spp. along with its semi-synthetic derivatives (1a–1e) using multidrug resistant *Escherichia coli* (MDREC). The test compounds did not show significant antibacterial activity of their own, but in combination, they reduced the minimum inhibitory concentration (MIC) of tetracycline (TET). In time kill assay, compound 1 and its derivative 1e in combination with TET reduced the cell viability in concentration dependent manner. Compounds 1 and 1e were also able to reduce the mutation prevention concentration of TET. Both compounds showed inhibition of ATP dependent efflux pumps. In real time polymerase chain reaction (RT-PCR) study, compounds 1 and 1e alone and in combination with TET showed significant down expression of efflux pump gene (yojI) encoding multidrug ATP binding cassettes (ABC) transporter protein. Molecular mechanism was also supported by the *in silico* docking studies, which revealed significant binding affinity of compounds 1 and 1e with YojI. This study confirms that compound 1 and its derivative 1e are ABC efflux pump inhibitors which may be the basis for development of antibacterial combinations for the management of MDR infections from inexpensive natural product.

Key words: 4-hydroxy-α-tetralone, ABC transporters, drug resistance reversal, efflux pump, *in silico* docking, multidrug resistant *Escherichia coli*

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The extensive use of antibiotics has raised serious health concern throughout the world due to development of resistance in bacteria against many classes of antibiotics (1). Multidrug resistant (MDR) bacteria not only exert additional burden of infection but also increase severity of infections both in the hospital and in the community (2). MDR bacteria over-express the efflux pumps, which expel a wide group of unrelated compounds and antibiotics before they reach the site of their action (3). In spite of outer membrane barriers, the gram negative bacteria utilize efflux pumps to achieve high degree of resistance (4). Hence, these bacterial efflux pumps could be potential targets to combat problematic infectious diseases caused by *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhimurium* and Methicillin resistant *Staphylococcus aureus* (5).

Plants have always been a source of novel drugs and play crucial role because their compounds may work as natural blueprint or may be directly used as phytomedicine (6). The plant secondary metabolites viz. terpenoids, alkaloids, glycosteroids, flavonoids, polyphenols, tetralones etc. have become alternate antimicrobial agents because of structural diversity. It has been observed that these plants either contain antimicrobial compounds or possess compounds that have no antibacterial activity of its own, but possess the ability to enhance activity of antibiotics (7). Synergism is known as the positive interaction of two agents in combination, which exerts inhibitory effect greater than the sum of their individual effects. This combination therapy has been useful (i) in the expansion of antimicrobial spectrum (ii) prevention of the emergence of resistant mutants and (iii) in minimization of toxicity. This synergistic combination therapy may be used as an alternative to monotherapy for treating patients with invasive and MDR infections (8).

The various species of genus *Ammannia* are frequently used in traditional Chinese and Indian medicine (9–11). *Ammannia baccifera* Linn. is widely used in traditional Chinese and Indian herbal formulations for the treatment of a number of diseases including spinal disease, haemorrhoids, common cold, human female infertility and gastroenteropathy

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(10). It has been reported to possess anticancer, antirheumatic, antidiuretic, antipyretic, antisteroidogenic, antimicrobial and rubefacient activities. *Ammannia baccifera* is also used as an external remedy for ringworm and skin diseases (11). In an earlier study, we reported bio-enhancing potential of extracts and the compounds isolated from *A. multiflora*, wherein the 4-hydroxy-α-tetralone and its various semi-synthetic acyl and aryl derivatives showed bio-enhancing potential against the nalidixic acid sensitive and nalidixic acid resistant strains of *E. coli* (12). This prompted us to carry out detailed biological investigations on 4-hydroxy-α-tetralone and various semi-synthetic acyl/aryl derivatives against the MDREC to investigate the possible mode of action. This study is in continuation to our search for drug resistance reversal agents from plants (12–14). The present study reports drug resistance reversal potential of 4-hydroxy-α-tetralone and its derivatives in combination with TET using MDREC. The most active combinations were further evaluated for time-kill kinetics, mutation prevention efficiency, efflux pump inhibition, ATPase inhibition, real time expression analysis and *in silico* docking studies.

Methods and Materials

Bacterial strains and culture media

Multi drug resistant *Escherichia coli* clinical isolates MDREC-KG4 and MDREC-KG1 were obtained from Department of Microbiology, King George Medical University Lucknow, India whereas MDREC-EM5 and MDREC-EM7 were the multi drug resistant mutants of *E. coli* isolated from drug sensitive strain MDSEC trough ethyl methyl sulfonate induced mutagenesis (13,14). Drug sensitive *E. coli* (MDSEC, MTCC 739) was procured from Microbial Type Culture Collection, Institute of Microbial Technology Chandigarh, India. Antibiotic resistance/sensitivity pattern of different *E. coli* strains is presented in Table 1. Standard Mueller–Hinton agar and broth (MHA and MHB, Hi-Media, Mumbai, India) were used as bacterial culture media. Mueller Hinton Broth No. 2 (Control Cations) was used for combination study. Colony counts were determined using MHA plates.

Test compounds and antibiotics

The compound 4-hydroxy-α-tetralone (**1**) a natural product was earlier isolated from of *Ammannia spp*. The chemical derivatization of compound **1** into its five acyl and aryl derivatives; myristoyl (**1a**), palmitoyl (**1b**), cinnamoyl (**1c**), m-anisoyl (**1d**) and 3, 4, 5-trimethoxy benzoyl (**1e**) was carried out according to method available in our earlier publications (10,12). Chemical structures are given in Figure 1.

