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Evaluation of Natural Coumarins as Potential Inhibitors of Efflux Pump Gene Expression in Carbapenem-Resistant Acinetobacter baumannii from Respiratory Infection Patients

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ABSTRACT

Objective: *This study investigates the potential of natural coumarins to inhibit efflux pump gene expression in carbapenem-resistant strains of Acinetobacter baumannii isolated from respiratory infections. Carbapenem resistance is a growing public health threat, and A. baumannii is a notorious pathogen associated with nosocomial infections. Efflux pumps are one of the mechanisms bacteria employ to develop resistance by actively removing antibiotics from the cell. This study investigates the efficacy of coumarins against certain mechanisms of resistance in certain bacteria.* ***Method:*** *Microtiter plate dilution assays were used to determine the minimum inhibitory concentration (MIC) of the antibiotic meropenem, a member of the class of carbapenems, along with coumarins. The study started from January to June 2023, a total (100) sputum specimens were collected from the Intensive Care Units (ICUs) of hospitals in Diwaniyah. Carbapenem-resistant A. baumannii had four isolates used in the study, each of the six experimental groups. Group 1 was a control and was subjected to a sub-MIC (6.25 µg/mL) of the carbapenem active antibiotic. Group 2 was the control and was subjected to 50 µg/mL of ethidium bromide (EtBr) to confirm the presence of functional efflux pumps. Groups 3 to 6 investigated the interaction of coumarins at a sub-MIC (62.5, 125, 250, and 500 µg/mL) with meropenem. A unique experimental control was used in this study which was real-time quantitative PCR (qPCR) to measure the fold change and the relative expression of two efflux pump genes, adeR and adeB, in all groups. Statistical methods that used were Chi-square at ($P < 0.01$) and LSD in one way ANOVA method at ($P< 0.05$) as well as histograms analysis were performed by using GraphPad Prism 7 Statistics software for estimation the difference between means in all groups of the experiment were used.* ***Results:*** *Given both genes had the same fold change in the control group, that change was determined to be 1.0. The corresponding relative changes in control values of the coumarin groups were 0.06 to 0.32 for adeR and 0.25 to 0.66 for adeB which means that both groups in the study were highly over-expressing the genes, exhibiting at least a 2-fold increase in the baseline expression, while the other group (the control) was also exhibiting very high baseline expression. EtBr group's increase was the most pronounced across the board and in tune with the most upregulation and the function of being an established efflux pump substrate. Statistical comparisons highlighted significant differences between ADEB gene expression in the sub-MIC control and all the coumarin treated groups. Interestingly, for the adeR gene, significant differences were observed only between the control and EtBr groups, not between the control and coumarin-treated groups themselves.* ***Novelty:*** *This study concludes that coumarin treatment at the tested concentrations significantly downregulates the expression of adeR and adeB efflux pump genes in carbapenem-resistant A. baumannii isolates. This downregulation potentially enhances the efficacy of meropenem against these multidrug-resistant bacteria.*

INTRODUCTION

Acinetobacter baumannii is classified as a Gram-negative coccobacilli non-glucose fermentative opportunistic pathogen [1]. *Acinetobacter baumannii* is considered to be a significant nosocomial pathogen because to its prolonged survival inside hospital