Determination of minimum inhibitory concentration

The test compounds 4-hydroxy-α-tetralone (**1**) and its derivatives were diluted into final concentrations of 1000–1.9 μg/mL and evaluated against test organisms. Minimum inhibitory concentration (MIC) values were determined by 2-fold serial dilution broth assay with starting inoculums of 5×10^5 CFU/mL, incubated at 37 °C for 24 h and detected from the observatory data as per CLSI guidelines (15). Experimental observations were performed in triplicate to rule out any error during the procedure. The antibiotics, tetracycline (TET) and ofloxacin procured from Sigma Aldrich (USA) were used as a positive control.

Table 1: Antibiotic resistance/sensitivity pattern of different cultures of *Escherichia coli*

	MIC (μg/mL) of different antibiotics against sensitive, mutants and clinical isolates of *E. coli*				
Used antibiotics	MDREC-KG4	MDREC-KG1	MDREC-EM5	MDREC-EM7	MDSEC
Penicillin	400	200	100	50	50
Ampicillin	400	200	25	12.5	3.125
Polymyxin-B	50	50	12.5	6.25	6.25
Nalidixic acid	400	200	25	25	6.25
Ofloxacin	50	25	1.56	0.78	0.39
Rifampicin	100	25	12.5	25	6.25
Streptomycin	200	200	12.5	12.5	12.5
Kanamycin	100	50	25	12.5	6.25
Tetracycline	800	400	25	12.5	6.25
Erythromycin	200	200	100	100	50
Imipenem	0.19	0.19	0.19	0.19	0.19

Combination study

Combination studies were performed by broth checkerboard method (16). Cation-adjusted Mueller-Hinton broth (150 μL) was added to each well of the 96-well plate. The last four columns served as controls for *E. coli* growth and plate sterility. The final concentrations ranged from 800 to 6. 25 μg/mL for TET and from 100 to 0.78 μg/mLfor test compounds. Thus, each of the 64 wells had a unique combination of antibiotics and test compounds. The final bacterial inoculum in each well was 5×10^5 CFU/mL, except the negative control. The plates were incubated at 37 °C for 24 h. The MIC for each combination was recorded as the last dilution without any turbidity as per CLSI guidelines. Results were recorded in terms of fold reduction and fractional inhibitory index. The interaction of each combination was based on the fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI). As classically defined, FICI of < 0.5 represented synergism, where as FICI of 0.5–4.0 represented typical additive action, while FIC index of > 4.0 represented antagonism (17). FICI was calculated by adding the FICs (MIC of drug A in combination with drug B divided by MIC of drug A alone).

Time kill assay

Time kill study of TET alone and in combination with compound **1** and its derivative **1e** against MDREC-KG4 was conducted using a previously reported method (18)

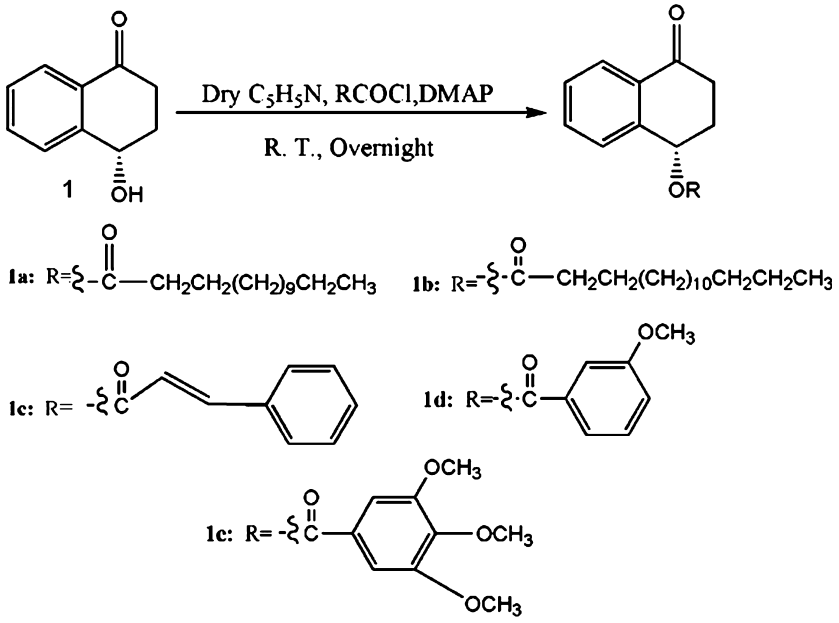

Figure 1: Chemical structure of natural compound (**1**) and its semi-synthetic derivatives (**1a**–**1e**).

at different concentrations (TET 100, 200, 400, 800, 1600 and 3200 μg/mL). These concentrations were equivalent to 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, 2MIC and 4MIC. Each analysis was done in triplicate with a control. Time kill curves were derived by plotting $\log_{10}$ CFU/mL against time (h). Time kill kinetics was also studied in combinations of antibiotic and the test compounds at the reduced concentrations at which maximum synergy was observed.

Selection of resistant mutants in vitro

The mutation prevention concentration (MPC) of TET against *E. coli* MTCC 739 was determined as described previously (19). A bacterial suspension of 10^{10} CFU (0.1 mL) was plated onto MHA containing TET concentrations equal to 2×, 4×, 8× and 16× MIC. The same concentrations of tetracycline were also tested in the presence of compounds **1** and **1e** at 25 mg/L. The mutation frequency was calculated by counting the total number of colonies appearing after 48 h of incubation at 37 °C on the drug-containing plate and then dividing the number by the total number of CFU plated.

Ethidium bromide efflux studies

The fluorometric determination of ethidium bromide efflux was performed as per reported method (20). To get metabolically active cells the culture of MDREC-KG4 was grown in 10 mL MHB (pH 7.3 ± 0.2) with optical density (OD) of 0.6 at 600 nm. The cells were collected by centrifugation at 16 060 × *g* for 3 min and washed with phosphate buffer saline. Ethidium bromide (25 μg/mL) wad added in the bacterial suspension and incubated for 60 min at 25 °C in the absence/presence of test compounds (**1** and **1e**) at 25 μg/mL each. The EB-loaded bacterial suspension were centrifuged at 16 060 × *g* for 3 min, the supernatant discarded and the pellet re-suspended in cold PBS (1×). The tubes then placed on ice. Aliquots of 0.095 mL of the bacterial suspension were distributed to 0.3 mL 96 well plates. Loss of fluorescence was recorded for 30 min at a regular interval of 1 min at the excitation and emission wavelength of 530 nm and 585 nm respectively using spectrofluorometer (FLUO star omega; BMG Labtech, Offenburg, Germany).

ATPase inhibitory assay

Bacterial membrane proteins were isolated by an earlier reported method (21). ATPase assay was carried out in a 96 micro plate format using Quantichrom™ ATPase Assay kit (BioAssay Systems, Hayward, CA, USA). Initially optimal enzyme concentration was determined by a series of dilutions of enzyme (membrane protein containing ATPase) in assay buffer. Enzyme and inhibitor was incubated first for a certain period of time, before adding the substrate. Reactions were set up to 40 μL containing 20 μL Assay Buffer, 5 μL enzyme(20 μg/μL), 5 μL Inhibitor, 10 μL (4 mM ATP) and control with no inhibitor in separate wells. At the end of reaction, 200 μL reagent was added and incubated for 30 min at room temperature. The improved malachite green reagent forms a stable dark green color with liberated inorganic phosphate(Pi), which was measured spectrophotometrically on a plate reader (620 nm) (22).

qRT– PCR analysis of efflux pump gene

The transcriptional profile of efflux pump gene (*yojI*) encoding multidrug ATP binding cassettes (ABC) transporter

protein was analyzed in treated and non treated cells of MDREC-KG-4. Cells were grown to mid-log phase in the presence of sub-inhibitory concentration (1/4 MIC) of tetracycline, compound **1** and its derivative **1e** alone and in combination. The real-time quantification of the RNA templates was analyzed by SYBR GreenER qPCR super mix (Invitrogen, Grand Island, NY, USA) using 7900HT fast real time PCR system (Applied Biosystems, Grand Island, NY, USA). Observations were recorded in terms of LogRQ after normalization of indigenous gene (GAPDH) expression.

In silico docking study

Protein structure prediction of YojI using homology modeling

Homology model was developed for YojI protein of *E. coli* (23), since no crystallographic data is available at present in Protein Data Bank (PDB) (www.rcsb.org) for molecular docking studies by employing GENO3D, an automatic web server for protein structure modeling (www.geno3d-pbil.ibcp.fr). Template protein maltose/maltodextrin transport ATP-binding protein (acc.No. NP_142200.1) showed 32.9% sequence identity with YojI a multidrug ATP binding cassettes (ABC) transporter protein (acc. No. YP_490449.1). Best model based on highest number of residues present in the allowed region of Ramachandran plot was selected.

In silico molecular modeling and docking parameters

Molecular docking, construction, geometry optimization and energy minimization of reserpine a well established efflux pump inhibitor was used as control (24–27) and the under study compounds **1** and its derivatives **1a**–**1e** was performed with YojI receptor by using the Sybyl-X v1.3 molecular modeling and drug discovery software (Tripos International, USA). All the molecules were initially designed in Sybyl, while the molecular construction, geometry optimization, and energy minimization process was performed by using HP XW4600 workstation, running the Red Hat® Enterprise Linux 4.0 (32-bit) operating system (Silicon Graphics Inc., Fremont, CA, USA). The Tripos force field with a distance-dependent dielectric and Powell gradient algorithm with a convergence criterion of 0.001 kcal/mol was used for optimization. Partial atomic charges were assessed by using Gasteiger-Hückel method. 2D structures were converted to 3D structures using the program Concord v4.0 and maximum number of iterations performed in the minimization was set to 2000. Further geometry optimization was done through MOPAC-6 package using the semi-empirical PM3 Hamiltonian method. To find the possible bioactive conformations of control, compound **1** and its derivative **1e**, the Sybyl-X v1.3 interfaced with Surflex-Dock program was used to dock the ligands into the active site of *E. coli* YojI protein. During docking procedure all parameters were assigned to there default values (28).

Results

Two clinical isolates and 2 mutants were found to be resistant to all the clinically used antibiotics except imipenem. The compound 4-hydroxy-α-tetralone (**1**) and its semi-synthetic derivatives (1**a**-1**e**) exhibited MIC in the range of 250–1000 μg/mL against multi drug resistant clinical isolates MDREC-KG4 and MDREC-KG1, multi drug resistant mutants MDREC-EM5, MDREC-EM7 and one multi drug sensitive MDSEC strain of *E. coli* and the results are presented in Table 2. When these compounds were tested in combination with TET, the MIC of TET was reduced up to eight fold. Further, these observations were validated by using another antibiotic ofloxacin against all multi drug resistant strains of *E. coli* wherein reduction in the MIC of ofloxacin up to 8 folds was recorded (Table S1). On the basis of FICI, compounds **1**, **1c** and **1e** showed synergistic interaction with TET (Table 2). Compound **1e** exhibited best interaction with TET in terms of reduction in the MIC against MDREC-KG4 and MDREC-KG1 that showed high level of resistance towards TET.