settings, its resistance to various antimicrobial agents, and its potential to colonize susceptible individuals who are undergoing treatment with broad-spectrum antibiotics [2]. The association of certain infections, urinary tract, skin and soft tissue, pneumonia, and bloodstream infections, is known among those with weakened immunity system/s [3,4]. As of today, there is a worldwide prevalence of MDR A.baumannii, and so far this phenomenon has been associated with high levels of deaths, illnesses, and expenses towards healthcare [5]. There is a global prevalence of A.baumannii which spans all continents such as Europe, North America, Latin America, and Asia [6]. A.baumannii and other related types of bacteria achieve this multidrug same (non)resistance through generation of β-lactamases, structural changes of outer membrane proteins, and (other) penicillin-binding proteins, and by the selective overactivity of efflux pumps [7]. As a result of previously regarding A. baumannii to be of low concern, the incidence of this organism has greatly increased in hospitals; in fact, this pathogen is known to cause as much as 20% of infections in Intensive Care Unit wards (8, 9). We must also add that Acinetobacter is and is a pathogen that withstands high levels of desiccation, high intensity ultraviolet (UV) light, most known chemical disinfectants, and detergents. The organism is able to tolerate dry environments and is located in multiple locations around the hospital, including backs, pieces of furniture, and clinical instruments. As a result, the main route of transmission is hand contact of healthcare workers, which can involve multiple origins. Acinetobacter baumannii has recently become more common as an endemic and epidemic organism in the hospital environment [10]. In the hospital, Acinetobacter baumannii presents a great deal of danger as it can selectively colonize and infect very immunocompromised individuals in the intensive care unit (ICU) [11-13]. Limited options are available for eradicating A. baumannii from commonly used medical equipment, notably catheter-related devices, in hospital settings[14]. The mechanisms of B-lactam resistance are diverse and encompass several well-characterized genes. An established process that is well recognized is the reduction of antibiotic build-up inside bacterial cells through the efflux pump and/or the decrease in permeability caused by outer membrane proteins. Efflux is a widespread mechanism that is linked to antibiotic resistance outside of bacterial cells. Efflux pumps are becoming increasingly important because they have a wide range of substances they can transport, which leads to resistance to multiple antibiotics. Additionally, efflux pumps can work together with other resistance mechanisms to further increase the level of resistance. Regrettably, several efflux pumps in A. baumannii have been found to decrease susceptibility to imipenem. These pumps, namely AdeABC [15], AdeFGH [16], and AdeIJK [17], belong to the resistance-nodulation-division (RND) family and have been linked to resistance against aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol, and trimethoprim. Additionally, increased transcription of three RND efflux pumps has been associated with carbapenem resistance in A. baumannii (CRAB) [18]. So far, several efflux pump inhibitors (EPIs) have been examined on *A. baumannii*, including Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), Phenylalanine-arginine β-naphthylamide (PAβN),

reserpine, omeprazole, 1-(1-Naphthylmethyl)-piperazine (NMP), and verapamil. Certain substances exhibited a notable impact when combined with antibiotics, whilst others showed a restricted impact [19,20]. In order to combat the effects of efflux and reverse imipenem resistance, researchers have created additional efflux pump inhibitors (EPIs). These inhibitors may serve as potent alternative adjuvant therapy options instead of Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which has demonstrated high efficacy but also has toxicity risks [19]. For more than ten years, plants or chemicals derived from plants have been shown to possess antibacterial properties that can suppress a wide range of infections [21]. The combination of essential oils and antibiotics has been recommended due to their ability to enhance antibiotic activity and reduce toxicity. This combination has shown promising synergy against Gram-negative bacteria, particularly cinnamon, thymol, clove, caraway, and other essential oils, as supported by previous studies [22-25]. The coumarin's Potential as an Inhibitor Coumarin shows potential as an inhibitor for efflux pumps in CRAB strains. While research is ongoing, some studies suggest it might downregulate efflux pump gene expression in *A. baumannii*. Cinnamon oil and trimethoprim regulate overexpressed efflux pump-encoding genes to eliminate clinical strains of Acinetobacter baumannii resistant to carbapenems. A study used Cinnamomum verum oil, which contains coumarin along with other compounds, and found it decreased the expression of specific efflux pump genes (adeB, adeC, adeK, and adeJ) in CRAB strains. This resulted in increased susceptibility to imipenem, a carbapenem antibiotic[26]. the aim of the study is to explore Potential Inhibitors of Natural Coumarins on Efflux Pumps gene expression of Carbapenem-Resistant *Acinetobacter baumannii* isolated from respiratory infection patients.

RESEARCH METHOD

Sample collection

Sputum samples were collected from patients who were admitted to the medical intensive care unit (MICU) between January 7, 2025, and June 21, 2025. During their stay in the intensive care unit (ICU), sputum samples were obtained from patients who received mechanical ventilation, using sputum traps.

Culture and Identification

The specimens were subjected to inoculation on MacConkey agar medium and *A. baumannii* Chrom agar. Subsequently, they were cultured for a duration of 24 hours at a temperature of 37 degrees Celsius. The identification of culture growth as *A. baumannii* growth was achieved through the observation of colony morphology as seen in Figure (1), and further validated by the detection of 16sRNA and blaOXA51 genes [27].