The treatment of MDREC-KG4 with TET at various concentration (1/8MIC, 1/4MIC, 1/2 MIC, MIC, 2MIC and 4MIC) reduced the viability of *E. coli* significantly at MIC, 2MIC and 4MIC (Figure 2A). However, the reduction in viability of MDREC-KG4 could be achieved at lower concentrations (1/4MIC and 1/8MIC) of TET when tested in combination with compound **1** and **1e** (Figure 2B).

TET at 50 μg/mL (8 × MIC) did not allow the growth of *E. coli* MTCC 739 indicating its mutation prevention concentration. However, MPC was reduced to 25 μg/mL and 12.5 μg/mL when tested in combination with compound **1** and **1e** respectively (Table 3).

To evaluate the efflux pump inhibitory effect, compounds **1** and **1e** were subjected to fluorescence based ethidium bromide efflux assay using MDREC-KG4. As evident from Figure 3, significant decrease in fluorescence was observed in non treated control cells. While in presence of compounds **1** and **1e**, the loss of fluorescence was significantly reduced (Figure 3).

Further, both compounds **1** and **1e** were found to inhibit ATPase at 25 μg/mL concentration at which optimal reduction in the MIC of TET was observed (Figure 4). The known drug reserpine also inhibited ATPase at 25 μg/mL indicating involvement of these compounds in the inhibition of ATP dependent efflux pumps.

ATP dependent efflux pump inhibition was further validated by studying the expression of multidrug ATP binding cassettes (ABC) transporter protein gene (*yojI*). As evident from Figure 5, treatment of TET (1/4 MIC) increased the expression of *yojI*. On the other hand, treatment of **1e** significantly

Table 2: *In vitro* interactions of plant compounds and TET against different cultures of *Escherichia coli*

Organisms	Agents	MIC (μg/mL)		MIC (mM)		FICI	Outcome	Reduction (n-folds) in the MIC of TET
		Alone	Combination	Alone	Combination			
MDREC-KG4	TET	800	–	1.801	–	–	–	–
	1	500	25/200	3.086	0.154/0.450	<0.5	Synergy	4
	1a	1000	50/400	2.688	0.134/0.900	0.5–4.0	Addition	2
	1b	1000	50/400	2.500	0.125/0.900	0.5–4.0	Addition	2
	1c	500	25/200	1.712	0.085/0.450	<0.5	Synergy	4
	1d	1000	50/400	3.378	0.169/0.900	0.5–4.0	Addition	2
	1e	250	25/100	0.702	0.070/0.225	<0.5	Synergy	8
	RES	800	25/400	1.314	0.041/0.900	0.5–4.0	Addition	2
MDREC-KG1	TET	400	–	0.900	–	–	–	–
	1	500	50/100	3.086	0.308/0.225	<0.5	Synergy	4
	1a	1000	50/200	2.688	0.134/0.450	0.5–4.0	Addition	2
	1b	1000	50/200	2.500	0.125/0.450	0.5–4.0	Addition	2
	1c	500	50/100	1.712	0.171/0.225	<0.5	Synergy	4
	1d	1000	50/200	3.378	0.169/0.450	0.5–4.0	Addition	2
	1e	250	25/50	0.702	0.070/0.112	<0.5	Synergy	8
	RES	800	50/200	1.314	0.082/0.450	0.5–4.0	Addition	2
MDREC-EM5	TET	25	–	0.056	–	–	–	–
	1	500	25/6.25	3.086	0.154/0.014	<0.5	Synergy	4
	1a	1000	50/12.5	2.688	0.134/0.028	0.5–4.0	Addition	2
	1b	1000	25/12.5	2.500	0.125/0.028	0.5–4.0	Addition	2
	1c	500	25/6.25	1.712	0.085/0.014	<0.5	Synergy	4
	1d	1000	50/12.5	3.378	0.169/0.028	0.5–4.0	Addition	2
	1e	250	25/6.25	0.702	0.070/0.014	<0.5	Synergy	4
	RES	800	50/12.5	1.314	0.082/0.028	0.5–4.0	Addition	2
MDREC-EM7	TET	12.5	–	0.028	–	–	–	–
	1	500	25/3.12	3.086	0.154/0.007	<0.5	Synergy	4
	1a	1000	50/6.25	2.688	0.134/0.014	0.5–4.0	Addition	2
	1b	1000	50/6.25	2.500	0.125/0.014	0.5–4.0	Addition	2
	1c	500	25/3.12	1.712	0.085/0.007	<0.5	Synergy	4
	1d	1000	50/6.25	3.378	0.169/0.014	0.5–4.0	Addition	2
	1e	250	25/3.12	0.702	0.070/0.007	<0.5	Synergy	4
	RES	800	50/6.25	1.314	0.082/0.014	0.5–4.0	Addition	2
MDSEC	TET	6.25	–	0.014	–	–	–	–
	1	500	25/3.12	3.086	0.154/0.007	0.5–4.0	Addition	2
	1a	1000	50/3.12	2.688	0.134/0.007	0.5–4.0	Addition	2
	1b	1000	50/3.12	2.500	0.125/0.007	0.5–4.0	Addition	2
	1c	500	25/3.12	1.712	0.085/0.007	0.5–4.0	Addition	2
	1d	1000	50/3.12	3.378	0.169/0.007	0.5–4.0	Addition	2
	1e	250	25/1.56	0.702	0.070/0.003	<0.5	Synergy	4
	RES	800	50/3.12	1.314	0.082/0.007	0.5–4.0	Addition	2

lowered the expression level of *yojI* in MDREC-KG4 alone and in combination with TET.