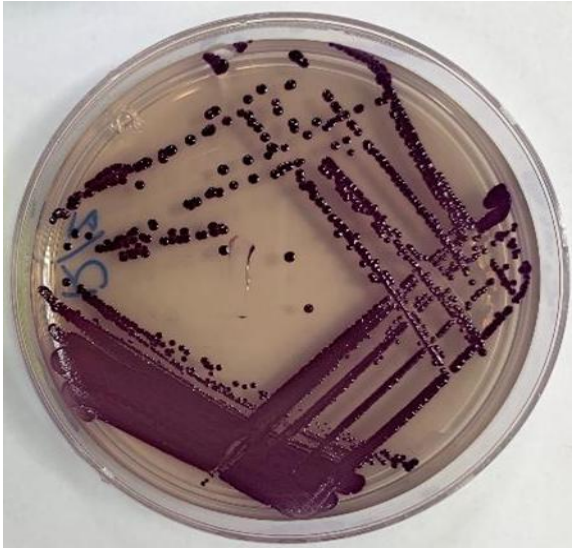

Figure 1. The morphology of *A. baumannii* colonies on *HiCrome Acinetobacter* medium after incubation at 37°C for 24 hours

Antimicrobial Susceptibility Test for *Acinetobacter* spp

The isolates underwent Kirby-Bauer disc diffusion sensitivity testing in accordance with the standards set forth by the Clinical and Laboratory Standard Institute (CLSI), utilizing Mueller-Hinton Agar. [28]. The anti-microbials were utilized: meropenem (10 µg), imipenem (10 µg), azithromycin (15 µg), ciprofloxacin (30 µg) and levofloxacin (5 µg) Acinetobacter confines with decreased susceptibility to meropenem (10 µg) anti-microbial disc (inhibition zone diameter ≤ 11 mm) as seen in Figure (2).

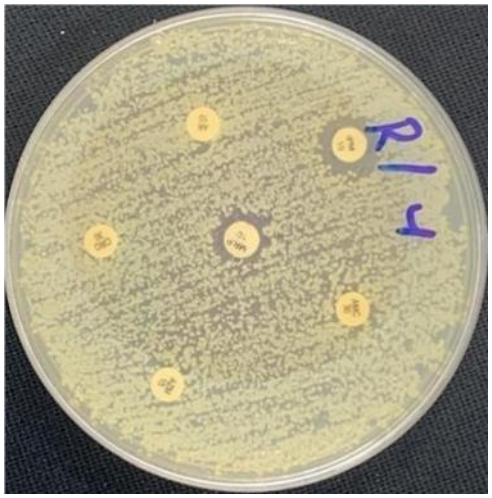

Figure 2. The morphology of *A. baumannii* colonies on Kirby-Bauer disc diffusion sensitivity testing after incubation at 37°C for 24 hours.

Determination of minimum inhibitory concentration (MIC) of meropenem, coumarin and ethidium bromide against carbapenem resistant Acinetobacter baumannii isolates

The minimum inhibitory concentration (MIC) of meropenem (MEM), coumarin (CUM), and ethidium bromide (EtBr) against Acinetobacter baumannii was evaluated using the broth micro-dilution technique [29]. The Acinetobacter baumannii bacterial

culture that was grown overnight in MH broth was diluted with sterile phosphate buffer saline (PBS) to achieve a turbidity corresponding to 0.5 McFarland standards. A bacterial suspension was made by diluting it 1:100 in sterile MH broth. Meropenem, coumarin, and ethidium bromide were diluted in sterile MH broth to concentrations ranging from 0.78 to 50 µg/mL, 1.93 to 1000 µg/mL, and 1.6 to 200 µg/mL, respectively. Each of the dilutions was performed in a sterile 10-well microplate. To each well, 50 µL of a freshly prepared bacterial suspension was added. Data were gathered subsequent to an 18-hour incubation period at 37°C. According to the guidelines, the minimum inhibitory concentration (MIC) of Acinetobacter baumannii was determined to be the lowest concentration of the potential antimicrobial that inhibited visible bacterial growth [29].

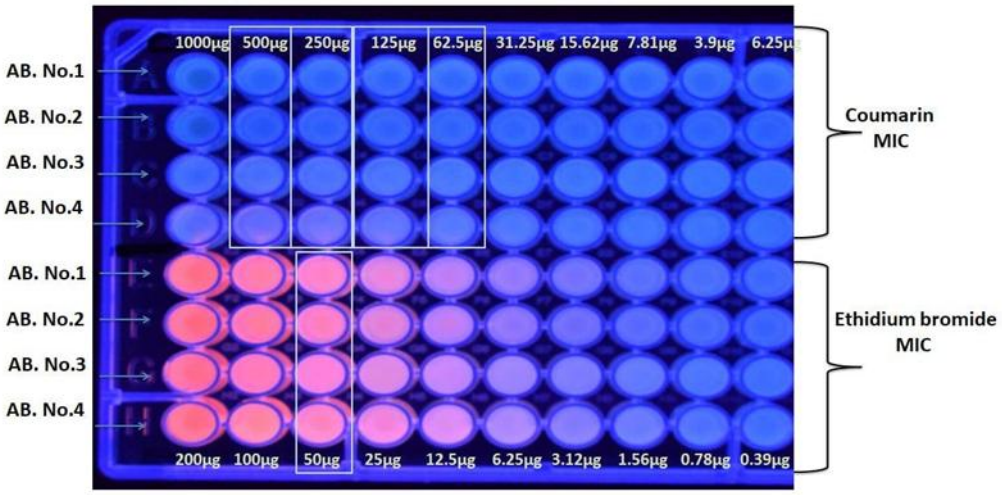

Figure 3. Minimal Inhibitory Concentration (MIC) of Coumarin (CUM) and Ethidium Bromide (EtBr) Using Micro-broth Dilution under UV Light

Extraction of RNA and its Analysis through qRT-PCR

The effect of coumarin on the expression of certain efflux pump (adeR and adeB) genes was determined through qRT-PCR. In this regard, 5 mL of Luria-Bertani (LB) broth was incubated with 0.5 mL of a bacterial suspension. The bacterial suspension was adjusted to a 0.5 McFarland standard. Following this, the suspension was incubated at 37 ^{o}C until the cells had grown over a period of 18 hours**.** The extraction of total RNA was performed using the GeneJET RNA purification Kit (Thermo Fisher Scientific Inc. Germany) according to the instructions provided by the manufacturer. 2 µg of total RNA was utilized to synthesize cDNA using reverse transcription employing random hexamer primers (ThermoFisher scientific, USA) in a final reaction volume of 20 µL, following the instructions provided by the manufacturer of SuperScript™ II RT (Invitrogen™, California, USA). The quantification of gene transcripts was performed using the PowerUp™ SYBR™ Green Master Mix from Applied Biosystems, Thermo Fisher Scientific, using the Agilent Stratagene Mx3005P qPCR Cycler System 5 Color 96-Well instrument located in Santa Clara, California, USA. The primers utilized for

quantitative real-time polymerase chain reaction (qRT-PCR) are specified in Table (1). The fold changes in gene expression were calculated using the comparative Ct (2−ΔΔCt) technique, with 16 S rRNA used as a reference gene.

Table 1. The primer sequence for *A. baumannii* of efflux pump genes (adeR and adeB)of qRT-PCR and there product size

Primer	Sequence 5′-3′	PCR product (bp)	NCBI Reference code
adeB -F	GCGAATAGTACGGAAGGCGA	554	NZ_CP043953.1
adeB -R	ATTGCTTCACCCGATGACGT		
adeR -F	TCCTGTGATCATGCTGACGG	449	NZ_CP043953.1:
adeR -R	CCACGCCACGCACATTAATT		

RESULTS AND DISCUSSION

Ethidium Bromide MIC Results for Acinetobacter baumannii Efflux Pumps

The microtiter plate dilutions of the Ethidium Bromide test are based on the fluorescence shown by Ethidium Bromide (EtBr) when it attaches to bacterial DNA and is exposed to UV light. The efflux pump activity in Acinetobacter baumannii was investigated by measuring the findings in the concentration range of 200-1.62µg/ml in EtBr wells. The efflux pump activity findings of an ethidium bromide-agar minimum inhibitory concentration (MIC) experiment for four isolates of Acinetobacter baumannii. The graphic displays the concentration of EtBr, ranging from 200µg/ml to 1.62 µg/ml, along the top. The isolates contain Ab isolate#1 to Ab isolate#4. The data shown in the figure1 illustrate that the 50 ug/ml dilution of EtBr is the best possible concentration for the evacuation of the EtBr for the active pump for each Ab isolate. At this concentration the bacteria were able to eject EtBr enough to stop EtBr from binding to the DNA and quenched fluorescence. Acinetobacter baumannii is a bacterium that is able to develop resistance to a large number of antibiotics. This makes it a dangerous organism in a clinical setting. A. baumannii develops resistance because of the ability of this organism to use evacuation carried out by specific efflux pumps, which is a system of proteins that eject toxic material from the cell. The Ethidium Bromide Minimum Inhibitory Concentration (EtBr-MIC) test is a standard test that many workers use to assess the potential of efflux pumps. This test uses the principal of EtBr, which is able to fluoresce under UV light when it binds to the DNA of a bacterium. In the EtBr-MIC test A. baumannii could be used to show the presence of four isolates of A. baumannii that have active efflux pumps [30]. To conclude the EtBr-MIC test is a valid method for studying efflux pumps in A. baumannii .