Molecular docking study of compound **1** with YojI receptor showed better binding affinity as indicated by Sybyl Surflex-Dock scores (total score) of 5.2635 which is similar to reserpine (control) and formed H-bonds (hydrogen-bonds) of length 2.0, 2.5 and 2.2 Å with the hydrophobic residues namely, ALA-140, ALA-142 and ARG-122. Similarly, molecular docking results for compound **1e** against target protein YojI, also showed good binding affinity as indicated by total score of 6.6932 which is similar to reserpine and form H-bond of length 2.0 Å with the hydrophobic residue ARG-146. The other derivatives showed varying degree of binding affinity (docking scores from 3.6546 to 4.5132) with the target protein YojI. Docking results for control compound reserpine against YojI showed significant binding affinity as indicated by total score of 6.5866 and formed two hydrogen bonds with ARG-122 and THR-48 of length 2.2 and 1.8 respectively (Table 4).

Discussion

MDR organisms are known to exhibit high degree of resistance to an array of drugs through the involvement of ATP-dependent and ATP-independent efflux pumps (2,29). In order to reduce the selection pressure of antibiotics on microbes and for the better efficacy of existing antibiotics, the need of the hour is to search for agents that are able

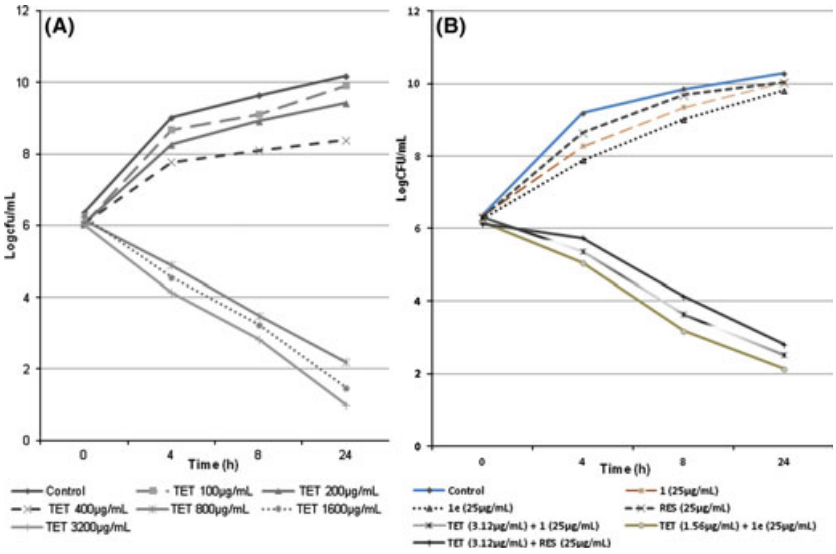

Figure 2: Time–kill curves of MDREC-KG4 showing the dose dependent bactericidal effect of (A) Tetracycline (B) Tetracycline in combination with compounds **1** and **1e**.

Table 3: Mutation frequency of *Escherichia coli* (MTCC 739)

	Mutation frequency of *E. coli* with TET alone and in combination with compounds				
Agents	MIC	2 MIC	4 MIC	8 MIC	16 MIC
TET (Alone)	3.7×10^{10}	1.2×10^{10}	0.3×10^{10}	$<10^{10}$	$<10^{10}$
TET + **1**	1.4×10^{10}	0.2×10^{10}	$<10^{10}$	$<10^{10}$	$<10^{10}$
TET + **1e**	0.8×10^{10}	$<10^{10}$	$<10^{10}$	$<10^{10}$	$<10^{10}$
TET + RES	2.3×10^{10}	0.5×10^{10}	$<10^{10}$	$<10^{10}$	$<10^{10}$

to reverse the drug resistance by inhibiting these efflux pumps. Such compounds, which do not possess antimicrobial activity of their own, but when given in combination with an antibiotic, enhances the activity and availability many folds (12,13,30,31). These compounds do not exert any selection pressure and on the other hand, reduce dosage of antibiotics many folds so that their ill effects are minimized. Thus, these compounds substantially delay the resistance development process and enhance the life-span of novel and existing antibiotics (13,32).

On the basis of MIC data, the compound **1** and the semi-synthetic derivatives **1a**–**1e** were found inactive against all tested organisms as their MIC was higher than that of stringent activity criteria (33). In a combination study, the compound **1** reduced the MIC of TET by four fold. This prompted us to carry out chemical transformation of **1** and study the structure activity relationship (SAR). Compound **1** was derivatized into five lipophilic acyl and aryl derivatives, of which, myristoyl (**1a**), palmitoyl (**1b**) and m-anisoyl (**1d**) derivatives reduced MIC of the TET by two fold i.e. the drug resistance reversal potential was reduced to half to that of the parent compound (**1**). However, substitution of hydroxyl group with cinnamoyl (**1c**) did not affect the drug resistance reversal potential of parent compound (**1**). But careful analysis of the combination study results revealed that further substitutions of anisoyl (**1d**) benzene ring by two more methoxy groups (3, 4, 5-trimethoxy benzoyl derivative, **1e**) exhibited two times increased drug resistance reversal potential. Hence it may be concluded that esterification of 4-hydroxy-α-tetralone (**1**) with 3, 4, 5-trimethoxy benzoyl group significantly enhanced the drug resistance reversal potential. On the basis of FICI, compound **1** and its derivatives **1c** and **1e** synergistically reduced MIC of TET, while other derivatives showed additive interactions. Several natural products and derivatives have been reported that they did not possessed antibacterial activity but in combinations they were able to reduce the dose of partner drugs many folds (34,35). Compound **1** and its derivative **1e** were further explored for their possible mode of action because they were found to be the best in terms of reducing the MIC of TET.

These compounds not only increased the intrinsic susceptibility of *E. coli* to TET, but also significantly reduced the emergence of tetracycline resistant mutants of *E. coli*. The combinations of TET with compounds **1** and **1e** had bactericidal effect and reduced the CFU, whereas TET alone exhibited the same effect at higher concentration. Other plant compounds such as piperine and capsaicin are known to possess these properties (35,36).

Accumulation and efflux of ethidium bromide are the indicators of efflux pumps involvement in multidrug resistance development, particularly in gram negative bacteria such as *E. coli* (37). In a fluorescence based ethidium bromide efflux assay, compound **1e** showed significant (even better than reserpine) inhibition of the efflux pump. The efflux pump inhibitors/modulators might be working through any one or the combinations of; (i) altering regulatory steps that govern the expression of efflux pumps, (ii) inhibiting the

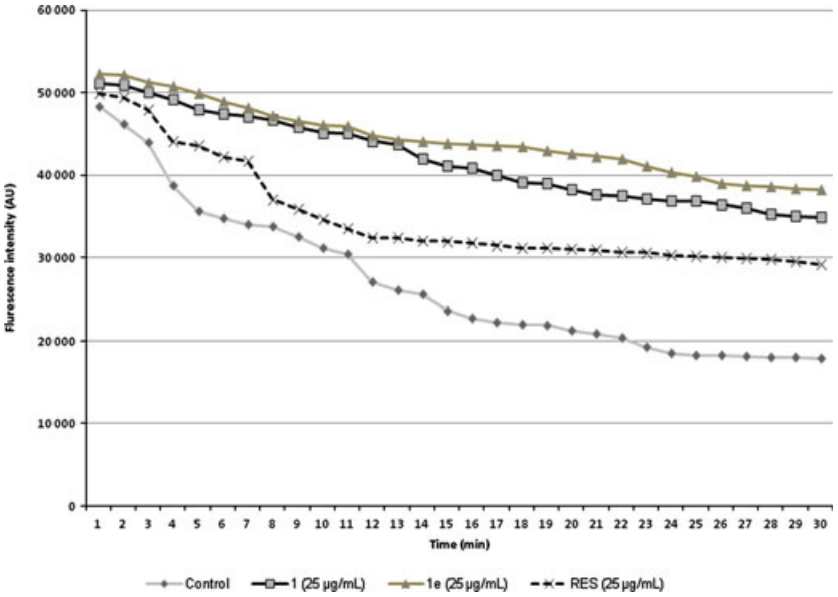

Figure 3: Inhibition of ethidium bromide efflux by compounds **1** and **1e**.

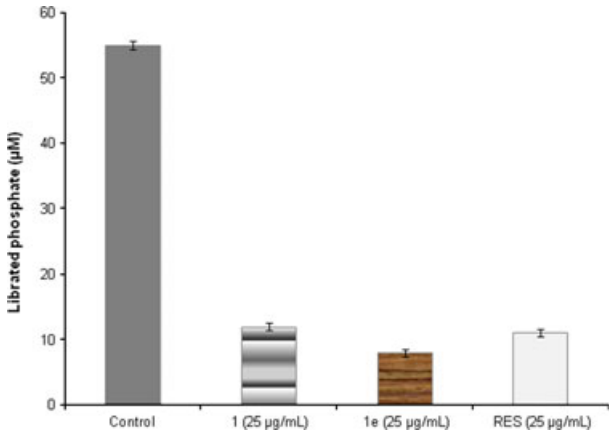

Figure 4: ATPase inhibitory activity of compounds **1** and **1e**.

functional assembly of the multi-component pump, (iii) blocking the outer membrane channel, (iv) collapsing the energy of efflux, (v) creating competitive or non-competitive inhibition with a non antibiotic molecule to the affinity sites of the efflux pump, and (vi) changing the chemical design of previous antibiotics (3). Many plant compounds have been reported to reduce the MIC of antibiotics through involvement of either one or the combinations of the above mechanisms (3,34–37).

Further, to understand whether these compounds interfere with ATP dependent efflux pump, compound **1** and its derivative **1e** were evaluated for ATPase inhibitory activity in clinical isolate MDREC-KG4 because it showed resistance to all the tested antibiotics. Compounds **1** and **1e** were found to inhibit ATPase, which indirectly measures the activity of efflux transporters. Among 74 ATP dependent transporters known so far in *E. coli*, 69 belong to ABC family efflux pumps while others are nominal such as P type ATPase, F type ATPases and H + –ATPase (38). The presence of higher number of ABC family efflux pumps in *E. coli* encouraged us to search ATPase inhibitors having similar properties like ouabain, reserpine and phenothiazine (39–41).