Estimation of Minimum Inhibitory Concentration (MIC) of Meropenem and Coumarin against Carbapenem-resistant Acinetobacter baumannii Isolates:

This study assesses the possible resistance mechanisms in some strains of Acinetobacter baumannii and examines the effect of Coumarin and Meropenem antibiotic against Carbapenem-resistant strains of Acinetobacter baumannii.

The microtiter plate dilution of the Meropenem antibiotic and Coumarin MIC assay is predicated on the bacterial growth on the various concentrations of Meropenem antibiotic and Coumarin. In this test, Acinetobacter baumannii was tested in the (1000-1.93µg/ml) concentration of coumarin wells and also tested for meropenem antibiotic with range (50-0.09µg/ml) as seen in Fig.(1)

Group No. 1: This group tests the effect of a sub-MIC concentration of meropenem (6.25 µg/mL) on four isolates of carbapenem-resistant Acinetobacter baumannii. MIC refers to the minimum inhibitory concentration, which is the lowest concentration of an antibiotic that can prevent bacterial growth. A sub-MIC concentration is a lower dose than the MIC.

Group No. 2: This group uses ethidium bromide at 50 µg/mL to investigate the presence of a efflux pump. Efflux pumps are mechanisms that can allow bacteria to pump antibiotics out of the cell, reducing their effectiveness. By including a group with ethidium bromide, which is a known inhibitor of efflux pumps, the researchers can assess whether this mechanism is contributing to carbapenem resistance in these isolates.

Group No. 3 & 4 & 5 & 6: These groups test the combined effect of coumarin (at 500 µg/mL , 250 µg/mL , 125 µg/mL and 62.5 µg/mL) with a sub-MIC concentration of meropenem (6.25 µg/mL) on four isolates each. Coumarin is added in order to see if it could be used in conjunction with meropenem to potentially be able to reverse meropenem's carbapenemase resistance. The diverse concentrations of coumarin are meant to see if there is a dose dependent response.

Gene Expression Results

The results of a quantitative real-time PCR (qPCR) experiment which aimed to measure the fold change of relative gene expression of the two efflux pump genes, adeR and adeB, in Meropenem resistant Acinetobacter baumannii isolates. The Livak method (2^-ΔΔCt) was used to measure the fold change. The experiment was used five experimental groups which were, a SubMIC (sub-minimum inhibitory concentration of Meropenem 6.25ug/ml) control, Ethidium bromide (Et.br) positive control, and 4 groups which were treated with different concentrations of (Coumarin) 62.5 µg, 125 µg, 250 µg, 500 µg which are used as efflux pump inhibitors and these concentrations were mixed with SubMIC Meropenem 6.25ug/ml to trigger the expression of efflux pump genes. The SubMIC group represents the control group where the bacteria were exposed to a sub-inhibitory concentration of meropenem, an antibiotic. The EtBr group is the positive control and serves as a baseline reference point for high efflux pump activity, as Ethidium bromide is a well-known substrate for efflux pumps in many bacteria.

The remaining groups (Cum 62.5ug, Cum 125ug, Cum250ug and Cum500ug) represent the bacteria treated with varying concentrations of coumarin, an efflux pump inhibitor.

The relative expression of the adeR and adeB genes appears to be downregulated in all experimental groups compared to the SubMIC control group. The

fold change in the SubMIC group is around 1.0 for both genes, while the fold change in the other groups ranges from 0.06 to 0.32 for adeR and 0.25 to 0.66 for adeB. The highest fold change, indicating the most up-regulation, is seen in the EtBr positive control group for both genes. There is clear dose-dependent response with increasing coumarin concentrations as seen in Figure (4).

Overall, the results suggest that coumarin treatment at the concentrations used in this experiment have a significant inhibitory effect on the expression of the adeR and adeB efflux pump genes in these Acinetobacter baumannii isolates throw acting as inhibitors of DNA topoisomerase type II, a key enzyme involved in DNA replication, thereby significantly inhibiting nucleic acid synthesis. Additionally, coumarins interfere with bacterial quorum-sensing mechanisms, preventing biofilm formation and the production of virulence factors. When consumed in high doses, coumarin can cause several adverse side effects, with the most notable being liver damage. The effect on the liver is typically reversible once exposure is stopped.

The statistical analysis compares the fold change of relative gene expression in efflux pump resistance adeR gene of resistant Acinetobacter baumannii isolates across five experimental groups. A Least Significant Difference (LSD) post-hoc test was performed to compare gene expression between groups. The analysis shows that there is a significant difference ($p < 0.05$) in gene expression between the SubMIC control group and the Ethidium bromide positive control group, as well as all Coumarin treated groups. There are no significant differences between the Coumarin treated groups themselves. The efflux pump resistance adeB gene expression analysis shows that there exists a statistically significant ($p < 0.05$) variance in gene expression between the SubMIC control and Coumarin treated groups (62.5 µg, 125 µg, 250 µg, and 500 µg) and between each of those groups.