Compounds **1** and **1e** not only enhanced the intrinsic susceptibility of MDREC-KG4 towards TET, but also significantly reduced the expression of ATP dependent efflux pump system. Tetracycline is known to induce higher expression of different efflux pump genes (42,43), but in presence of **1** and **1e** the expression level of *yojI* was significantly down regulated when used alone or in combination with TET. YojI was studied because it is one of the important ATP dependent multidrug ATP binding cassettes (ABC) transporter proteins in *E. coli* (38). In our study, compound **1** and **1e** have affected the transcriptional regulation of *yojI*. It might be possible that the compounds have additional effect either on functional assembly of the multi-component pump, outer membrane channels, or collapsing the energy of efflux pumps. RND efflux pumps (tripartite complex) have been reported as key players for multidrug resistance in gram negative bacteria but other efflux pumps are also crucial for the discovery of efflux pump inhibitors/modulators (38,42–44).

In order to further support the above observation, molecular docking studies of compound **1** and its derivatives **1a**–**1e** were carried out by exploring the binding site interacting residues. In docking site of compound **1** with YojI receptor, the chemical nature of binding site residues within a radius of 4 Å from bound compound were: acidic (polar, negative charged) e.g. GLU-94, GLU-144 (Glutamic acid); basic (polar, hydrophobic and positive charged) e.g. ARG-122, ARG-146 (Arginine), LYS-96, (Lysine); and

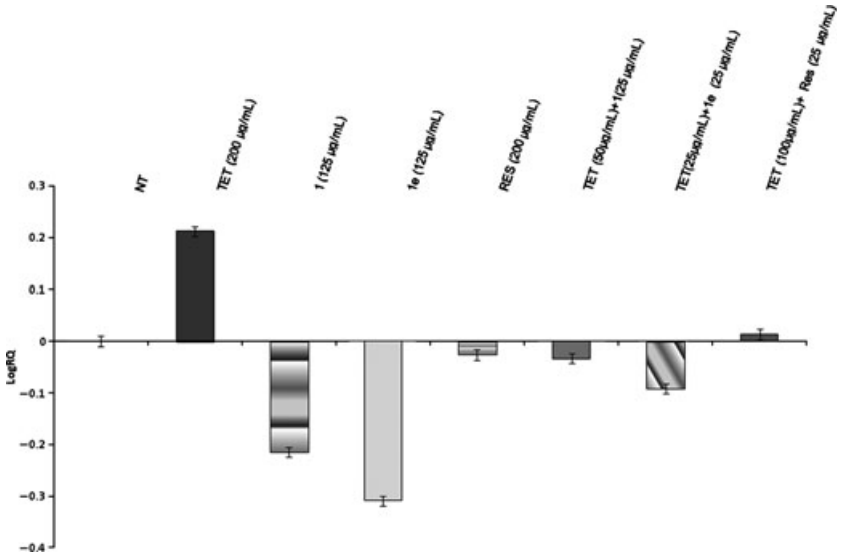

Figure 5: Expression pattern of *yojI* transcript of MDREC-KG4 in presence of TET, compounds **1** and **1e** alone and in combinations.

Table 4: Comparison of docking score and binding site residues of studied compounds against the antibacterial target YojI receptor

S. No.	Compound	Total score	Biding pocket residue in (4 Å)	Length of hydrogen bond (Å)	Amino acid residue involved in Docking interaction	No. of Hydrogen Bond (H)
1.	Compound-**1**	5.2635	LEU-86, GLU-94, LYS-96, ARG-122, ILE-123, ALA-140, ALA-141, ALA-142, ALA-143, GLU-144, ARG-146	2.0 2.5 2.2	ALA-140 ALA-142 ARG-122	3
2.	**1a**	4.5132	THR-48, LEU-50, ARG-74, PHE-77, SER-78, ALA-79, PHE-81, TRP-85, LEU-86, ALA-141, GLU-144, ARG-146	–	–	–
3.	**1b**	3.7412	THR-48, LEU-50, ARG-74, LYS-75, PHE-77, SER-78, ALA-79,VAL-80, PHE-81, TRP-85, LEU-86, ALA-141, GLU-144, ARG-146	–	–	–
4.	**1c**	4.2403	THR-48, LEU-50, SER-78, ALA-79, PHE-81, TRP-85, LEU-86	–	–	–
5.	**1d**	3.6546	MET-45, THR-48, LEU-50, SER-78, ALA-79, PHE-81, TRP-85, LEU-86	–	–	–
6.	**1e**	6.6932	SER-78, ALA-79, PHE-81, TRP-85, LEU-86, GLU-94, LYS-96, ARG-122, ILE-123, ALA-137, ALA-140, ALA-141, ALA-143, GLU-144, ARG-146.	2.0	ARG-146	1
7.	Reserpine	6.5866	MET-45, THR-48, LEU-50, ARG-74, SER-78, ALA-79, VAL-80, PHE-81, TRP-85, LEU-86, GLU-94, LYS-96, LEU-107, ARG-122, ILE-123, ALA-137, ALA-140, ALA-141, ALA-143, GLU-144, ARG-146	2.2 1.8	ARG-122 THR-48	2

Surflex-Dock scores (total scores) were expressed in –log10(Kd) units to represent binding affinities.

hydrophobic e.g. LEU-86 (Leucine), ILE-123 (Isoleucine); ALA-140, ALA-141, ALA-142, ALA-143 (Alanine), thus compound **1** showed good binding affinity and strong hydrophobic interactions which may lead to more stability and activity (Figure 6A, Table 4).

In docking sites of compound **1e** with YojI receptor, the conserved binding site pocket amino acid residues within a selection radius of 4 Å from bound ligands were: nucleophilic (polar, hydrophobic) e.g. SER-78 (Serine); hydrophobic e.g. LEU-86 (Leucine), ILE-123 (Isoleucine); ALA-137, ALA-140, ALA-141, ALA-143(Alanine); acidic (polar, negative charged) e.g. GLU-144 (Glutamic acid); basic (polar, hydrophobic and positive charged) e.g. ARG-122 (Arginine); aromatic (hydrophobic) e.g. PHE-81, (Phenylalanine); acidic (polar, negative charged) e.g. GLU-144 (Glutamic acid); basic (polar, hydrophobic and positive charged) e.g. ARG-146 (Arginine), LYS-96, (Lysine); and hydrophobic amino acid TRP-85 (Tryptophan), therefore compound **1e** showed good binding affinity and strong hydrophobic interaction with YojI receptor, indicating enhanced stability and activity of this compound (Figure 6B).

However, in case of docking pose of reserpine (positive control) with target protein YojI, the conserved binding site pocket amino acid residues within a selection radius of

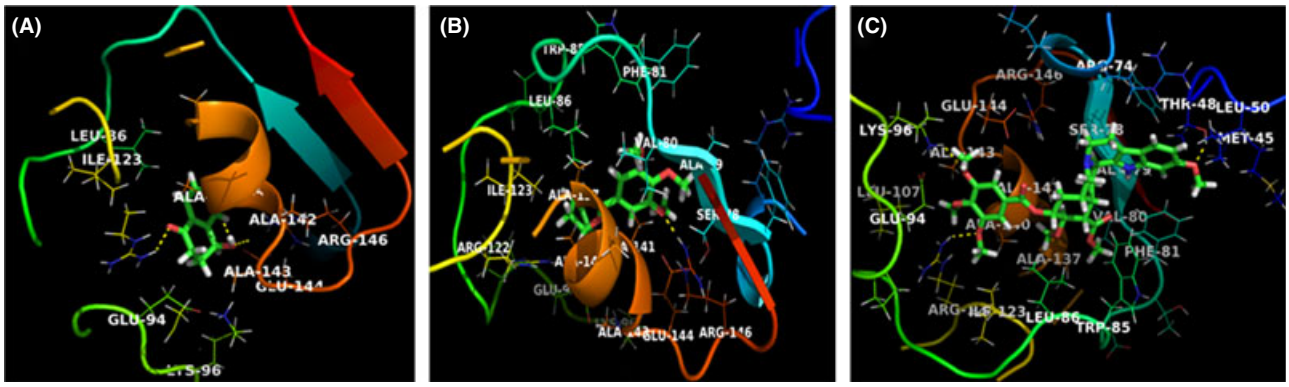

Figure 6: *In silico* molecular docking showing the binding site residues of YojI protein within a selection radius of 4 Å from bound ligands revealing the hydrophobic pocket of active conformation. Binding sites of: (A) compound **1**, (B) compound **1e** and (C) reserpine.

4 Å from bound ligands were nucleophilic (polar, hydrophobic) e.g. SER-78 (Serine); hydrophobic e.g. LEU-50, LEU-86, LEU-107 (Leucine), ILE-123 (Isoleucine), MET-45 (Methionine), THR-48 (Threonine), VAL-80 (Valine); ALA-79, ALA-137, ALA-140, ALA-141, ALA-143 (Alanine); acidic (polar, negative charged) e.g. GLU-94, GLU-144 (Glutamic acid); basic (polar, hydrophobic and positive charged) e.g. ARG-74, ARG-122, ARG-146 (Arginine), LYS-96, (Lysine); and aromatic (hydrophobic) e.g. PHE-81 (Phenylalanine), TRP-85 (Tryptophan). When docking results of reserpine were compared with that of compounds **1** and **1e**, it was revealed that the basic and hydrophobic residue arginine was common in all while interacting with target protein (Figure 6C).

The observations recorded in this study justify the hypothesis according to which, the molecules that target the cell membrane or cell walls are most likely to synergize with conventional antibiotics or antiseptics by inhibiting the efflux pumps, weakening the cell envelope and increasing cellular permeability (45). As already mentioned by Higgins (2007), probably, the time has come not to fight on different types of drug resistance but to find alternatives to avoid and avert it (46). In this regard, these plants based compounds will be a promising candidate as ABC transporter blockers to manage the multidrug resistance.

Conclusions and Future Direction

For the first time, drug resistance reversal potential and mechanism of natural compound **1** and its derivative **1e** were deduced through inhibition of ATP dependent efflux pumps. Inhibition of efflux pumps by these inhibitors may be useful in: (i) lowering the dose of antibiotics; (ii) reducing the drug resistance development frequency; and (iii) increasing the efficacy of antibiotics against multidrug resistant *E. coli* strains. These results may be of great help in the development of inexpensive and dose economic antibacterial drug combinations from a very common and widely distributed herb, *A. multiflora*.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Time–kill curves of MDSEC showing the dose dependent bactericidal effect of (A) Tetracycline (B) Tetracycline in combination with compound **1** and its derivative **1e**.

Figure S2. *In-silico* molecular docking showing the binding site residues of YOJI protein within radius of 4 Å from bound ligands revealing the hydrophobic pocket of active conformation.

Table S1. Minimum inhibitory concentration (MIC) of ofloxacin with and without natural compound (**1**) and its derivatives (**1a**–**1e**) against different cultures of *Escherichia coli*.