There is comparably no statistically significant difference between the expression of the SubMIC control and that of Ethidium Bromide Positive Control.

This study assesses the impact of coumarin, an efflux pump inhibitor, on the expression of the two efflux pump genes, adeR and adeB, in carbapenem resistant Acinetobacter baumannii. The analysis indicates that the expression of the two genes is significantly downregulated by the coumarin, thereby restoring the activity of meropenem that is otherwise rendered ineffective by the resistance mechanisms of the bacteria.

The study comprised of six groups, which included a SubMIC of meropenem control, an Ethidium Bromide (EtBr) positive control, and three groups treated with different concentrations of Coumarin in combination with the SubMIC meropenem. The SubMIC group control was primarily aimed at gauging the gene expression, while the EtBr group, a known positive control for efflux pumps, provided evidence for a substantial efflux activity.

From the qPCR results, it was evident that in the groups treated with Coumarin, there was a significant downregulation of expression of both genes, compared to the SubMIC control which was not treated with coumarin.

The fold change in the control group for both genes was about 1.0, which corresponded to their basal expression. On the other hand, in the coumarin groups the fold change was 0.06 to 0.32 for adeR and 0.25 to 0.66 for adeB, indicating the fraction loss in gene expression was of considerable magnitude. Notably, the group of EtBr had the highest fold change, which was the most considerable case of upregulation and was consistent with the action of this group as an efflux pump substrate as shown in tables (2) and (3).

The observed dose-dependent response also adds further support to the findings. With increasing coumarin concentration, the fold change reflecting gene downregulation also increased. This indicates the concentration dependent action of coumarin on the expression of efflux pump genes.

These observations were confirmed by the statistical analysis with the Least Significant Difference (LSD) post-hoc test. For adeR expression, there was a significant difference ($p < 0.05$) observed between the SubMIC control, the EtBr group, and all coumarin treated groups. However, there were no significant differences among the coumarin groups themselves. This indicates that all concentrations of coumarin suppressed adeR expression to a similar extent.

The statistical analysis for the expression of adeB, however, followed a slightly different pattern. While a statistically significant difference ($p < 0.05$) was present between the SubMIC control and all groups with coumarins, the SubMIC control and the EtBr group did not differ significantly. This indicates that in these A. baumannii isolates, the expression of adeB, unlike adeR, may not be directly activated during EtBr exposure.

The rise of multidrug-resistant A. baumannii presents a concern to public health. Among the major contributors to this resistance, the efflux pumps, protein channels, remove the antibiotics from the cell [31]. This study aims to assess the extent of coumarin which is a natural and known efflux pump inhibitor antifungal, and to alleviate this growing concern in A. baumannii isolates. Efforts to alleviate the problem warrant the use of coumarin to act as a potential novel approach to therapy by targeting exopolysaccharide (EPS) efflux pumps. Many studies demonstrate the potential of coumarin. Martin et al. (2023) [30]. showed that and suggest the inhibition of efflux pumps, are synergistic with coumarin derivatives and antibiotics against Staphylococcus aureus. A similar observation was reported by Viveiros et al., One of the coumarin derivatives was also reported to overcome the efflux pumps inhibition of tetracycline resistance in Escherichia coli [32]. Moreover, Andrade et al. research.

Coumarin-related compounds were found to selectively act upon the NorA efflux pump in S. aureus, potentiating the effect of norfloxacin [33]. This possibility is further supported by preliminary studies. In recent studies conducted by Avakh et al., the investigators demonstrated activity of some coumarin derivatives against the MexXY efflux pump of the other extensively drug resistant (XDR) pathogen, Pseudomonas aeruginosa [34]. These studies, however, do not come without limitations. Martin et al. have pointed out that certain coumarin derivatives would exhibit no direct antibacterial

activity [30]. In addition, the in vivo effects of these compounds have yet to be properly elucidated. The pharmacokinetic properties of these compounds, along with their possible toxicity, also remain to be elucidated [35]. In summary, these studies illustrate the great promise represented by coumarin derivatives in the treatment of drug resistant infections. The unique nature of these compounds along with their demonstrated ability to inhibit efflux pumps make these compounds worthy of further investigation. The refinement of these compounds, particularly relating to their biological activity, method of introduction into the body, and toxicity constitute a priority in the treatment of multi-drug resistant (MDR) pathogens. The evidence that coumarin compounds downregulated the expression of the efflux pump genes, adeR and adeB, of meropenem resistant Acinetobacter baumannii, is unequivocal. The statistically significant results demonstrate the effect is both real and of practical importance, and the difference between the coumarin treated and the control groups in terms of the gene expression shows this is a dose-dependent effect.

Although there is no understanding of the precise actions of coumarin, the results of the test of the proposed anti-acinetobacter baumannii infection coumarin derivative suggest a potential for the development of adeB efflux pump inhibitor strategies used in anti A. baumannii. Moreover, there remains to be enough dexriptive actions to test coumarin alongside meropenem in vitro.

Table 2. Relative expression of efflux pump resistant adeR genes in meropenem resistant in Acinetobacter baumannii isolates using the Livak expression method ($2^{-\Delta\Delta CT}$).

No. sample	CT (adeR)	CT (16SrRNA)	ΔCT: T	ΔCT: C	ΔΔCT	Fold change (2^-ΔCT)	Mean± St. error
SubMIC	16.34	16.37	-0.03	-0.33	0.30	0.81	1.03±0.27
SubMIC	15.38	16.11	-0.73	-0.33	-0.40	1.32	
SubMIC	15.29	15.86	-0.57	-0.33	-0.24	1.18	
SubMIC	16.52	16.51	0.01	-0.33	0.34	0.79	
Et.br 50ug	20.14	16.14	4.00	-0.33	4.33	0.05	0.14±0.09
Et.br 50ug	19.14	16.67	2.47	-0.33	2.80	0.14	
Et.br 50ug	17.06	15.46	1.60	-0.33	1.93	0.26	
Et.br 50ug	19.82	16.91	2.91	-0.33	3.24	0.11	
Cum500ug	20.17	16.69	3.48	-0.33	3.81	0.07	0.06±0.02
Cum500ug	19.02	14.9	4.12	-0.33	4.45	0.05	
Cum500ug	19.67	16.27	3.40	-0.33	3.73	0.08	
Cum500ug	20.34	15.61	4.73	-0.33	5.06	0.03	
Cum250ug	17.78	15.21	2.57	-0.33	2.90	0.13	0.09±0.04
Cum250ug	19.2	15.96	3.24	-0.33	3.57	0.08	
Cum250ug	19.92	15.55	4.37	-0.33	4.70	0.04	
Cum250ug	18.57	15.87	2.70	-0.33	3.03	0.12	
Cum 125ug	18.24	16.74	1.50	-0.33	1.83	0.28	0.20±0.15
Cum 125ug	19.22	16.47	2.75	-0.33	3.08	0.12	
Cum 125ug	19.54	15.14	4.40	-0.33	4.73	0.04	

Cum 125ug	17.21	16.06	1.15	-0.33	1.48	0.36	0.32±0.19
Cum 62.5ug	17.53	16.49	1.04	-0.33	1.37	0.39	
Cum 62.5ug	17.22	15.25	1.97	-0.33	2.30	0.20	
Cum 62.5ug	17.33	14.8	2.53	-0.33	2.86	0.14	
Cum 62.5ug	17.06	16.58	0.48	-0.33	0.81	0.57	
Mean C	15.8825	16.2125	-0.33				

Table (3). Fold change of gene expression in efflux pump resistant adeB gene using Livak ($2^{-\Delta\Delta CT}$) in Meropenem resistant Acinetobacter baumannii isolates.

No. sample	CT (adeB)	CT (16SrRNA)	ΔCT: T	ΔCT: C	ΔΔCT	Fold change (2^-ΔCT)	Mean± St. error
SubMIC	15.3	16.37	-1.07	-0.69	-0.38	1.31	1.02±0.22
SubMIC	15.35	16.11	-0.76	-0.69	-0.07	1.05	
SubMIC	15.54	15.86	-0.32	-0.69	0.37	0.78	
SubMIC	15.92	16.51	-0.59	-0.69	0.10	0.94	
Et.br 50ug	15.39	16.14	-0.75	-0.69	-0.06	1.05	0.66±0.48
Et.br 50ug	15.89	16.67	-0.78	-0.69	-0.09	1.07	
Et.br 50ug	18.29	15.46	2.83	-0.69	3.52	0.09	
Et.br 50ug	17.46	16.91	0.55	-0.69	1.24	0.42	
Cum500ug	18.32	16.69	1.63	-0.69	2.32	0.20	0.25±0.15
Cum500ug	18.54	14.9	3.64	-0.69	4.33	0.05	
Cum500ug	16.95	16.27	0.68	-0.69	1.37	0.39	
Cum500ug	16.45	15.61	0.84	-0.69	1.53	0.35	
Cum250ug	17.04	16.21	0.83	-0.69	1.52	0.35	0.33±0.18
Cum250ug	17.5	15.96	1.54	-0.69	2.23	0.21	
Cum250ug	17.35	15.55	1.80	-0.69	2.49	0.18	
Cum250ug	16.98	16.87	0.11	-0.69	0.80	0.58	
Cum 125ug	16.42	16.74	-0.32	-0.69	0.37	0.78	0.50±0.26
Cum 125ug	17.36	16.47	0.89	-0.69	1.58	0.34	
Cum 125ug	16.58	15.14	1.44	-0.69	2.13	0.23	
Cum 125ug	15.97	16.06	-0.09	-0.69	0.60	0.66	
Cum 62.5ug	16.44	16.49	-0.05	-0.69	0.64	0.64	0.41±0.16
Cum 62.5ug	16.47	15.25	1.22	-0.69	1.90	0.27	
Cum 62.5ug	15.52	14.8	0.72	-0.69	1.41	0.38	
Cum 62.5ug	17.44	16.58	0.86	-0.69	1.55	0.34	
Mean C	15.5275	16.2125	-0.685				

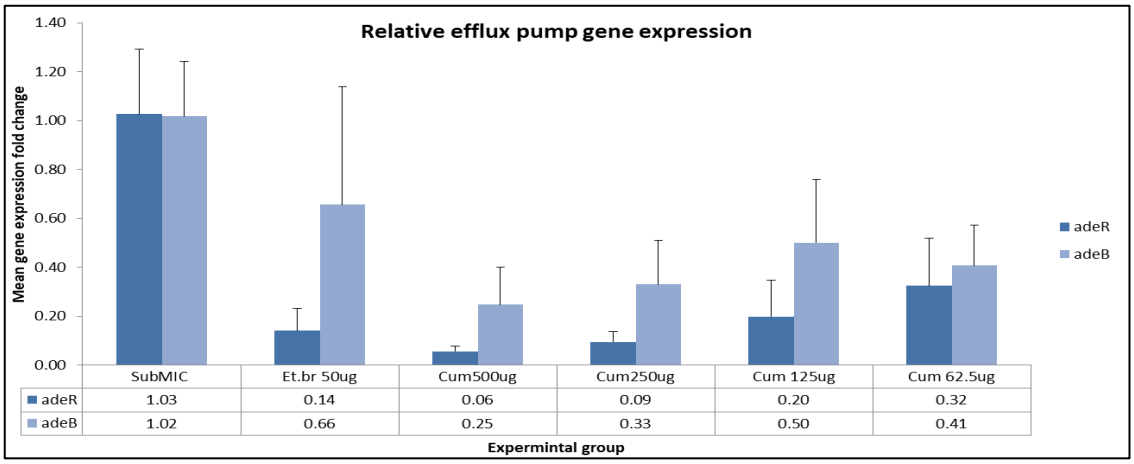

Figure 4. The average of fold change gene expression of efflux pump antibiotic resistance adeR and adeB genes in Meropenem resistant Acinetobacter baumannii isolates

CONCLUSION

Fundamental Finding : The relative expression of the adeR and adeB genes appears to be downregulated in all experimental groups compared to the SubMIC control group. The fold change in the SubMIC group is around 1.0 for both genes, while the fold change in the other groups ranges from 0.06 to 0.32 for adeR and 0.25 to 0.66 for adeB. There is clear dose-dependent response with increasing coumarin concentrations. The analysis indicates that the expression of the two genes is significantly downregulated by the coumarin. **Implication :** This indicates the concentration dependent action of coumarin on the expression of efflux pump genes. The statistically significant results demonstrate the effect is both real and of practical importance, and the difference between the coumarin treated and the control groups in terms of the gene expression shows this is a dose-dependent effect. **Limitation :** The in vivo effects of these compounds have yet to be properly elucidated. The pharmacokinetic properties of these compounds, along with their possible toxicity, also remain to be elucidated. When consumed in high doses, coumarin can cause several adverse side effects, with the most notable being liver damage. **Future Research :** The unique nature of these compounds along with their demonstrated ability to inhibit efflux pumps make these compounds worthy of further investigation. The refinement of these compounds, particularly relating to their biological activity, method of introduction into the body, and toxicity constitute a priority in the treatment of multi-drug resistant (MDR) pathogens.

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